Iron Status in Norwegian Blood Donors

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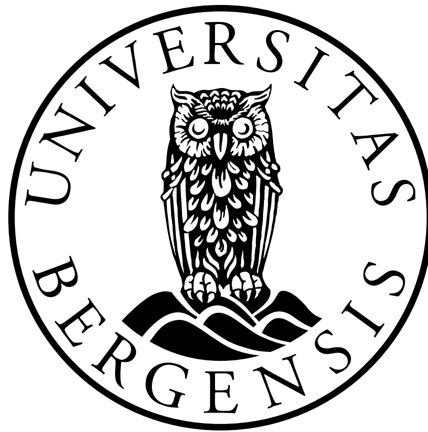

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This thesis became a reality through discussions with Tor Hervig, the head of Blood Bank at Haukeland University Hospital. He proposed the theme “Iron status in Norwegian blood donors”. In working with blood donors I agreed that important matters concerning the iron status in blood donors were not fully explored. Also in teaching transfusion medicine, changes in iron status for blood donors while donating was ambiguous and I found it suitable to refine this area. We have had interesting discussions about who should be offered iron supplementations and who would be harmed by additional iron. This raised ethical issues, and these ethical discussions we have not really finished.

The incitement to this thesis was the opportunities given by Ålesund University College, through an invitation to participate in courses in ethics and philosophy at the Vest Norsk Nettverk (VNN). After making contact with the Department of Immunology and transfusion medicine (earlier name Blood Bank) and the Laboratory of Clinical Biochemistry (LKB) at Haukeland University Hospital (HUS) this study began to develop. The study was supported financially by Western Norway Regional Health Authority, the Norwegian Plasma Fractionation Project, and Ålesund University College.

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Abstract

Background and aims: Blood banks in Norway struggle to close the gap between need and supply of blood. The overall objective of this study was to investigate the iron status in blood donors. The aims of this thesis were first to compare the iron status in new blood donors in 1993-97 and in 2005-06, to describe possible changes in iron status. To describe the effect of four consecutive blood donations without iron supplementation for newly recruited donors was the second aim. The third objective was to study the effect of iron supplementation during the first week after blood donation. The fourth aim was to describe the distribution of HFE variants in blood donors with hereditary hemochromatosis, and compare changes in serum ferritin after blood donations between HFE variants and also between HFE variants and wild types.

Results: Hb was decreased by 0.1 g/dL and serum ferritin was decreased by 4µg/L for women, both significant changes in the studied decade. This corresponded to the significantly increased new donor-rejection, from 14 % to 24 % for women. The differences in ferritin were most obvious for young women 18 - 45 years. Hb and serum ferritin decreased for women and serum ferritin decreased for men, with number of donations. This decrease was less prominent for longer intervals between donations, particularly between the third and fourth donations. Short term iron supplementation had a beneficial effect on iron status after donation. Donors with a serum ferritin below 50 µg/L should be offered iron, while donors with a serum ferritin above 80 µg/L do not need extra iron. Donors in the interval between these limits should be treated individually. Differences in serum ferritin were not statistically significant for women or men between HFE variants groups, and were significantly higher in the HFE variant-group than for wild types for women, but not for men. The serum ferritin ratio (ferritin at last donation divided by ferritin next to the last donation) was not significantly different between the HFE variant groups for any gender. The serum ferritin ratio was significantly larger for HFE variants than for wild types men, but not for women.

Conclusion: The study revealed the need for more knowledge about the iron status in donors, the effect of donation interval, serum ferritin measurements and iron supplementation, differentiated to gender and age. Iron supplementation had a significant impact on the restoration of iron status after donation. The donors with hereditary hemochromatosis can augment the blood supply.

Publications

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Abbreviations

A	Adenine (nitrogenous bases), purine
ALAT	Alanin aminotransferase
ALP	Alcaline phosphatase
ANOVA	Analyses of variance
ASAT	Aspartate aminotransferase
ATP	Adenosine triphosphate
C	Cytosine (nitrogenous bases), pyrimidine
(c845G→A)	G to A transition at nucleotide 845 (results in C282Y mutation) in the HFE protein
(c187C→G)	C to G transition at nucleotide 187 (results in H63D mutation) in the HFE protein
C282	Hemochromatosis gene wild type
C282Y	Substitution of tyrosin for cystein at amino acid 282 (Cys282Tyr) in the HFE protein
CHr	Concentration of hemoglobin in reticulocytes
CRP	C- reactive protein
CV	Coefficient of Variation
DNA	Deoxyribonucleic acid
FDA	Food and Drug Administration, Rochville, MD, USA
G	Guanine (nitrogenous bases), purine
γ-GT	Gammaglutamyltransferase
H63	Hemochromatosis gene wild type
H63D	Substitution of aspartic acid for histidin at amino acid 63 (His63Asp) in the HFE protein
Hb	Hemoglobin concentration
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HFE	The official symbol for the hemochromatosis gene located at chromosome 6p21.3
HH	Hereditary hemochromatosis
HiCN	Cyanmethaemoglobin
HIV	Human immunodeficiency virus
HUNT	Helseundersøkelsen i Nord Trøndelag
ICSH	International Council for Standardization in Haematology
MEIA	Microparticle Enzyme Immunoassay
nlme	Non-linear and linear mixed effects model
PCR	Polymerase chain reaction
R	Statistical program

SD	Standard Deviation
SPSS	Statistical Package for Social Sciences
SNP	Single nucleotide polymorphism
sTfR	Soluble transferrin receptor
TfR 1	A specific cell-surface transferrin receptor
TfR-F index	sTfR/ log of serum ferritin

Introduction

The blood donors

In Norway, blood transfusions are regarded as safe [1, 2]. The blood bank surveillance system (hemovigilance) is handling information on complications of transfusions as well as suggestions for improvement of the transfusion service. In addition to the data collected, the system also draws attention to the field of blood safety. The objective of the system is to supervise all complications related to blood transfusions, both from the donor's and the patient's perspectives. Furthermore the field of transfusion medicine is exposed to detailed regulations listed in directives and guidelines [1].

To donate blood is also safe, but despite a positive attitude to donating blood [3], only 2 % of the eligible Norwegian population are donors [4]. The frequency of blood donations is about 2.4 per year per donor [5, 6]. A standard whole blood donation is 450 ml. The number of registered donors was 91 341 in 1999 and 92 985 in 2005, representing an increase of 1 %. In the same period, the number of transfused units of erythrocytes increased from 172 288 to 189 432 [5], an increase of 9 %, showing that there is an increasing gap between demand for blood transfusions and available units of erythrocytes. The guidelines for accepting blood donors are quite strict in Norway, and in line with the Council of Europe's guidelines [7]. Requirements related to iron status is a part of these regulations. According to the Council of Europe the inclusion criteria are Hb ≥ 12.5 g/dL for women and ≥ 13.5 g/dL for men [7]. Locally, additional requirements are related to iron stores. In Bergen inclusion criteria for serum ferritin is ≥ 18 µg/L. At first-time registration an increasing number of healthy volunteers are rejected because they fail to meet the inclusion criteria for Hb [8] and serum ferritin [9].

A substantial amount of work is related to exclusion of subjects unsuitable for donations. The regulations may be too strict, making it difficult to cover the demand for blood supply. The average age in the Norwegian population is increasing, and the need for blood transfusion rises due to increased prevalence of disease, accidents and surgery; thus expanding the gap between need and supply.

This study is focusing on iron status when recruiting and retaining blood donors, as a reduction of deferrals due to iron status may have great impact on the donor situation and on the blood supply.

Recruiting new and retaining established donors

Recruiting a sufficient number of new blood donors is a huge challenge in many countries [3, 6, 10, 11]. In Norway one of the major sources for recruiting new donors is the fact that people are invited and informed by established donors, often among friends and family-members [8]. At Haukeland University Hospital in Bergen, the rejection of new donors was about 25 % in 2003 (Knut Liseth, personal communication). One of the main reasons for rejection was failing to meet the Hb criteria, predominantly amongst young women [9].

To keep the blood supply sufficient, an increased pressure is laid upon established donors. The donors with a high frequency of donations are at risk for iron deficiency. In Norway both genders are allowed to donate four times a year [12]. Mean number of donations in Bergen in 2007 was 2.6 per year for women and 2.9 for men [13]. By comparison, women of childbearing age in United States may donate only one unit of blood while men may donate four or more units per year [14]. Eligible donors who are willing and altruistic should be retained as good donors, without aggravating their iron status.

