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

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Chapter 6

General discussion
The research described in this thesis provides new insights in associations of genetics and lifestyle behaviors with iron and erythrocyte parameters in Dutch blood donors. This final chapter describes an overview of the main findings, followed by methodological considerations and implications for both current practice and future research.

**Main findings**

In Chapter 2 we described the Donor InSight (DIS) study population, objectives and methods, and showed that the DIS-I sample was representative of the total donor population in terms of age, body mass index (BMI), hemoglobin (Hb) level, blood pressure, blood type and donation behavior.

In Chapter 3 we showed that dietary iron intake was associated with Hb levels, and this association was mediated by ferritin levels. Donors who reported a higher heme iron consumption, as well as those reporting lower non-heme iron consumption, generally had higher Hb levels, which was mediated by higher ferritin levels. Associations of physical activity were negligible and not mediated by ferritin levels. Similar, but less pronounced, associations were found between lifestyle behaviors and Hb trajectories.

Chapters 4 and 5 focused on the contribution of genetic factors to iron and erythrocyte parameters. In our systematic review (Chapter 4) we showed that only a limited number of single nucleotide polymorphisms (SNPs) in a total of fourteen genes were associated with erythrocyte parameters in more than one study and/or across erythrocyte parameters, indicating that these might play an important role in erythropoiesis. Indeed, *ABO*, *TMPRSS6*, *HFE*, *MYB*, *HBS1L* and *HBD* are known to be involved in erythropoiesis, but the role of the remaining eight genes is unclear. The large number of SNPs and associated genes identified in associations with erythrocyte parameters, indicate a complex interplay of multiple genes in erythropoiesis and its regulation.

Finally, Chapter 5 presents results of a genome-wide association study (GWAS) using DIS-III data. A limited number of independent SNPs reached the threshold suggestive (p<1x10⁻³) for an association with Hb trajectories (n=12), Hb levels (n=20) and ferritin levels (n=24). Different SNPs were involved in each of the outcomes. Five previously identified SNPs in our systematic review were associated with Hb levels (p<1x10⁻³) in this GWAS, namely rs9610638 (near *KCTD17*), rs4820268 (in *TMPRSS6*), rs2076085 (in *TMPRSS6*), rs2413450 (in *TMPRSS6*) and rs2072860 (in *TMPRSS6*). Only one SNP reached genome-wide (GW) significance (p<5x10⁻⁸) for an association with ferritin
levels in Dutch donors, namely rs112016443, which is known to influence gene expression of \( WDSUB1 \). This association needs to be replicated in a larger cohort because it comprises a rare variant with a small effect size.

**Methodological considerations**

In order to enable proper interpretation of the results from this thesis, several strengths and limitations need to be considered. We therefore address considerations in study design, generalizability, measurements and research methodology regarding internal and external validation.

**Study design**

DIS-III is an observational cohort study among Dutch donors and was specifically set up in 2014 to study the research questions of this thesis, namely to study genes and lifestyle behaviors in association with iron and erythrocyte parameters in Dutch blood donors. This study design did best fit our research questions, as we aimed to examine the influence of multiple determinants (i.e. genes and lifestyle behaviors) on multiple outcomes (i.e. iron and erythrocyte parameters) without intervening.\(^1\) Other large studies among blood donors have been performed, such as INTERVAL in England, the Danish Blood Donor Study in Denmark, and the RISE study in America.\(^2,4\) INTERVAL is a randomized controlled trial (RCT) and randomly assigned donors to different donation intervals (i.e. males: 12-week (standard) versus 10-week versus 8-week; females: 16-week (standard) versus 14-week versus 12-week) in order to study whether donation intervals could be safely and acceptably be shortened to optimize blood supply.\(^2\) This RCT showed that a shorter donation interval was beneficial for the number of blood units collected, without affecting quality of life, physical activity or cognitive functioning, but was disadvantageous for donation-related symptoms, mean Hb and ferritin levels and low Hb deferrals.\(^5,6\) The Danish Blood Donor Study, a prospective cohort and bio-bank study, was set up to study differences in health and donation history between donors, but also to serve as cohort for collaborative research.\(^3\) Last, the RISE study was a prospective cohort study investigating four groups of donors (i.e. male and female first-time and reactivated blood donors) in order to study the effect of blood donation intensity on iron and Hb levels.\(^4\) These other blood donor cohorts have different study designs and populations, fitting their specific research questions. In our case, we intended to observe what happens in reality, and therefore we chose for an observational cohort study.

