Associations of environmental characteristics and lifestyle behaviours with donor blood parameters

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- CHAPTER SIX -

Dietary intake of haem iron is associated with ferritin and hemoglobin levels in Dutch blood donors: results from Donor InSight

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Abstract

Introduction

Whole blood donors, especially frequently donating donors, have a risk of iron deficiency and low haemoglobin levels, which may affect their health and eligibility to donate. Lifestyle behaviours, such as dietary iron intake and physical activity, may influence iron stores and thereby haemoglobin levels. We aimed to investigate whether dietary iron intake and questionnaire-based moderate-to-vigorous physical activity were associated with haemoglobin levels, and whether ferritin levels mediated these associations.

Methods

In Donor InSight-III, a Dutch cohort study of blood and plasma donors, data on haem and non-haem iron intake (mg/day), moderate-to-vigorous physical activity (10 minutes/day), haemoglobin levels (mmol/L) and ferritin levels (μg/L) were available in 2,323 donors (1,074 male).

Results

Donors with higher haem iron intakes (regression coefficients (β) in men and women: 0.160 and 0.065 mmol/L higher haemoglobin per 1 mg of haem iron, respectively) and lower non-haem iron intakes (β: -0.014 and -0.017, respectively) had higher haemoglobin levels, adjusted for relevant confounders. Ferritin levels mediated these associations (indirect effect (95% confidence interval) in men and women respectively: 0.074 (0.045 to 0.111) and 0.061 (0.030 to 0.096) for haem and -0.003 (-0.008 to 0.001) and -0.008 (-0.013 to -0.003) for non-haem). Moderate-to-vigorous physical activity was negatively associated with haemoglobin levels in men only (β: -0.005), but not mediated by ferritin levels.

Conclusion

Higher haem and lower non-haem iron intake were associated with higher haemoglobin levels in donors, via higher ferritin levels. This indicates that donors with high haem iron intake may be more capable of maintaining iron stores to recover haemoglobin levels after blood donation.

Introduction

A whole blood donation results in the loss of approximately 225-250 mg of iron[1]. Therefore, frequent whole blood donations may lead to iron depletion and a subsequent decline in haemoglobin levels[2,3]. In order to ensure donor health and blood product quality, donor eligibility criteria are set[4]. In many countries, including the Netherlands, minimum haemoglobin levels are mandated at each donation (in the Netherlands 8.4 mmol/L (135 g/L) for men and 7.8 mmol/L (125 g/L) for women). A study among blood donors has shown that donors differ in haemoglobin level recovery after blood donation, with some donors showing relatively stable haemoglobin trajectories over time, while other donors show declining trajectories[5]. This latter may be due to the fact that for those donors a donation interval of 56 days, which is the minimum interval in many countries including the Netherlands, is too short to restore haemoglobin levels[2,6,7]. Several factors, including sex, age, season and number of donations are established determinants of haemoglobin levels[8-12]. It may be speculated that differences in lifestyle behaviours between donors, such as dietary iron intake and physical activity, may influence haemoglobin levels as well.

Iron homeostasis is tightly regulated and maintained by recycling iron from old erythrocytes, by replacing lost iron with dietary iron, and by mobilizing stored iron when necessary[1,13,14]. In blood donors dietary iron intake may be even more important in order to maintain iron homeostasis and thereby haemoglobin levels given the iron loss associated with blood donation. A diet generally contains haem iron (present in animal foods) with high bioavailability (15-35%) and non-haem iron (especially present in plant-based foods) with 1-20% bioavailability[14,15]. Haem iron generally constitutes only about 15% of the total dietary iron intake[14,15]. Two previous studies among blood donors did not find associations between intake of iron-rich food items and iron stores or haemoglobin levels[16,17], while one study among blood donors found mainly meat intake to be associated with iron stores[18]. To our knowledge, it is unknown whether dietary haem and non-haem iron intake are positively associated with haemoglobin levels and iron stores in blood donors.

Physical activity may influence haemoglobin levels as well. Available literature suggests two general hypotheses with regard to this relation. First, physical activity may decrease haemoglobin levels through iron loss via sweat, urine, and the gastrointestinal tract, as well as by exercise-induced haemolysis or hemodilution[19-21]. Second, physical activity may increase haemoglobin levels as physical activity requires increased amounts of oxygen to be transported throughout the body by haemoglobin[22-24]. The number of studies investigating the effect of physical activity on ferritin levels (i.e. a measure representing iron

stores)[14,25] are limited, particularly in blood donors, and the results of these studies are inconclusive[26-29].