Iron status in blood donors

Important questions about the impact of donations on the donor's iron status are:

- Who will develop iron deficiency?
- What is the ideal frequency of donation?
- What is the effect of iron supplementation to donors?

The increasing percentage of young female donors further underlines the importance of controlling the iron status as the physiological iron loss is high in menstruating females [14]. One whole - blood donation removes about 250 mg of iron (4-10 % of the body iron) and leads to a reduction of 0.5 g/dL in the venous Hb immediately after donation [15]. The lowest point is reached 2-3 days after donation and Hb regains after 30-40 days [16, 17]. Short intervals between donations may increase the risk of iron depletion, while longer intervals

and/or iron supplementation after donations may prevent iron deficiency [17-21]. Ferrous sulphate is the most used formula to compensate for the loss of iron in blood donors [22, 23]. Unless donors are taking iron supplementation regularly, even two donations per year is associated with a 28 % incidence of iron deficiency [24]. Bianco *et al* found that 13 % of donors were rejected and that 41 % of all deferrals were explained by low Hb-values [25]. The reduction of iron in donors is not sufficiently compensated by a normal diet [26], and it is discussed whether iron supplementation after donations will restore the iron status more quickly [23, 27, 28]. Furthermore, iron supplementation is a controversial issue as a donor may suffer from unknown, hereditary hemochromatosis in the preclinical stage, and it may be considered unethical to offer iron supplementation to these donors [25].
Genetic screening for hemochromatosis is not a routine. This is the most frequent disorder of inborn metabolism in the Caucasian population with a prevalence of homozygotic expression of five to seven per thousand [29]. Taken this into consideration there is a certain possibility to give iron supplementation on wrong indications to persons who have contraindications for using iron [30, 31]. At the opposite, young female blood donors who are vulnerable to iron deficiency, should be offered iron supplementation. In general, to avoid a possible harm, it is preferential that the iron status of a donor has been normalized prior to a subsequent donation of blood [32]. Increased frequency of donation may cause stress in the donor. Better knowledge of adjusting the frequency of donations both for donors with low iron status and for donors having iron overload will take better care of the donor on an individual basis.

Biological function of iron

Iron is an essential nutrient for all living organisms as all cells need iron for vital biological processes [33]. Erythropoiesis is quantitatively the main consumer of iron. New erythrocytes need about 20-25 mg of iron per day to synthesize hemoglobin molecules [34]. About 1-2 mg of iron is absorbed from the gut, and the rest comes from breakdown of erythrocytes in the macrophages. Iron is involved in oxygen transport by hemoglobin and myoglobin, in electron transport during mitochondrial respiration and ATP synthesis, in DNA synthesis, etc. An adult normally has roughly 4-5 g of iron, depending on body weight and the size of accumulated reserves. Women of reproductive age have lower iron stores than men, due to menstrual blood loss. The body's iron status is balanced remarkably well, by a fine regulatory mechanism at the molecular level for iron absorption in the gut enterocytes. Roughly speaking there is an inverse relationship between the need for iron and the rate of iron absorption. Thus

iron absorption is greater in iron depleted individuals than in those with iron overload [35, 36]. The molecular iron metabolism is complex, and it is beyond the scope of this thesis to describe it in detail.
Hepcidin, a 25 amino-acid peptide secreted into the plasma from the hepatocytes, is probably a key regulator of intestinal iron absorption and also of iron recycling via macrophages [37]. Hepcidin production is up-regulated in iron overload leading to reduced absorption of iron, and down-regulated in iron deficiency, causing increased iron absorption. The liver protein, hemochromatosis protein (HFE), regulates the activation of hepcidin synthesis [35]. Mutations in the HFE gene reduce the synthesis of hepcidin and iron absorption is increased. This is a fundamental mechanism in hereditary hemochromatosis. Measurements of hepcidin in serum may be a future method to assess fluctuations in the body's iron balance.

Bioavailability and absorption of iron

Most iron absorption takes place in the proximal duodenum. Bioavailability of iron is dependent on its chemical form in the food and the presence of other food items that enhance or inhibit the absorption. An average diet may contain 10-20 mg of iron per day, but only 1-2 mg is absorbed [38]. Dietary iron consists of two components, heme iron and non-heme (inorganic) iron. To be absorbed from food, iron must either be in the form of heme or converted to soluble ferrous salts and small molecular iron chelates (ascorbate, citrate, and other organic acids and amino acids). From heme iron, 15-35 % is absorbed [35]. Non-heme iron is found in non-meat sources such as legumes and leafy vegetables, and accounts for approximately 90 % of dietary iron, but only 2-20 % will be absorbed, depending on the iron status of the individual and the ratio of enhancers and inhibitors in the diet [39]. Non-heme iron is absorbed in the ferrous form (Fe^{2+}).

The iron related proteins in plasma

Ferritin, a storage protein of iron, is a globular molecule that can harbor up to 4500 iron atoms within its protein mantel (apoferritin) protecting the cells from the reactive cations [33]. Stored ferritin in the tissues is normally in equilibrium with a small amount of ferritin leaking into the blood stream. In healthy individuals iron stores can indirectly be quantified by measuring serum ferritin (μg/L). Both the macrophages and the hepatocytes have large capacity for iron storage.

Transferrin is a carrier protein for iron in blood plasma. Ferric iron (Fe^{3+}) bound to transferrin is carried via the circulatory network to hematopoietic and other tissues. Transferrin keeps ferric iron soluble and prevents iron from reacting with other molecules by attenuating its redox activity. It aids in delivery of iron to cells by binding to a specific cell-surface transferrin receptor, TfR 1.

Transferrin receptor is present in low concentrations in all cells, but is expressed at high levels by cell types with large needs of iron such as developing erythroid precursors. The higher the iron requirement of the cells, the higher is the density of receptors at the cell membrane. Consequently, the major mass of receptors is contained in hematopoietic bone marrow. A soluble part of the TfR is measurable in serum with levels increasing in the presence of iron deficiency, as well as in conditions with increased erythropoiesis [40]. Synthesis of transferrin receptor is induced by iron deficiency. Synthesis of ferritin increases with high iron levels, while synthesis of TfR 1 is reduced. The opposite occurs in individuals with a low serum ferritin level [35].

The above mentioned proteins are important for diagnosing compromised iron metabolism.

Iron deficiency

As long as iron can be mobilized from body iron stores to compensate for insufficient intake, the iron supply to tissues remains normal [41]. According to severity iron deficiency is divided into three stages [42]. In the first stage, iron depletion, the iron stores are depleted without restricted supply of iron to the tissues, and hemoglobin synthesis is unaffected. In the second stage, iron deficient erythropoiesis, transport and functional compartments are affected. There is a reduced incorporation of iron into Hb, but the Hb and erythrocyte -indices in blood are still within normal range. In the third and most severe stage, iron deficiency anemia, Hb falls below normal, eventually accompanied by symptoms and clinical signs.

Blood donors have an increased risk of iron deficiency when blood is let regularly without replacement therapy [43]. Iron absorption seems to be somewhat more efficient than in comparable non-donors [44], but blood donors generally have lower iron stores and an increased prevalence of depleted iron stores compared to non-donors [45].

Iron overload

The classical iron overload disorder in hemochromatosis is caused by mutations in the HFE gene. This is an autosomal recessive iron-overload disease. The location of the mutations, which was discovered in 1996, is on chromosome 6. In most cases the mutation is a single-base change, a G to A transition at nucleotide 845 (c845G→A). This results in the substitution of tyrosine for cysteine at position 282 of the HFE protein (C282Y) [46]. A second missense variant is a C to G change (c187C→G), resulting in a histidine to aspartic acid substitution at position 63 of the HFE protein (H63D). HFE- gene variants are described as C282Y homozygosity, C282Y heterozygosity, C282Y/H63D compound heterozygosity, H63D heterozygosity and H63D homozygosity [46-48]. The mutation originated by chance in north-western Europe about 2000 years ago. Frequencies of C282Y homozygosity are 0.5 % -0.7 % in Norway and in people of northern European descent [30, 49-51], while frequencies of C282Y heterozygosity and C282Y/H63D compound heterozygosity accumulated are 15 %. Subjects diagnosed as hemochromatotic, accumulate iron from excessive dietary absorption [30]. It is impossible to predict to what extent the mutation will be phenotypically expressed [52]. Adams *et al* [53] studied populations of multiple ethnicities with asymptomatic C282Y homozygosity, and they stated that subjects in these populations do not develop iron overload. Individuals diagnosed with hereditary hemochromatosis (HH) are at increased risk of liver cancer, and other diseases as arthritis/joint pain, impotence, heart-failure and liver cirrhosis [54, 55]. Individuals with iron overload are treated by phlebotomy, and therefore they can be regarded as valuable blood donors [56]. In Norway it is discussed whether or not these subjects could be registered donors. At Haukeland University Hospital there is a local agreement about accepting subjects diagnosed with HH as blood donors after they have been bled to normal serum ferritin concentrations and after complying with the inclusion criteria for blood donors.

