The role of genes and lifestyle behaviors in iron and erythrocyte parameters in blood donors

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Chapter 1

General introduction
Iron homeostasis is tightly regulated in the human body, as iron is necessary for the formation of functional hemoglobin (Hb) and is thereby involved in the transport of oxygen throughout the body and most of the carbon dioxide back to the lungs, but free iron is toxic.1, 2 Because of its toxicity, iron is bound to the iron-transport protein transferrin when it is transported through the body.1 Hb is a protein found in erythrocytes and it consists of four globin protein subunits, each binding a heme group.3 These heme groups all contain an iron atom, which can bind either oxygen or carbon dioxide, giving Hb its oxygen-binding capacity.3 Low iron availability could be a limiting factor for the production of functional Hb. Under normal circumstances, systemic iron homeostasis is maintained by (1) recycling iron from degraded erythrocytes, (2) absorbing iron from the diet by the intestines and (3) mobilizing iron from iron stores when necessary.1, 4, 5 Ferritin levels represent iron stores and low levels are considered to indicate iron deficiency.4, 6, 7 In the literature, hepcidin is described as the key regulator of iron homeostasis. Hepcidin is a hormone produced by the liver, which inhibits iron absorption by ferroportin, the only known mammalian cellular iron exporter.8, 9 Hepcidin production is regulated by erythroferrone (ERFE), which is produced by erythroblasts in the marrow and extramedullary sites and suppresses hepcidin production when iron levels are low.8, 9

**Blood donation and iron**

Blood donors lose approximately 250 mg of iron with every whole blood donation (~500 mL), in addition to the regular 1-2 mg of iron loss per day.5, 6 Iron homeostasis can be restored by a more efficient absorption of dietary iron and by mobilizing iron from iron stores, which is possible due to decreasing hepcidin levels.5, 6, 11, 12 The amount of dietary iron absorbed in the intestines is limited and depends on body iron status and diet composition (i.e. presence of enhancers/inhibitors).13 Total body iron has been assessed to be ~4000 mg in men (estimated to be 50 mg/kg) and ~2500 mg in women (estimated to be 40 mg/kg).6, 12 Iron stores, i.e. ferritin, are estimated to comprise ~20% of total body iron, what would amount to ~800 mg in men and ~500 mg in women.6, 12 It has been estimated that the amount of iron lost with a blood donation is comparable with ~28% of the total non-heme iron stores for men and between 42-60% for women.6 Comparing the amount of iron lost with a blood donation with the total body iron shows a loss of 6% for men and 10% for women. Studies among blood donors have also shown that it takes 24-26 weeks (168-182 days) to replenish iron stores after a blood donation.6, 14, 15 This is much longer than the currently allowed minimal donation intervals in many countries.16 As a result, frequent blood donations may result in depleted iron stores (i.e. iron deficiency erythropoiesis) and subsequently declining Hb levels (i.e. iron-deficiency anemia).14, 17 The INTERVAL study - a randomized-controlled trial (RCT) performed in the United
Kingdom- showed that shorter donation intervals (12-week (standard) versus 10-week versus 8-week intervals in men and 16-week (standard) versus 14-week versus 12-week intervals in women) were associated with more donation-related symptoms, low Hb deferrals, and iron deficiency.\textsuperscript{18} Iron deficiency -defined as the loss of storage iron (ferritin <15 ng/mL)- is common among whole blood donors and is estimated to be between 25-35\%.\textsuperscript{6} In the Netherlands this would correspond with 85,000-120,000 whole blood donors, based on the numbers of registered donors in 2018. It has been suggested that iron deficiency in blood donors could result in a variety of symptoms associated with anemia, such as fatigue, decreased physical endurance and work capacity, impairment in cognitive functions (e.g. attention, concentration) and restless leg syndrome (RLS, a neurological disorder with irresistible need to move the legs).\textsuperscript{6, 19-27} There is a debate on the consequences of iron deficiency due to blood donation, as only a few RCTs have been performed among healthy blood donors.\textsuperscript{6, 18, 28} One RCT investigated the clinical effect of iron supplementation versus placebo in female donors over a four-week period and found no effect on fatigue, depression, exercise performance or quality of life (QoL).\textsuperscript{28} The INTERVAL study reported no effect of shorter intervals on QoL, physical activity and cognitive function, however they did find shorter intervals to be associated with more donation-related symptoms, such as fatigue, shortness of breath, dizziness, weakness and RLS.\textsuperscript{18} A recent systematic review on the association between iron deficiency and iron-deficiency related symptoms in whole blood donors concluded that convincing evidence for an association was only available for RLS in female whole blood donors with low iron stores. (manuscript in preparation: Zalpuri et al. Iron deficiency-related symptoms in whole blood donors: a systematic review)