DIS-III included a large sample of 3,046 blood donors, which can be considered a strength for the (mediation) analyses carried out in Chapter 3 of this thesis. However, the same sample size can be considered relatively small for the GWAS performed
in Chapter 5, as large numbers of tests are performed in GWASs requiring a lower p-value (i.e. adapted to the number of tests performed) and thus a much larger sample size to get adequate statistical power.\(^7\)

**Generalizability**

For the extrapolation of the results from the studies carried out as part of this thesis to the Dutch donor population, factors that might have affected the generalizability (i.e. external validity) should be considered, namely (1) the lack of new donors, (2) the inclusion of inactive donors, (3) the healthy donor effect, and (4) the lack of non-Caucasian donors. First of all, the study population of DIS-III (2015-2016) consisted of donors who participated in earlier rounds of DIS in 2007-2009 and/or 2012-2013. One should keep in mind that therefore the study population did not include any donors who started donating after 2013. Furthermore, three groups of DIS participants were invited, namely a group of donors with stable Hb trajectories, a group with declining Hb trajectories, and a randomly selected group of donors from DIS-I and/or DIS-II participants. In the selection process we aimed to get similar sized groups of men and women with a known Hb trajectory and a similar sized random sample, which has resulted in an oversampling of donors with declining Hb trajectories. As a result, the DIS-III population is less representative of the Dutch donor population as compared with DIS-I. However, adding interaction terms between group (i.e. stable Hb trajectory, declining Hb trajectory and random sample) and lifestyle behaviors showed that associations of lifestyle behaviors and Hb levels were similar for the three groups of donors (data not shown).

Secondly, the DIS-III sample included inactive donors (n=738; i.e. donors who had donated in the past, but not in the previous 24 months). This subset of inactive donors had higher ferritin levels compared with the rest of the donor population, as the time since their previous donation was longer (data not shown). Hb levels and allele frequencies were similar between active and inactive donors (data not shown). This may have confounded our results, but we took this into account by adjusting our analyses for the number of donations in the past two years and for time since last donation. By doing so, we basically end up with estimations of effects of lifestyle behaviors and SNPs on Hb and ferritin levels, corrected for differences in donation history and time since the last donation between individual donors. In the INTERVAL study, donation intervals are randomly assigned to donors and Hb level has been measured at the start and end of the study.\(^5\)\(^6\) In this study lower Hb and ferritin levels were indeed seen in donors with shorter donation intervals.\(^5\)\(^6\) The randomly assigned donation intervals in this study would provide the opportunity to externally validate our results in donors with well-controlled donation intervals, but without the availability of Hb measurements at every donation.
Thirdly, blood donors are generally a healthier subset of the general population as a result of both self-selection by the donor as well as the selection criteria for donation applied by the blood supply organization, which results in a bias known as the healthy donor effect. Additionally, we included a particularly motivated group of donors who already participated in earlier rounds of DIS and who may be more health-conscious than non-participants. As a result from these types of bias, variation in Hb and ferritin levels may be missed, as donors who became ill or had low Hb levels were deferred for donation and thereby missed in this study. This would mean that the results from this thesis may have been underestimated and stronger associations may have been found if these donors would have been included.

Finally, our study was mainly performed in Caucasians (non-Caucasian: n=24) and results should therefore not be extrapolated to non-Caucasian populations. As in most blood supply organizations, the Dutch blood supply organization is experiencing difficulties in recruiting donors from ethnic minority populations. We would therefore expect comparable results in other European blood donor populations, but would recommend to carefully reassess all results before generalizing to non-Caucasian populations.