The need for new tests to assess iron deficiency

Iron deficiency and consequently iron deficient anemia is detected by measurements of Hb and serum ferritin. Several studies have confirmed that blood donation is associated with a decrease in serum ferritin [20, 21, 38, 57]. However, by comparing serum ferritin and sTfR in frequent donors, Punnonen *et al* [58] found that while 63 % of the women had serum ferritin below the cut-off level for empty iron stores (defined as serum ferritin < 12 μg/L), only 17 %

had pathologically increased sTfR (defined as sTfR > 4 mg/L), indicating iron deficient erythropoiesis. In men the percentages were six and eight, respectively.
The use of sTfR (soluble transferrin receptor) has enabled differentiation between iron deficient erythropoiesis and empty iron stores assessed by analysis of serum ferritin. The diagnostic sensitivity of detecting iron deficiency in anaemic patients was found to be increased by using the TfR-F index which is the ratio between the sTfR and the log of serum ferritin [59]. Increase of sTfR followed by a reduction of CHr are early steps in the development of iron deficient erythropoiesis, while Hb-reduction and increased percent of hypochrome red cells appear later in the process [60]. The lack of clear cut-off values with the lower reference limit for Hb varying between 11.5 and 12.3 g/dL [61] and for serum ferritin between 12 and 27 μg/L [62], has stimulated the search for new markers to improve assessment of the iron status [63]. Also, the use of serum ferritin is hampered by its non-specific response as an inflammatory acute phase reactant [64]. Thus, subjects with borderline values of Hb and serum ferritin would profit by tests which more precisely respond to the transition from iron replete to iron deficient erythropoiesis. In this regard, the potential of new tests such as the Hb-content of reticulocytes (CHr), the fraction of hypochrome red cells (percent hypochrome red cells) and the soluble transferrin receptor in serum (sTfR) are of interest.

The present research

Aims of study

The overall objective was to investigate the iron status in blood donors.
The aims in this study were

- To compare the iron status in new blood donors in 1993-97 and in 2005-06 to study possible changes in iron status
- To study the effects of donating blood without iron supplementation
- To study the effect of iron supplementation during the first week after blood donation
- To describe the distribution of HFE variants and evaluate the effect of blood donation on the iron status in donors with hereditary hemochromatosis

Materials and methods

Design

The design used in each of the four papers is presented in Table 1, including number of subjects (n) in each population, inclusion criteria and interventions.

Table 1 *Study designs, groups of subjects, inclusion criteria and interventions*

Paper	Study design	Subjects	Inclusion criteria	Interventions
Paper I	Retrospective for group one Prospective for group two	Group one: n = 943 (55 % women) Group two: n = 1013 (63 % women)	All new donors, healthy subjects, eligible for donation, but not yet tested for iron status Informed consent	None
Paper II	Longitudinal	Start: 651 women, 375 men Completed: 133 women, 117 men	New blood donor, willingness to donate without iron supplementation for 4 donations in one year. Informed consent	Blood donations without iron supplementation
Paper III	Prospective longitudinal with randomization to groups receiving iron supplementation or not	Start 198 women, 200 men Completed 164 women, 154 men	Established blood donor with at least one earlier donation, serum ferritin > 20µg/L, willingness to donate without or with iron supplementation and to return 6-10 days after donation. Informed consent	Iron supplementation Niferex 100 mg once a day for 10 days (registered as Clinical Trial)
Paper IV	Retrospective	147 donors diagnosed with HH (40 women, 107 men) and comparison group 87 donors (43 women and 44 men)	Accepted as blood donor, diagnosed with HH. Comparison group: Included in study II, donated four times without iron supplementation and HFE wild type Informed consent	None

Hematological and Biochemical methods

Hematological and biochemical methods used to describe iron status in the present study are shown in Table 2, including dates for method-changes. Iron status was assessed by Hb (g/dL) and serum ferritin (µg/L) [42]. Hb was tested by HemoCue B only for group one in Paper I. Hb was later tested by HemoCue 201+ and Advia 120, all venous tests. There was no change in reference interval (women 11.5-15.5 g/dL and men 12.5-16.5 g/dL), but HemoCue 201+ was found to give 0.11 g/dL lower measurements for Hb than HemoCue B [9].

New tests for early detection of iron deficiency were included from 05.06.2005 (Papers I, II

and III). CHr was analysed by Advia 120 with CV 1.5 %. Reference interval for CHr was 31.5 – 35.5 pg for both genders. Percent hypochrome red cells were analysed by Advia 120 with CV 3.7 %, and reference interval 0.1-1.1 % for both genders.

Soluble TfR was analysed by Dade Behring, N latex sTfR method until 29.01.2007, with reference interval 0.84 – 1.54 mg/L for both genders. The analytical method for sTfR was changed 29.01.2007 to Tina Quant Soluble Transferrin Receptor method, with CV 3.5 - 4.5 %. Reference interval was changed to 1.9 - 4.4 mg/L for women and 2.2 - 5.0 mg/L for men (Table 2). Serum ferritin was analysed by MEIA (Abbot) and Bayer Immuno I for group 1 in Paper I. The serum ferritin method was changed by the start of prospective data collection from 05.06.2005 to Modular PP, Tina Quant, with reference interval: Women, pre menopause 10-150 µg/L and post menopause 20-250 µg/L and men 20-250 µg/L. From 29.01.2007 the serum ferritin method was changed to Modular E, including new reference interval: 18-240 µg/L and 34-300 µg/L for women and men respectively.

Table 2 *Biochemical methods and relevant analytical instruments used in Papers I-IV*

	Instrument used from 01.01.1993 to 31.12.1997	Instrument used from 05.06.2005 to 29.01.2007	Instrument used from 29.01.2007
Paper	I	I, II, III, IV	II, III, IV
Hb (venous)	HemoCue B, (HemoCueAB, Angelholm, Sweden)	HemoCue 201+ Advia 120 (Bayer Diagnostics, Tarrytown, NY) Women 11.5-15.5 g/dL Men 12.5-16.5 g/dL	HemoCue 201+ Advia 120
Serum ferritin	Abbot (Abbott Laboratories, Abbott Park, IL, USA), CV 3 % Bayer Immuno 1 (Bayer Diagnostics, Tarrytown, NY), CV 4 %	Modular PP, Tina Quant (F. Hoffman- La Roche Ltd, Basel, Switzerland), CV < 4 % Women, pre menopause 10-150 µg/L post menopause 20-250 µg/L Men, 20-250 µg/L	Modular E (ECLIA, electrochemi-luminescence immunoassay, Roche Diagnostics GmbH, Mannheim, Germany), CV < 4 %. Women18-240 µg/L Men 34-300 µg/L
sTfR	-	Dade Behring (Behring Nephelometer II, Dade Behring Marburg GmbH, Germany) CV 4 % 0.84 – 1.54 mg/L for both genders	Tina Quant Soluble Transferrin Receptor method (ACN 665, Roche Diagnostica, Modular PP, Japan) CV 3.5 - 4.5 % Women 1.9 - 4.4 mg/L Men 2.2 - 5.0 mg/L
CHr	-	Advia 120 CV 1.5 % 31.5 – 35.5 pg for both genders	Advia 120
Percent hypochrome mature red cells	-	Advia 120 CV 3.7 % 0.1-1.1 % for both genders	Advia 120

To exclude inflammation, liver and kidney disease, subjects in Papers I (group 2), II and III were tested for C-reactive protein (CRP), alanine aminotransferase (ALAT), aspartate aminotransferase (ASAT), alcaline phosphatase (ALP), gamma glutamyltransferase (γ-GT) and creatinine, analysed by Modular PP and Modular E. In Paper IV, C-reactive protein (CRP) and alanine aminotransferase (ALAT) were tested at every donation in donors with hemochromatosis to exclude inflammation and liver disease.