Sanquin, the only blood supply organization in the Netherlands, collects blood on a voluntary non-remunerated basis. In 2018, 341,509 persons were registered as either whole blood, plasma, or platelet donor at Sanquin and 412,682 units of whole blood were collected.\textsuperscript{29} Sanquin has set several criteria in order to ensure donor health and blood product quality, which is also the case for most other European countries.\textsuperscript{30} First, capillary Hb levels (HemoCue\textsuperscript{\textregistered} AB, 201+ analyzer, Ängelholm, Sweden) are measured before each donation using a finger stick and donors with Hb levels below a certain threshold (7.8 mmol/L for women and 8.4 mmol/L for men) are temporarily deferred from donating. Although capillary Hb levels measured with the HemoCue are known to be overestimated when compared with venous Hb levels, it is considered a reliable method for blood donor screening.\textsuperscript{31, 32} In 2018, 3.5\% of the male and 6.6\% of the female whole blood donors that visited a donation site, got temporarily deferred for donation because of low Hb levels. Second, there is a minimum donation interval of 56 and 122 days and a maximum of five and three donations per year, for men and women respectively. Last, a policy of ferritin-guided extension of donation intervals
is currently being introduced in the Netherlands, following a stepwise approach from 2017 onwards. Ferritin levels are measured after the new donor screening (i.e. the first visit of a donor during which no blood is drawn for donation), after each fifth donation (i.e. fifth, tenth, fifteenth etc.) and at the first visit after deferral for low ferritin levels. Donors are deferred for respectively six and twelve months if ferritin levels are ≤30 and <15 µg/L. Studies described in this thesis, however, are based on data collected before implementation of ferritin-guided donation interval extension, and are therefore not influenced by this policy.

Measures -minimum Hb and ferritin levels and donation intervals- to protect donors from iron deficiency and related side-effects are universal and apply to all donors, while there are already variables known that influence Hb levels and of which information is available in blood bank information systems. These variables include sex, age, season and number of donations. Prediction models for low Hb deferral in whole blood donors have been made with these variables combined with previous Hb levels. A study by Nasserinejad et al. (2015) showed that indeed differences exist between blood donors in Hb levels and Hb level recovery after blood donation, resulting in different Hb trajectories after multiple donations. In this study about one-third of the whole blood donors showed relatively stable Hb levels over time, while two-thirds showed declining Hb trajectories. So, there seem to be different groups of donors, which might be due to the influence of different factors, such as sex, age, season and number of donations, already described in this paragraph. The current donation criteria can possibly be improved by using this information to determine more precisely and more individually who needs to be postponed for donation.

**Lifestyle behaviors**

The iron status of donors might be influenced by their lifestyle. As discussed previously, absorption of dietary iron plays a central role in iron homeostasis. Nonetheless, results of previous studies on associations between dietary iron intake and iron parameters in blood donors are inconclusive. Dietary iron consists of heme iron from animal foods constituting approximately 15% of the diet and non-heme iron mainly from plant-based foods constituting approximately 85% of the diet. These two types of iron show a different bioavailability, i.e. 15-35% versus 1-20% for heme and non-heme iron, respectively. Research on the separate influence of heme and non-heme iron intake on iron parameters in blood donors is lacking.

Besides dietary iron intake, it has been suggested that physical activity may also influence Hb levels. Negative as well as positive associations with Hb levels have been described in literature. Negative associations between physical activity and Hb levels
are suggested to be due to the loss of iron via sweat, urine and the gastrointestinal tract and hemodilution.\textsuperscript{43-45} Whereas, a positive association with Hb levels has been suggested as increased amounts of oxygen need to be transported in endurance sports, potentially stimulating erythropoiesis.\textsuperscript{46-48}

Overall, in addition to the variables of which information is already available in blood bank information systems, dietary iron intake and physical activity may influence Hb levels in blood donors. Therefore, it is important to gain insight into whether these lifestyle behaviors could further explain the differences in Hb levels between donors.