In sum, our DIS study population is quite representative of the Dutch donor population, with potentially some bias towards Caucasian, relatively more healthy donors. This makes our study population generalizable to other European blood donor populations, but requires great caution when extrapolating results to the general population and to non-European blood donor populations.

**Measurements**

DIS-III was specifically set up to study the research questions of this thesis and measurements were thus especially performed for this study. We have therefore opted for the most valid measurement method that best suited our study design for each determinant and outcome. For example, venous Hb level, which is considered the most precise method for the measurement of Hb level, was assessed using a hematology analyzer (XT-2000, Sysmex, Kobe, Japan) instead of using the routinely measured capillary Hb level data (HemoCue® AB, 201+ analyzer, Ängelholm, Sweden). Currently, the COMPARE study is investigating different Hb testing strategies for the blood bank in a large group of 31,000 donors across England (unpublished data). Ferritin levels were measured in plasma samples from lithium heparin tubes using validated methods by the National Screening Laboratory of the Dutch blood supply organization Sanquin (procedure D-VR-587-NL-009720 on the Architect Ci8200, Abbott Laboratories, Illinois, U.S.A). A limitation related to our
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ferritin measurement is that we did not measure C-Reactive protein (CRP) in the blood; a protein synthesized by the liver in case of inflammation. Plasma ferritin levels can be increased in case of inflammation and by not measuring CRP this could not be taken into account in our analyses. However, as blood donors are generally healthy and deferred for donation if not, the problem of high ferritin levels due to inflammation can be considered negligible. Indeed, a previous study has shown that CRP levels did not explain variation of ferritin levels in blood donors.

Dietary iron intake was measured using a food frequency questionnaire (FFQ) focusing on iron intake, because we were interested in usual dietary intake and the FFQ allowed us to assess this in the most simply, fast and cost-effectively way in our large study population. The FFQ enabled us to study heme and non-heme iron intake separately and to show different associations with Hb levels for each (Chapter 3).

Next, moderate-to-vigorous physical activity (MVPA) was measured subjectively by the validated International Physical Activity Questionnaire - Short Form (IPAQ-SF), as questionnaires provide the most cost-effective way to measure physical activity in large samples such as DIS-III. However, as the IPAQ-SF is limited by social desirability and recall bias, we also objectively measured MVPA in a subgroup of 741 DIS-III participants using accelerometers (wGT3X-BT and GT3X Actigraph, Pensacola, FL, USA). This enabled us to conduct sensitivity analyses in Chapter 3 to assess the robustness of the investigated associations.

Last, DIS-III was the first study to fit stable and declining Hb trajectories in order to get an estimate of Hb level recovery after repeated blood donations. These Hb trajectories were fitted before the start of DIS-III by classifying donors into groups based on routinely measured pre-donation capillary Hb levels using latent-class growth analyses. Donors who were most alike with regard to Hb trajectories were jointly captured in one group, while distinguishing them from donors with different Hb trajectories, assigned to other groups. Although Hb trajectories were fitted before the start of DIS-III, we expect them to have remained stable during the course of DIS-III, because the trajectories were based on multiple Hb level measurements over time and were independent of number of previous donations and donation interval. It is striking that declining trajectories were mainly seen in donors with high initial Hb levels, while stable Hb trajectories were mainly seen in those with low initial Hb levels, probably because this last group had little room to decline as a result of the minimum pre-donation Hb levels (7.8 mmol/L for women and 8.4 mmol/L for men) applied by Sanquin. In our studies we did not find strong associations with Hb trajectories. This may be due to the fact that these Hb trajectories may represent the
initial Hb level of a donor more than the Hb recovery of a donor. The prediction of Hb trajectory has been shown to be quite robust, with Hb trajectories being almost certain after two donations after the screening visit. So, Hb trajectory may not reflect Hb recovery as they are mainly driven by initial Hb levels and therefore may say more about initial Hb levels than about Hb recovery. A possibly better measure for Hb recovery might be current Hb levels adjusted for time since the previous donation and number of previous donations, as done in this thesis, because this approximates Hb levels of donors after multiple donations, thereby correcting for imbalances between donors in their donation history. Furthermore, one should keep in mind that Hb trajectories were known for a smaller group of donors, and that Hb trajectories are a dichotomization of many data points into stable and declining Hb trajectories, which resulted in a loss of data and thereby lower power to detect associations.