Correlations between old and new methods

Adjustments of analytical methods from various time periods were necessary because different methods were carried out with various instruments and reference intervals during the study period. This was the case for Hb and serum ferritin, in study I, for ferritin in study IV, and for sTfR in study II. Equations for adjustments are included in Table 3.

Hb adjustments

From 1993 to 1997 Hb was analysed by the first generation of the HemoCue B-Haemoglobin system [65]. From 2005 Hb was tested by the HemoCue 201+ system [66]. Both instruments were factory-calibrated with blood against the cyanmethaemoglobin (HiCN) standard method recommended by the International Council for Standardization in Haematology (ICSH) [67], and controlled daily with a commercial cyanmethaemoglobin (HiCN) standard solution from Eurotrol [68]. As shown by Bäck *et al* [66], both the correlation and linearity within the relevant Hb-range, between the two HemoCue versions, were excellent with $r = 0.998$ and $y = 0.98x – 0.138$, respectively. It was not possible to perform correlations between the two HemoCue systems, because the old instrument was removed before this study started. The analytical coefficient of variation (CV) for HemoCue 201+ was 0.75 %. On average HemoCue 201+ gave 0.11 g/dL lower results than the HemoCue B-Haemoglobin device. Hb from the period 2005-2006 was adjusted to older Hb values (1993-97) by increasing the values in study I by 0.1 g/dL [13] (Table 3).

Serum ferritin adjustments

The correlation and linearity between the Abbot Imx and the Bayer Immuno 1 methods, were

excellent with r = 0.99 and y = 1.03x – 1.8 in the relevant range. Thus, the two methods were regarded as equal with respect to analytical performance (Table 2 and 3).
The correlation between the methods used in 1993-97 (group 1) and 2005-06 (group 2) was assessed by comparing pair-wise data obtained by the Bayer Immuno 1 method and the Modular PP method. Based on the linear regression, the following formula was derived to adjust the serum ferritin values of group two to the analytical level of group one: lnImmuno 1 = [-0.4 + 1.07*ln (serum ferritin for group two)]. This provided a good model fit throughout the ferritin range, Figure 1.

From 2005, serum ferritin was analyzed by Modular PP, Table 2. In the relevant range with ferritin values up to 100 µg/L, the correlation and linearity between the Modular PP and the Bayer Immuno 1 methods was very good with R^2= 0.974 and y (Modular) = x (Immuno 1) + 3.8. From ferritin values around 150 µg/L up to 500 – 600 µg/L, the correlation points scattered more and the relationship between the two methods changed with Immuno 1 giving increasingly 5 - 14 % higher values than the Modular PP method. To eliminate the influence of methodological bias in comparison of the groups from different time periods (group one from 1993 to 1997 and group two from 2005 to 2006) as described in Paper I, we chose the methods used in the earliest period as reference methods, and recalculated serum ferritin measurements in the latest period. Serum ferritin values < 100 µg/L which included 96 % of the women, and 56 % of the men in the period 2005-2006, were lowered by 3.8 µg/L. In this group, pathological high ferritin values above the inclusion limits (see above), were increased according to the percentage difference between the two methods in the range 150 - 600 µg/L, as noted above. Adjusted values were only used in comparison of the groups.

sTfR adjustments

sTfR results obtained before 29.01.2007 were multiplied by a factor 1.5 (based on calculations from the test laboratory).

Table 3

Equations used for correlation between measurements from old and new analytical instruments

	From instrument	To instrument	Correlation equation	Regression equation after testing paired data (or factor used)
Hb	HemoCue B	HemoCue 201+	r = 0.998 and y = 0.98x – 0.138	Increasing the Hb values from HemoCue 201+ by adding 0.1 g/dL
Hb	HemoCue 201+	Advia 120		None
Serum ferritin	Abbot Imx	Bayer Immuno I	r = 0.99 and y = 1.03x – 1.8 in the relevant range	None
Serum ferritin	Bayer Immuno I	Modular PP, Tina Quant	r= 0.987 and y = x + 3.8. y = modular x = Immuno I	Exp [-0.401 + 1.073·ln (ferritin by Modular PP)] This formula was used to equalize the ferritin measurements in group two (2005-06), to those in group one (1993-97)
Serum ferritin	Modular PP, Tina Quant	Modular E		Equation for transforming ferritin values by Modular PP to values comparable to measurements by Modular E: ferritin by modular E = exp [-0.004 + 1.069·ln (ferritin by Modular PP)], where exp is the exponential function.
sTfR	Dade Behring, N latex sTfR method	Tina Quant Soluble Transferrin Receptor method		sTfR results obtained before January 29th 2007 were multiplied by a factor 1.5 (based on calculations from the test laboratory)

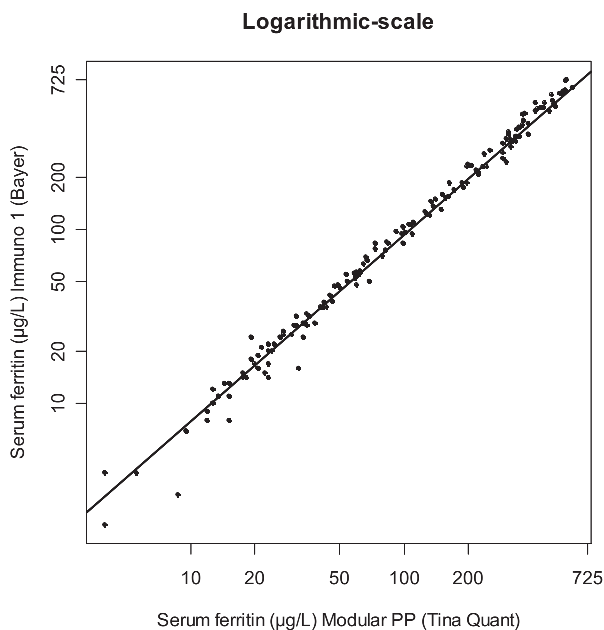

Figure 1 Regression line for pair-wise data-set, serum ferritin

Genetic screening methods

In Paper III genetic screening for the classical HFE-mutations (C282Y and H63D) of hereditary hemochromatosis was performed for all subjects who returned after 8 ± 2 days. The analysis was done at HUNT bio bank, Levanger, Norway, by Qiagen kit, where DNA was purified using QIAamp DNA Blood Mini Kit (QIAGEN, Düsseldorf, Germany)[69]. Genotyping of the two SNPs H63D / rs1799945 and C282Y / rs1800562, were performed by 7900HT Fast Real-Time PCR (Polymerase chain reaction) System using TaqMan® SNP Genotyping Assays (Applied Biosystems, Life Technologies Corporation, Carlsbad, CA, USA)[70] as recommended by the manufacturer.

In Paper II and IV, genetic screening for the classical HFE-mutations (C282Y and H63D) of hereditary hemochromatosis was performed at Centre for medical genetics and molecular medicine, Haukeland University Hospital, Bergen, from 01.11.1997 to 28.05.2008. DNA was purified using methods from Applied Biosystems from 01.11.1997 to 01.09.2002 [70], and Qiagen from 01.09.2002-28.05.2008 [69]. Genotyping of the H63D and C282Y variants were

performed by PCR, using restriction enzymes on agar electrophoresis from 01.11.1997 to 31.12.2002. After this period the genotyping was performed by using PCR methods from Applied Biosystems, all methods were performed as recommended by the manufacturer.