**Genetic variation**

The use of genetic data is emerging, due to the increasing affordability of DNA analysis and the growing potential applications. Even blood supply organizations can no longer ignore it. The production of arrays for the genotyping of blood groups is in progress and these arrays are expected to be faster, better, more comprehensive and ultimately also cheaper than serological typing. These arrays are suitable for many more single nucleotide polymorphisms (SNPs) besides the blood groups, and thus offer great opportunities for applications regarding iron status/-metabolism in the future.

In literature several gene variants have been associated with iron and erythrocyte parameters, namely SNPs in the \textit{HFE}, \textit{TMPRSS6}, \textit{TFR2}, \textit{BYSL-CCND3}, \textit{FBX07}, and \textit{SMIM1} genes.\textsuperscript{49-51} So, differences in Hb levels, Hb level recovery after blood donation (i.e. Hb trajectories) and ferritin levels between blood donors could also be due to genetic predisposition. Genome-wide association studies (GWAS) provide a hypothesis-free approach to simultaneously study associations between millions of SNPs and phenotypes, in our case iron and erythrocyte parameters.\textsuperscript{52}

**Donor InSight-III**

Donor InSight-III (DIS-III), a cohort study among Dutch donors, was set-up in 2014 to study lifestyle and genetic factors in association with iron and erythrocyte parameters in Dutch blood donors. Other large studies among blood donors were already set-up in other countries (i.e. Danish Blood Donor Study in 2015, INTERVAL in 2012 and The REDS-II Donor Iron Status Evaluation (RISE) study in 2007), however these studies had different study populations and different study aims. Unlike the other studies, stable and declining Hb trajectories (i.e. Hb recovery after blood donation) have been determined for DIS-III, according to methods described in Nasserinejad et al. (2015).\textsuperscript{38} These trajectories were determined in order to get a measure for Hb level recovery after blood donation (Figure 1.1)\textsuperscript{38}, and were based on routinely measured pre-
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donation capillary Hb levels using latent-class growth analyses. With these analyses, we defined two groups of donors by jointly capturing donors who were most alike with regard to Hb trajectories in one group, while at the same time assigning donors who differ most from this group with regard to Hb trajectories to another group.\textsuperscript{38,53} This resulted in one group with declining Hb trajectories (i.e. with a negative slope) and one group with stable Hb trajectories (i.e. with a flat/slightly rising slope).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{Hemoglobin (Hb) level profiles for male (A) and female (B) donors. The solid and dashed lines indicate different types of Hb trajectories: Donor I represents a stable Hb trajectory and Donor II represents an unstable trajectory (reproduced with permission of K. Nasserinejad; source: Nasserinejad et al. Transfusion 2015;55;1955-1963;DOI:10.1111/trf.13066).}
\end{figure}

\textbf{Scope and outline this thesis}

In this thesis it is studied how genetic and lifestyle factors are associated with iron and erythrocyte parameters in Dutch blood donors. The majority of the studies described in this thesis are performed in an observational cohort study among Dutch blood donors; Donor InSight (DIS). DIS was originally set up to study donor characteristics, donor motivations and health effects in a representative cohort of Dutch donors.\textsuperscript{54} In 2015, we started the data collection for DIS-III, specifically aiming to assess associations of genetic and lifestyle determinants with iron parameters in Dutch blood donors. In total, 2,868 (47\%) of the invited donors provided informed consent for DIS-III.\textsuperscript{54}
In Chapter 2, the aims and set-up of Donor InSight (DIS) are described. In addition, the DIS population is characterized in detail and its representativeness is compared with the general Dutch donor population. Chapter 3 presents associations between lifestyle behaviors and Hb levels and trajectories, using detailed data on dietary iron intake (heme and non-heme), physical activity (self-reported and objectively measured), Hb levels and ferritin levels, all collected as part of DIS-III. Furthermore, mediation effects of ferritin levels are investigated using multiple regression analyses. Chapter 4 presents a systematic literature review on associations between SNPs and erythrocyte parameters, including Hb, in humans. In this chapter, all available literature on these associations was searched, described and summarized using a narrative approach. Chapter 5 identifies SNPs associated with Hb levels, Hb trajectories and ferritin levels in DIS-III participants. DIS-III data-collection was especially set-up for this genome-wide association study (GWAS) and a unique aspect of this study is the Hb trajectories phenotype. Finally, a general discussion of the main findings and implications of this thesis is provided in Chapter 6.
References