**Research methodology**

We have applied a broad spectrum of different research methods, adjusted to the research question and the nature of the data, in order to investigate the aim of this thesis. The applied methods ranged from a systematic literature review to linear and logistic regression analyses, mediation analyses and a GWAS. In Chapter 3 we used multiple regression analyses instead of using the traditional causal steps approach for our mediation analyses. Advantages of this approach are that (1) it does not focus only on the significance of effects, (2) it estimates a mediated effect, and (3) it takes into account inconsistent mediation. In Chapter 4 we studied genetic factors associated with iron and erythrocyte parameters by means of a systematic literature review. We used a narrative approach, as the large numbers of and variety in studied SNPs and erythrocyte parameters caused too much heterogeneity to enable a meta-analysis. In Chapter 5 a GWAS was performed as this was the best approach to gain insight into these associations in our donor population. The power of a GWAS lies in that fact that all known SNPs are studied at the same time for an association with a phenotype, in our case iron and erythrocyte parameters. This entails the opportunity to find SNPs and genes with unsuspected relevance and of unknown function, and thereby giving the opportunity to unravel new underlying mechanisms. GWASs usually use a Bonferroni corrected p-value of <5x10⁻⁸ to account for multiple testing. As a result, large sample sizes are necessary to find weak associations. Previously published SNPs for Hb levels did not reach GW-significance in our GWAS, but they did reach p<0.001 and showed similar directions of effects and effect sizes. This suggests that these SNPs are indeed associated with Hb levels. We conducted the first GWAS investigating SNPs associated with Hb and ferritin levels in blood donors, as all previous studies among blood donors towards these associations were restricted to only a limited number of SNPs (two to eleven).
Implications

The findings reported in this thesis have several implications. First, the results of Chapter 3 suggest that donors who consume more heme iron have higher Hb levels. This does not imply that dietary advice will be beneficial for blood donors and studies towards the usefulness of dietary advice show conflicting results regarding its usefulness.49-45. Besides, encouraging meat consumption does not fit the current spirit of the time with concerns about bio-industry, nitrogen, climate change, animal welfare and potential negative health effects of meat consumption. In addition, wanting to adjust dietary patterns of donors is difficult to reconcile with voluntary non-remunerated donorship. In Chapter 3 we also showed that adjusting for phytate-rich and polyphenol-rich food items, which are both known to inhibit iron absorption, indeed diminished the negative associations between non-heme iron intake and Hb levels.46-48 More precise measurements of amounts and timing of total phytate and polyphenol intake, instead of food item consumption, would have enabled us to make more accurate adjustments for substances known to promote or inhibit iron absorption. We therefore recommend follow-up studies to focus on heme and non-heme iron intake separately, to measure phytate and polyphenol intake more precisely, and to measure food combinations (as iron absorption is influenced by other food items).49

The results of Chapter 4 and Chapter 5 of this thesis showed that only a limited number of SNPs have been reported repeatedly to be involved in erythrocyte parameters, and only one SNP (rs112016443), which has not been described before, was identified in our donor population to be associated with ferritin levels. It is known that this SNP influences WDSUB1 gene expression, but it remains unclear how WDSUB1 could be connected to ferritin levels in blood donors. Therefore, this SNP and gene should be target for further investigation, such as replication studies and studies on the role of this SNP in ferritin and iron homeostasis (e.g. by knock-out mice). For Hb trajectories and Hb levels, no GW-significant SNPs were identified in our donor population. For Hb levels we were, however, able to replicate repeatedly found SNPs in our GWAS.50 These previously reported SNPs for Hb levels (i.e. rs17342717 in SLC17A1, rs1800562 in HFE, rs5756504 in TMPRSS6 and rs4820268 in TMPRSS6) showed similar directions of effects and effect sizes in our GWAS. As we were the first to study Hb trajectories, we could not compare our GWAS results on this outcome with previous studies and vice versa. Nonetheless, results for Hb trajectories were not the same as those for Hb levels, which indicates that these are two distinct features. Furthermore, Hb trajectories may be more donation history-dependent and environmentally determined than genetically determined. SNP based heritability scores indicated that 49% (p-value: 0.10; 95% confidence interval: 0.0-1.0) of the variation in Hb...
trajectories in our cohort of Dutch donors could be explained by common SNPs (Chapter 5). However, this heritability score did not reach statistical significance, and therefore we cannot reject the null-hypothesis that the heritability score is equal to zero. Other explanations for the lack of associations could be (1) the loss of data and thereby power to detect associations as a result of the dichotomization of many data points into stable/declining Hb trajectories, or (2) the relatively small sample size for a GWAS, especially regarding Hb trajectories, and thus the limited power to detect associations. Therefore, it is recommended to redo our GWAS in a larger sample of blood donors. Overall, we were able to identify one GW-significant SNP for ferritin levels and no GW-significant SNPs for Hb trajectories and levels, however additional GWASs in larger samples are recommended to validate these findings.