Statistical methods

Serum ferritin was log transformed because of skewness, and presented as geometric mean. To be able to compare methods from different periods of time, regression analysis between paired data was performed in study I, II and IV as described in the segment about biochemical tests, and shown in Table 3. Two sample t-tests were used for data without a marked skewness or after a log transformation. Exact Mann Whitney tests were used elsewhere. Exact Chi-square tests were used for the comparison of dichotomous or nominal variables between groups. Pearson correlation coefficients were calculated for bivariate associations of Hb and serum ferritin with the other blood tests in study I. To discover a difference of at least 0.2 g/dL for Hb, power calculation was based on a SD = 0.8 g/dL. For serum ferritin, power calculation based on SD = 38 µg/L for women and 56 µg/L for men discovered a change of at least 7 µg/L and 20 µg/L for women and men, respectively. To obtain a 5 % significance level and 80 % power, it was required a minimum of 253 subjects of each gender for Hb, and a minimum of 464 women and 125 men for serum ferritin, in each group [71], Paper I.
In study II the time course of Hb and log-transformed serum ferritin from first to last donation was modelled by linear mixed effects models. We adjusted for baseline values, and also used age and the TfR-F index as baseline covariates. In addition the number of donations and time since last donation were within-subject covariates. Interactions of the number of donations with time since the previous donation and the TfR-F index were included. The models were estimated separately for each gender.
In Study III we performed univariate and multivariate analyses for iron status variables for each gender separately. To compare changes in dependent variables (Hb, serum ferritin, sTfR, CHr and percent hypochrome mature red blood cells) between groups, each final iron status variable in the two groups was compared by regression analyses adjusted for baseline value and number of days after donations. Also, within the iron supplementation group, pre and post values were compared by paired sample t tests (except for percent hypochrome red blood cells where a paired sample Wilcoxon test was used). To discover a difference of at least 0.3 g/dL for Hb, power calculation was based on SD = 0.8 g/dL. For serum ferritin, power calculation based on SD = 38 µg/L for women and 56 µg/L for men, discovered a change of at least 7

μg/L and 20 μg/L for women and men, respectively. Calculations, based on two-sided t-tests for two samples with a 5 % level and 80 % power, required a minimum of 71 subjects of each gender within each group of donors. To explore possible different effects of iron supplementation dependent on HFE mutations or not, the regression analyses above were repeated with an interaction between randomisation group and HFE variant. Reappearance of 164 women and 154 men was acceptable. Required significance was set at 0.05.

In study IV, data were gathered from archives from 1997 to 2008, and included several time-specific biochemical analytical methods, and factorisation was used to equalise the values as described above. For descriptive comparisons the geometric mean and range were reported for serum ferritin levels and ratios. The serum ferritin ratio between the two last donations was compared between three HFE gene variant groups for men, and also between two HFE gene variant - groups for women, by linear regression for log transformed ratios with adjustment for days between the two last donations and age at the last donation. Similar comparisons were made for serum ferritin values at the last two donations, and for the mutated HFE groups also at the first two donations. Age and donations per year were computed by t tests and ANOVA.

SPSS 15-17 (SPSS Inc., Chicago IL, USA) and R (The R Foundation for Statistical Computing, Vienna, Austria) was used for the data-analysis, with the R package "nlme" for the mixed effects models (nlme = nonlinear and linear mixed effects model) [72, 73].

Ethical concerns

Informed consent was obtained from all participants in all studies (Appendix 5). The study was accepted by Regional Medical Research Ethics Committee of Western Norway. To let the donors donate without offering any iron supplementation, was one of the important ethical concerns (Papers II and III). We informed the participants that the study was supervised by two medical specialists. Donors with iron deficiency anemia would be transferred to iron-treatment group. Criteria for transferring subjects to treatment group was Hb < 12.5 and < 13.5 g/dL for female and male donors respectively. These subjects could continue participating in the study, but time of transferring, treatment-description, dose and interval were noted. In study III they were still analyzed in the group they were randomized to. All subjects could withdraw from participating at any time. Another ethical concern was to offer iron supplementation to donors with risk of increasing their iron overload. The HFE status and serum ferritin at the time of inclusion were unknown (Paper III). Admission to donate is based

on medical history and earlier tests for Hb and serum ferritin. Hb-measurements (HemoCue 201+) are performed at the donation site, to inform the donor of their Hb-status. We considered the ethical concerns to be less prominent than the importance of studying effects of donation with or without iron supplementation.

Summary of papers I-IV

Paper I

Iron status in Norwegian blood donors: Comparison of iron status in new blood donors registered in 1993-1997 and in 2005 - 2006

We experienced increased deferral of new donors due to Hb below inclusion limit. The purpose of the study was to find an answer to why this deferral increased. The aim was to describe changes in iron status in newly recruited prospective donors in two time periods, a decade apart, and compare the groups: group one from 1993-97 and group two from 2005-06. We hypothesized that a possible deterioration of iron status could explain the increase in rejection of new donors.

In women, there was a small but statistically significant reduction of the mean Hb in women from 13.2 g/dL in group one (1993-97) to 13.1 g/dL in group two (2005-2006) ($P = 0.040$, Table 4). For women, both in young (18-30 years) and older (31-45 years) new donors there was a shift towards lower Hb-values in group two (Paper I). In group two, 2.2 % of the women versus 0.6 % in group one, were anaemic with Hb < 11.5 g/dL, while 19.5 % of the women in group two versus 13.2 % in group one had $11.5 \leq$ Hb < 12.5 g/dL ($P = 0.001$ for age 18-30 years and 0.043 for age 31-45 years, chi-square tests in subgroups), i. e. below the threshold for blood donors. There were no significant differences in Hb between the two age groups in women in group one or two.

In women there was a highly significant reduction in the geometric mean of serum ferritin from 30.9 µg/L in group one to 26.9 µg/L in group two ($P = 0.001$, Table 4).

When comparing group two versus group one, 20.7 % versus 13.2 % of the women had empty iron stores defined as serum ferritin < 15 µg/L, while 34.1 % versus 36.9 % had small iron stores defined as $15 \leq$ serum ferritin ≤ 30 µg/L ($P < 0.001$ for age 18-30 years and 0.456 for age 31-45 years, chi square tests in subgroups). The shift of serum ferritin towards lower values in group two was most obvious in the young women between 18 and 30 years of age (Paper I). In men there were no significant changes in Hb or serum ferritin concentrations.

Table 4

Comparison of Hb (g/dL) and serum ferritin (µg/L) between new donors registered in 1993-97 and 2005-06

	Women				Men			
	Group 1[a] n = 521 Mean(SD) (min-max)	Group 2[b] n = 641 Mean(SD) (min-max)	Diff. (CI)[d]	p[c]	Group 1[a] n = 422 Mean(SD) (min-max)	Group 2[b] n = 372 Mean(SD) (min-max)	Diff. (CI)[d]	p[c]
Hb	13.2 (0.7) (10.6-15.6)	13.1 (0.8) (10.8-16.1)	0.1 (0,0.2)	0.040	14.8 (0.8) (12.9-17.1)	15.0 (0.9) (12.1-17.7)	-0.2 (-0.3,-0.1)	0.007
	Group 1 n = 521 Geometric mean (min-max)	Group 2 n = 628 Geometric mean (min-max)	Diff (CI)[f]	p[e]	Group 1 n = 422 Geometric mean (min-max)	Group 2 n = 369 Geometric mean (min-max)	Diff (CI)[f]	p[e]
Serum ferritin	31 (1-357)	27 (3-220)	4.0 1.7, 6.3	0.001	95 (16-390)	87 (10-784)	7.3 -0.5, 14.7	0.067

[a] Group 1 consists of retrospectively gathered data from archives in period 1, 1993-1997
[b] Group 2 consists of prospectively gathered data in period 2, from June 2005 to June 2006
[c] t-tests
[d] Difference between group means, with 95 % confidence intervals
[e] t-tests for log transformed variable
[f] Bootstrap BCa confidence intervals for differences between geometric means

The rejection rates of new donors in the two periods was 23.7 % in group two (2005-06) compared to 13.8 % in group one (P < 0.001) for women, Table 5.

Table 5

Rejection [a] of newly recruited women who failed to meet the donor inclusion criteria for Hb. Number (n) and percentage (%) of rejected donors in group 1 and 2, and P value for differences between groups (Paper I, Table 2).

	Women		
Group	1	2	P
n, total	521	641	
n, rejection code 1	72 (13.8 %)	152 (23.7 %)	< 0.001

[a] Rejection code 1 Hb < 12.5 g/dL for women

In sum, we found that the Hb was decreased by 0.1 g/dL and serum ferritin was decreased by 4 μg/L for women, both significant changes. This corresponded to the significantly increased new donor-rejection, from 14 % to 24 % for women. New donors' age range was 18-45 years and the differences in iron status in these age groups were of interest, and presented in Figures 1-4 in Paper I.

Paper II

The effect of blood donation frequency on iron status

The aim for this study was to develop a model that made it possible to predict safe donation intervals. The model described newly recruited donors at each of four subsequent donations during one year, without iron supplementation, and provided information about the interaction between frequency of donation and iron status. The model adjusted for time since previous donation, age and baseline values. Coefficients and P values for interactions from this model are presented in Paper II, Table 1. The changes of Hb and serum ferritin for the first four donations are shown in Figures 2 and 3. The main results were significant interactions between the number of donations and time since the previous donation on Hb ($P = 0.027$) and serum ferritin ($P < 0.001$), for women. Baseline age was positively related to serum ferritin ($P < 0.001$), but not to Hb ($P = 0.056$), for women. For men there was significant interaction between the number of donations and time since the previous donation for serum ferritin ($P < 0.001$).

Hb and serum ferritin decreased with number of donations for women and serum ferritin decreased with number of donations for men. This decrease was less prominent for longer intervals between donations, particularly between the third and fourth donations.

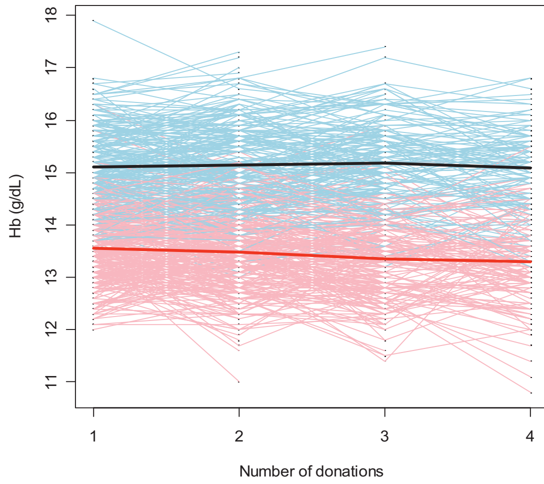

Figure 2 Hb (g/dL) changes for both genders at four donations without iron supplement (red = women, blue = men).

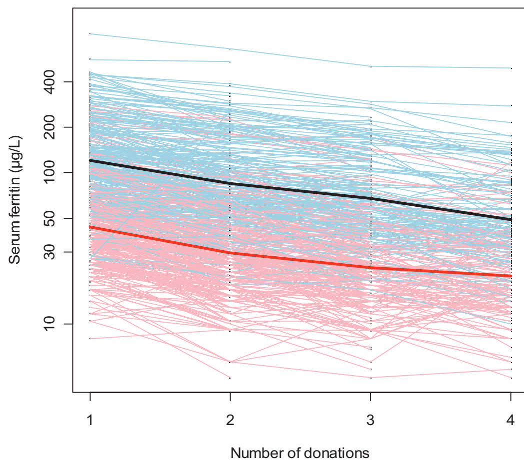

Figure 3 Serum ferritin (µg/L) changes for both genders at four donations without iron supplement (red = women, blue = men).

Paper III

Effect of iron supplementation on iron status during the first week after blood donation

At Haukeland University Hospital the routine is to offer blood donors 100 mg iron supplementation (Niferex ferroglycin sulphate complex, Niferex Schwartz Pharma, UCB group, Brüssel, Belgium), to be taken once a day for 10 days. The aim of this study was to compare the short time change in the iron status of established donors randomized to iron supplementation or no iron after a donation of one unit of blood (463 ml). A second objective was to evaluate the effect of iron supplementation in donors with the classical HFE- gene of hereditary hemochromatosis, compared to a HFE wild type control group.
Groups of both genders (199 women and 200 men), randomized to iron supplementation or no additional iron, were compared. Return was at 8 ± 2 days after donation, and reappearance of 164 women and 154 men was acceptable.
Iron supplementation did have a beneficial effect on iron status, one week after donation. Adjusted for baseline values there was a significant between group-difference in all iron status parameters tested, for both genders, generally with less favourable results in the group without iron supplementation. CHr seemed to be a useful test to control that the erythropoietic activity is responding normally about one week after a blood donation. Knowledge of the pre-donation serum ferritin value is important to decide if a donor should be offered iron supplementation or not after the blood donation. The findings supported our suggestions, that donors with a serum ferritin below 50 μg/L should be offered iron, while donors with a serum ferritin above 80 μg/L do not need extra iron. Donors in the interval between these limits should be treated individually.
The short time is regarded to give a realistic picture of the “real donor situation” as many donors do not comply with the recommended iron supplementation regime.
There was significant difference between baseline and end values in both randomized groups for all parameters, except for CHr. This parameter was not significantly changed from baseline in the group receiving iron, for both genders.
In subgroups with baseline serum ferritin < 50 μg/L and given iron, the fall in serum ferritin after 8 ± 2 days was 0 % for women and 13 % for men.
In subgroups with baseline serum ferritin < 50 μg/L there was a significant difference in serum ferritin reduction between the randomized groups (P < 0.001 for both genders).

The interactions between randomization group and the HFE status (except C292Y homozygosity) were insignificant, except for CHr (P = 0.010) in women. Thus in women with HFE variant the increase in CHr was higher (P < 0.001) in women with, than in women without, iron supplementation. High serum ferritin was not associated with any of the present HFE variants. Regrettably C282Y homozygosity was not found.

Paper IV

Blood donors with hereditary hemochromatosis

In the present study we aimed to describe the distribution of the classical HFE genotype in a cohort of donors with phenotypically expressed hemochromatosis. A second aim was to evaluate if the abnormal HFE gene variants influenced the change in iron status expressed as change in serum ferritin between the two last donations.

All subjects were earlier diagnosed as having hemochromatosis based on genetically and biochemical criteria. They were first bled therapeutically to a serum ferritin about 50 µg/L before starting as regular blood donors. Retrospective data from 147 donors (40 women and 106 men) registered in "hemochromatosis-blood donor-archives" at the blood bank at Haukeland University Hospital in Bergen were collected from their two last donations after informed consent was given. Mean number of donations per year was 3.3 for women and 3.7 for men. Donors in general, not tested for HFE, donate on average 2.6 and 2.9 times per year for women and men respectively [13]. The distributions of HFE variants, age and donations per year are presented in Paper IV, Table 1, including descriptions of risk groups defined after supposed risk for increased iron absorption.

The blood donors with hereditary hemochromatosis were compared to HFE wild types. This comparison group of new donors (43 women and 44 men) had donated four times in one year without iron supplementation (study II). Donors with HFE wild types had significantly lower serum ferritin concentrations overall than donors with HFE-variants for women, but not for men (Paper IV, Table 2). The overall difference in serum ferritin ratio between HFE gene variant group and the HFE wild types group was significant for men ($P < 0.001$), but not for women ($P = 0.676$), Table 6.

Blood donors with hereditary hemochromatosis sustained the donations well. No seroconversions (HIV, HBV, HCV) were found in the study period. The hemochromatosis-donors were not different from regular donors, and augmented the blood supply by higher donation-frequency than regular donors.

Table 6

Ferritin ratio (geometric mean and range) at two consecutive donations (T1 and T2) in donors with hemochromatosis and in HFE wild type donors.

Risk groups [a]	n	Ferritin ratio[b]	P value [c]	P value [d]
Women				
3	34	0.91 (0.07 – 1.89)	0.627	
1+2	6	1.00 (0.38 – 1.62)		
1+2+3	40	0.93 (0.07 – 1.89)		0.676
HFE wild type	43	0.83 (0.30 – 1.77)		
Men				
3	63	1.00 (0.08 – 5.09)	0.684	
2	13	1.05 (0.73 – 1.63)		
1	31	1.04 (0.40 – 2.92)		
1+2+3	107	1.02 (0.08 – 5.09)		< 0.001
HFE wild type	44	0.74 (0.26 – 1.56)		

[a] Risk group, defined after supposed risk for increased iron absorption

1 = low risk, C282Y or H63D heterozygosity
2 = medium risk, compound heterozygosity or H63D homozygosity
3 = high Risk, C282Y homozygosity

[b] Ferritin ratio is the ferritin concentration at T2 (last donation) divided by the ferritin concentration at T1 (next to last donation) within groups.

[c] P value: Comparison within the HFE gene variants subgroups by linear regression for log transformed ratio, adjusted for days between donations and age.

[d] P value: Comparison between the hemochromatosis group and the HFE wild type group by linear regression for the log transformed ferritin ratio, adjusted for days between donations and age.

Short summary of results

1. Iron status deteriorated with a 0.1 g/dL Hb-fall (P = 0.040) and a decrease in serum ferritin from 30.9 µg/L to 26.9 µg/L (P = 0.001) in new donors (women) from 1993-97 to 2005-06. This reflected an increased rejection of new donors (women) in the same period. For men the iron status did not deteriorate in this period.

2. The mixed effect model, developed to decide about safe donation intervals, showed that for women there was a significant interaction between the number of donations and time since the previous donation on Hb and serum ferritin for women. Baseline age was positively related to serum ferritin, but not to Hb for women. For men there was a significant interaction between the number of donations and time since the previous donation for serum ferritin, but not for Hb.