Lifestyle behaviors and genetic variation are both determinants which can possibly distinguish between donors more or less susceptible to low Hb and ferritin levels. Policy that can be conducted based on this information can consist of tailored donation intervals\(^5,\) \(^51-53\) based on lifestyle and genetic information and/or iron supplementation\(^54-56\). Questions on heme iron or meat consumption could for example relatively easily be incorporated in the donor health questionnaire that is completed by each donor before a donation, and this information can subsequently be considered for example by tailoring donation intervals. Information on genetic predisposition can be considered similarly to information on dietary intake. Arrays to determine the genetic profile of people become increasingly affordable and this genetic information could be very valuable for blood supply organizations. Information on the genetic profile of donors could for example be used to select donors who can donate more frequently and donors who should donate less frequently.

Before lifestyle and genetic information can be incorporated in blood bank policy, a number of follow-up steps to the studies described in this thesis are required in order to decide whether this information should be taken into account in the donation process and if so, how. With regard to lifestyle behaviors, we recommend to redo the prediction models for low Hb deferral, which recently showed that questionnaire-based predictors, among which food consumption, did not substantially improve these models.\(^51\) However, in this study food consumption was not measured using validated methods and only a limited number of food items were measured (i.e. dairy, fruit, vegetables, whole-wheat products, meat and fish).\(^51\) We recommend to further study what the main sources of heme iron intake were in DIS-III and subsequently whether it would be useful to distinguish between donors based on these sources, for example by incorporating them into prediction models for low Hb deferral.
With regard to genetic information, a consortium of studies among blood donors, among which INTERVAL and the Danish Blood Donor Study, currently collaborates to investigate SNPs in association with iron and erythrocyte parameters. This is in line with what we would recommend, namely to redo our GWAS in a larger sample of blood donors by collaborating with other blood donor cohorts in a large consortium, in order to increase power to find associations and to be able to replicate identified SNPs. Then, further studies are recommended to find out whether donation intervals based on lifestyle and/or genetic information of a donor is useful and worthwhile for blood supply organizations, thereby taking into account that genetic testing is also valuable for the matching of blood groups.

An alternative approach to tailored donation intervals could be the use of iron supplements. Iron supplementation has been shown to be effective in improving Hb and ferritin level recovery in blood donors. However, also for this approach further studies are necessary in order to gain insight into the optimal iron supplementation protocol, effects on donation-related symptoms and side-effects, and donor perception and behavior.

**Conclusion**

In this cohort, representative of the Dutch donor population, we investigated whether lifestyle and genetic factors were associated with iron and erythrocyte parameters, and found that heme iron intake has a positive impact on ferritin and Hb levels. One SNP was associated with ferritin levels in our DIS-III population, and a small number of SNPs and genes were repeatedly associated with erythrocyte parameters in population-based studies including our DIS-III population. These results provide starting points for further research on the role of lifestyle behaviors and gene variants in iron and erythrocyte parameters in blood donors. Eventually this may help to develop blood bank policies incorporating lifestyle and genetic information, for example by tailoring donation intervals.
References

General discussion