3. Iron supplementation the first week after donation had a beneficial effect for both genders. CHr seems to be a useful test and shows that erythropoietic activity is responding normally about one week after donation. Donors with serum ferritin below 50 µg/L should be offered iron supplementation, while donors with serum ferritin between 50 and 80 µg/L should be offered iron supplementation on individual basis. Donors having serum ferritin above 80 µg/L do not need iron supplementation. HFE heterozygosity variants did not differ on iron supplementation effect.

4. Differences in geometric mean of serum ferritin were not statistically significant for women or men between HFE groups. The geometric mean of serum ferritin was significantly higher in the HFE variant-group than for wild types for women but not for men. The serum ferritin ratio (ferritin at T2 divided by ferritin at T1) was not significantly different between the HFE variant groups for any gender. The change in serum ferritin was significantly larger for wild types than for HFE variants for men, but not for women.

Discussion

Aggravation of iron status

This study suggested that young women aged 18-31 are the most vulnerable to negative effects of blood donation, while men are not affected. Small iron stores are common in fertile, healthy women in industrialized countries, and iron deficiency is the leading cause of anaemia [74, 75]. Our finding of a negative trend in iron status in women is supported by evidence from other studies that the iron status of young people is compromised by the modern lifestyle in industrialized countries [74, 76]. The increased rejection of new donors through the last decade corresponds to the aggravated iron status in women. Reasons for the decrease in Hb and serum ferritin in women could be change of diets over time and a more sedentary daily life style [77, 78]. Change from oral contraceptives with a positive influence on iron status to medication with no influence on iron may also play a role [79, 80].

The lower serum ferritin in women, being around 30 μg/L and on average only about one third of the concentration in men suggests that the female population is permanently at the brink of iron deficiency. Thus there is ample evidence to assume that in some subjects the iron stores may be empty already with serum ferritin at 30-35 μg/L or even higher values. The evidence is most convincing for women where iron deficient erythropoiesis may be found when serum ferritin concentration is around 30 μg/L [62, 64].

Tests for detection of early signs of iron deficiency

Measurements of Hb and serum ferritin have been described in other studies as not sufficient to detect iron deficiency in blood donors [58, 81]. The reasons are the wide reference interval for both tests and the increase of serum ferritin caused by acute phase reactions as inflammation.

Given the inaccuracy of Hb and serum ferritin to detect early signs for iron deficiency and the transition to iron deficient erythropoiesis, it was of interest to examine the diagnostic potential of alternative iron status markers [60]. In connection with blood donations, it is of importance to describe the situation in subjects with Hb concentration around the lower acceptance limit. The question about iron deficient erythropoiesis particularly concerns women with Hb around the donor cut-off limit of 12.5 g/dL. In women in the “grey zone interval” with Hb 11.5 - 12.4 g/dL, the mean values of sTfR and CHr were within their respective reference intervals and

significantly different from the corresponding, pathological mean values in anaemic women with Hb < 11.5 g/dL (Paper I). On the other hand, in women with signs of iron deficient erythropoiesis expressed by either pathological sTfR or CHr, these tests correlated significantly to pathologically low Hb (Paper I). Thus, the results in Paper I indicate that either sTfR or CHr may be valuable supplementary tests in women with borderline values of Hb and serum ferritin, which is often the case when recruiting new young female donors. Elevation of sTfR is a sensitive indicator of iron deficient erythropoiesis, while fall in serum ferritin is a marker of iron deficiency and depleted iron stores [58]. Combined in the TfR-F index (sTfR / log of serum ferritin), these indicators have been suggested to increase the diagnostic sensitivity of iron deficiency with or without concomitant anaemia [59, 82, 83]. Boulton *et al* [31], stated that the TfR-F index could not predict the individuals who will develop iron deficiency after blood donation, while our studies showed that higher values of the basic TfR-F index made women more vulnerable to the negative effect of subsequent donations (Paper II).

Effect of frequency of donations on iron status

As the pressure on regular blood donors increases, it is necessary to establish a donation regimen that prevents the donors from acquiring health problems related to iron deficiency and anaemia. Simultaneously, an adequate blood supply must be ensured. In the EU Blood directive, Hb is the only test required to assess the iron status of blood donors [7]. Our model in Paper II showed that in female donors, prolonged intervals between donations may prevent decrease in Hb, whereas shorter donation intervals may be acceptable for selected male donors (Paper II). Serum ferritin is used by some blood banks as an additional marker of the iron status [9]. If serum ferritin at any time is below the commonly used cut-off level of 18 μg/L, this indicates empty iron stores and the donor may be deferred or given iron supplementation. Alternatively, the time intervals between donations may be prolonged. The model presented in Paper II, underlines the need to adjust blood donation frequency based on Hb and serum ferritin data. This is especially important if iron supplementation is not considered. Our findings (Paper II) are supported by other studies, but some investigators have used red cells indices or the TfR-F index to predict and evaluate iron deficiency in blood donors [18, 58]. Contrary to the study of Milman *et al* [19] where the serum ferritin level was decreased from the first to the second donation and then stabilized, we found a continuous fall in serum ferritin concentrations from the first to the fourth donation. The fact that the

prevalence of iron depletion is higher in menstruating women than after menopause [19, 84], further underlines the option to adjust donation intervals based on the iron status of the female donor.

Our model for the effect of donation intervals in donors not receiving iron supplementation used baseline covariates (Hb and serum ferritin) to predict results from subsequent measurements of Hb and serum ferritin. Since it comprises only four consecutive donations, the model is not useful to extrapolate for further blood donations. Nevertheless, the model in Paper II shows effects of independent variables (number of donations, time interval between donations and baseline age, Hb, serum ferritin and sTfR) on dependent variables (Hb and serum ferritin at each donation). Thus, the model points to factors important for donation surveillance. This model may be helpful and further developed to avoid unnecessary loss of donors and to design iron supplementation protocols for blood donors.

The effect of iron supplementation

The relationship between iron status and the need for iron supplementation in repeat blood donors is still controversial. Although it is not without problems to advocate use of iron medication to healthy individuals in order to make them able to donate, iron supplementation facilitates blood donations [23, 38, 57]. In Paper III the observation period after a blood donation was only about one week. In the present study, the short period was sufficient to find a significant change from baseline values in serum ferritin and sTfR like the changes seen in individuals during developing iron deficiency, as is also shown by others [22, 38]. The biochemical change in iron status was present whether or not the subjects were supplemented with iron although, as expected, the negative influence was less in those receiving iron. Compared to baseline value, the CHr, which is a rapid responder [60] of iron deficient erythropoiesis, was reduced in subjects without iron supplementation. In contrast CHr tended to increase in subjects receiving iron. In this regard, change in CHr concentration is a good marker to judge if the iron supplementation is sufficient to maintain hemoglobin synthesis, which is the ultimate point of the physiological process to replace blood loss. Thus we may conclude that iron supplementation did not completely prevent aggravation of iron status after donation, but certainly counteracted it by maintaining an almost normal erythropoiesis the first week after a blood donation. The failure of a large, daily dose of 100 mg iron to completely prevent a negative impact of blood donation on the iron status is explained by

physiological limits to absorption of iron from the gut and the inability even in iron deficiency, to speed up the rate of erythropoiesis by increasing the intake of iron above normal [57, 85, 86].

Interestingly, in subjects supplied with iron, we found an association between the baseline value of serum ferritin and its percentage reduction about one week after a blood donation (Paper III). The reduction in serum ferritin with baseline values above 80 µg/L was at the same level independent of whether iron was supplied or not. At this level the iron stores of the body are large enough to supply the iron needed to restore at least a fall in hemoglobin of 1.0 g/dL [86]. On the other hand, serum ferritin below 50 µg/L denotes small or empty iron stores [31, 75]. When the iron stores are small or empty it is compensated by an increasing power of the body to absorb supplied iron. This explanation is supported by the finding that in subjects with baseline value below 50 µg/L, the percentage reduction in serum ferritin was at the same level as in subjects with baseline serum ferritin above 80 µg/L, in subjects not receiving iron supplementation (Paper III). In this situation supplied iron is preferred, perhaps because it is more readily available than the endogenous residual iron which also most likely is too scanty to cover the extra need for hemoglobin synthesis. Our explanation is in line with present physiological knowledge about the relation between size of iron stores and iron absorption [86]. However, more studies are needed to confirm our results.

Iron supplementation is offered to blood donors in Norway, but this is not a routine procedure in many other countries [87]. Supplying donors with iron may be a controversial ethical issue because of the risk of inducing or worsen pathological iron overload in donors with unknown hemochromatosis [38]. For this reason it has been suggested to screen donors for the HFE-variants to identify those who could be harmed by intake of extra iron [88]. However, the penetrance of the HFE - variants is highly variable and the variants themselves are not a guarantee for generating pathological iron overload [53]. Therefore the cost-benefit of HFE-screening is indeed questionable. There was no different effect of iron supplementation on serum ferritin in donors with wild type alleles and the included HFE – variants (C282Y and H63D heterozygosity, C282Y/H63D compound heterozygosity and H63D homozygosity, no C282Y homozygosity was found in the included subjects, Paper III). However, the positive effect of iron supplementation on CHr was more pronounced in women with HFE - variants than in women with the wild type gene. The present study indicates, in line with suggestions from others [56], that it may be most correct to treat persons with HFE - variants, other than

C282Y homozygosity (who regrettably were not included in this study), as regular blood donors.

Hereditary hemochromatosis and blood donation

The relationship between donors with genetic HFE variants (C282Y or H63D homozygosity or heterozygosity and compound C282Y/H63D heterozygosity) and regular blood donors is described in Paper IV. Careful donor selection is a major precaution to obtain safe blood. There are arguments pro et contra using subjects with hereditary hemochromatosis as blood donors. The blood donors are altruistic, and as blood donors with increased iron stores have a positive health gain from removing excess body iron, they have been regarded as unsuitable for blood donations [56]. A major breakthrough for accepting donors with hemochromatosis was when such donations were approved by FDA [89]. Arguments in favour of this view had been provided by several other groups [90, 91] and the useful, though rather minor contributions from these donors were confirmed in later publications [56, 92, 93]. It is shown in US- studies that these donors represent the same risk for transmitting diseases as others. Some countries use this donor group also in the phlebotomy-therapy-period [56]. Donors with increased serum ferritin can be treated as ordinary donors independent of HFE-genotype. As Norwegian blood donors are not screened for HFE variants, there certainly may be donors who donate without knowing that they have a HFE variant.

As known from several studies [30, 94] the HFE variants C282Y homozygosity and C282Y/H63D (compound heterozygosity) are linked to increased iron stores. It should be emphasized that the heterozygote HFE gene variants were as effective as the homozygote C282Y to counteract a fall in serum ferritin between two donations in our study. Normally, loss of blood stimulates uptake of endogenous iron to compensate for the loss of iron [32-36]. Our results indicate that this process is not influenced by the different variants of the HFE genotype when the donors are selected on basis of a high initial serum ferritin concentration. Both the experience from Leitman's paper [56] and the Sanchez report [95] indicated that it should not be necessary to discard blood obtained during the period of therapeutic iron depletion. It may even be wise to prolong the iron depletion phase as the risk of organ damage due to iron overload is minimal as long as the serum ferritin level is below 500-600 µg/L [96, 97].

According to guidelines it is a goal to keep the serum ferritin concentration in hereditary hemochromatosis subjects around 50 μg/L. In several subjects the serum ferritin concentration was far above the desired concentration (50 μg/L) after the therapeutic phlebotomy phase. This is partly due to delay of phlebotomy after donor acceptance, but is also partly indicating that the intended therapeutic program was not fulfilled or completely well organized.

Limitations of this thesis

A valid cut-off limit for Hb is necessary for safe enrolment of new donors. However, as has been shown in this study, one should be careful to use generally recommended cut-off values irrespectively of the quality of the analytical device. Methodological bias complicates comparison of Hb measurements between different instruments. To eliminate a bias effect when comparing results from different instruments that cannot be calibrated by the user, like the widely used HemoCue system, is either to factorize the results from one of the instruments as we did or to use different cut-off limits. When comparing the rejection rates, we used the real Hb- and serum ferritin values obtained in each group. Some of the increased rejection of women in group two can simply be explained by the analytical bias between the HemoCue instruments. However, this correction for differences in Hb measurements did not change the fact that the rejection of women was significantly higher in 2005-06 than in 1993-97.

A double blinded design in Paper III would have given information from subjects not knowing the content of the received supplementation. The production of placebo iron supplementation was too expensive for our budget and beyond our possibilities due to the costs.

The small number of subjects in sub-categories of baseline values of serum ferritin and in groups with HFE gene variants was a third limitation. A larger number in our subgroups would have stabilised the results in Papers III and IV. In Paper IV, we aggregated different HFE gene variants in line with known clinical effects of the gene variants. If the study group had been larger, such aggregation would be unnecessary.

The follow up time after donation was only 8 ± 2 days in study III. The short observation time did not allow conclusions about the time required to obtain full restitution of iron status to baseline values, which would have been of great interest also.

Clinical consequences

As the majority of new blood donors are women, the results presented in Paper I represent a challenge to the blood banks. To avoid donor insufficiency, a strategy to deal with new donors appearing with low Hb and low serum ferritin is now implemented.

Calculated from baseline measurements of Hb and serum ferritin, our blood donation interval model may be used to determine blood donation intervals in order to prevent anaemia and/or iron deficiency in donors not receiving iron supplementation. We suggest that donors with serum ferritin between 18 and 30 μg/L should donate maximum two times per year. Established donors falling below serum ferritin inclusion limits can donate plasma. New donors with relatively low iron status (Hb < 12.8 g/dL for women/13.8 g/dL for men or serum ferritin < 25 μg/L) can donate this way, and avoid rejection.

Secondly, we observed the need for a better administration of iron supplementation to blood donors according to their serum ferritin values. Measurements of serum ferritin once a year is needed to supervise the iron supplementation. Donors with pre donation serum ferritin below 50 μg/L should be offered iron after donation, while donors with serum ferritin above 80 μg/L do not need additional iron. Donors with serum ferritin between these limits must be judged individually. Measurements of serum ferritin regularly can detect iron overload. If iron overload is present, it will be considered malpractice to provide iron supplementation. Paper III further underlines the need for a more adjusted regimen for iron supplementation.

Subjects with hereditary hemochromatosis may be eligible as blood donors if they are otherwise accepted according to inclusion criteria. These subjects may safely be advised to donate blood four times per year and sometimes even more often, depending on their actual iron status. In Paper IV, our results support the idea that subjects with high serum ferritin concentration and HFE gene variant may be useful donors. However, as some of these donors may develop organ damage if the phlebotomy program is not complied with, there must be a system to identify subjects who do not show up for regular donations. Our data showed that more strict regimes for follow-up are needed regarding subjects with HFE gene variants. In order to reduce the risk of iron overload in donors with HFE gene variations, a follow-up program picking up “drop-outs” is considered to be necessary. We have implemented a system with yearly control of all “hemochromatosis blood donors”.

Conclusions

- During the last decade the prevalence of young healthy Norwegian women with depleted iron stores has increased. The decrease in iron status in young women explains the increased rejection rates of women who volunteer to serve as blood donors. The young women aged 18-31 were most vulnerable to low iron status and following risk for rejection as blood donor. There is a need for continuous recruitment to blood donation among subjects of all ages (18-65). The study revealed the need for more knowledge about the effect of donation interval, serum ferritin measurements and iron supplementation, differentiated to gender and age.

- In healthy Norwegian blood donors, repeated donations without iron supplementation led to significant reduction in Hb in female donors only, and decreased serum ferritin concentration in both female and male donors. Increased interval between donations will be of benefit to female donors of childbearing age.

- Knowledge of the pre-donation serum ferritin value is decisive for iron supplementation after the blood donation. Iron supplementation had a significant impact on the restoration of iron status during the first week after donation.

- Subjects with hemochromatosis represent a valuable and safe contribution to the blood donor corps independent of their C282Y and H63D genotype. In general, the ferritin level tended to be higher in those with hemochromatosis than in wild types, especially in women. The negative influence of a blood donation on iron status, tended to be less in those with hemochromatosis than in wild types, especially in men.

Future studies

Longitudinal studies are needed to improve knowledge of the time required to obtain full restitution to baseline iron status values after blood donation. This knowledge is needed to design a safe donation regime with optimal donation intervals for the individual donor, and for improving the iron supplementation protocol. More long-term studies are necessary to clarify the relationship between HFE- variants and regular blood donation.

By using a double blinded design, including placebo for iron supplementation and blinded to the researcher, the findings would be more realistic than in the present study. We suggest a repeat of study III, with double blinded design.

Including erythrocyte-apheresis as donation method would have given a great possibility to compare effects of such donation to the 450 mL standard donation on iron status. This was not possible due to very few apheresis donations at the site in our study-period, 2006-2008.

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