Insights into associations between lifestyle behaviours and haemoglobin levels are valuable for blood supply organizations. Lifestyle behaviours can potentially be taken into account in order to prevent haemoglobin deferrals, for example through tailored donation intervals or lifestyle advice. In addition, studying the mediating role of ferritin levels in the associations between lifestyle behaviours and haemoglobin levels will help to gain insight into whether iron stores could indeed be the limiting or enabling factor that links lifestyle behaviour to haemoglobin level recovery after donation. In a Swiss study, donors who were low in haemoglobin or ferritin levels could choose one or more of the three following strategies: (1) iron supplementation, (2) extension of the donation interval, and/or (3) suggestions of dietary changes[30]. This study found that these measures contributed to an increase in haemoglobin level[30]. The Dutch Donor InSight-III (DIS-III) study provides both questionnaire-based and accelerometry-derived data on physical activity, as well as data on both haem and non-haem iron intake using validated questionnaires[31]. This in combination with measurements of haemoglobin and ferritin levels provides a unique opportunity to study how lifestyle behaviours are related to haemoglobin levels in blood donors. Hence, we investigated 1) associations between dietary iron intake and physical activity with haemoglobin levels and haemoglobin trajectories, and 2) to what extent these associations are mediated by ferritin levels. We hypothesized that a higher intake of haem iron, and to a lesser extent of non-haem iron, is associated with higher ferritin and haemoglobin levels. Additionally, we hypothesized a potential positive association between MVPA and ferritin and haemoglobin levels.

Methods

Study population

Data were collected as part of DIS-III (2015-2016), a cohort study among blood and plasma donors in the Netherlands. DIS-III aimed at gaining insight into donor characteristics, health and behaviour. Details of DIS-III have been described elsewhere and "Supplemental Methods" elucidate haemoglobin trajectories[32].

Participants completed a general questionnaire and food frequency questionnaire (FFQ) one week prior to providing blood samples for DIS-III. Blood samples were either taken from the sampling pouch of a blood bag or, if not combined with a regular donation, through venepuncture. These blood samples were used to do a full blood count and to store samples to measure ferritin at a later moment (see "measurements"). A total of 2,552 (42%

response rate) donors provided blood samples and completed the general questionnaire. For the current analyses, donors with self-reported diagnosis of hemochromatosis (n=6), who used iron supplements/-medication (n=221) and who were pregnant during DIS-III (n=5) were excluded (n=229, 9% in total), resulting in 2,323 participants. The Medical Ethical Committee of the Academic Medical Center (AMC) in the Netherlands, and Sanquin's Ethical Advisory Board approved DIS-III and all participants gave their written informed consent.

Measurements

Haemoglobin levels and erythrocyte parameters (red blood cell (RBC) count, hematocrit, mean cell volume (MCV), mean cell haemoglobin (MCH), mean cell haemoglobin concentration (MCHC) and red cell distribution width (RDW)) were measured for DIS-III using a hematology analyzer (XT-2000, Sysmex, Kobe, Japan) in an EDTA whole blood sample within 24-hours after blood donation[33]. Collected lithium heparin tubes were centrifuged within 24-hours after DIS-III blood collection and resulting plasma was subsequently stored at -80^{0}C[34]. Ferritin levels were measured within a year after blood collection, using the stored plasma sample from lithium heparin tubes (Architect Ci8200, Abbott Laboratories, Illinois, U.S.A.)[34].

Dietary haem and non-haem iron intake (mg/day) were measured with a FFQ[31] adapted to assess iron intake. The FFQ assessed usual dietary consumption in the past four weeks. Physical activity and sedentary behaviour were questionnaire-based as well as accelerometry-derived. Questionnaire-based assessments were done by using the validated International Physical Activity Questionnaire (IPAQ) - Short Form[35,36]. Sedentary behaviour was checked for confounding (see "Supplemental Methods"). Time spent in moderate-to-vigorous physical activity (MVPA) and sedentary behaviour was expressed in minutes/day. In a subset of DIS-III participants (n=654), these were also objectively measured with accelerometers (wGT3X-BT and GT3X Actigraph, Pensacola, U.S.A.) and data were handled using Troiano (2008) cut-off points[37]. See "Supplemental Methods" for details on possible confounders.

Statistical analyses

Descriptive statistics are presented as mean ± standard deviation (SD), or in case of a skewed distribution as median and interquartile range (IQR). Associations between lifestyle behaviours (i.e. haem/non-haem iron intake and questionnaire-based MVPA) and haemoglobin levels and mediation analyses of ferritin levels as mediator of this association were studied using multiple linear regression analyses. Complete case analyses were performed and in case

of non-linear associations with skewed variables, the dependent variables were log-transformed. All models were constructed for men and women separately and adjusted for relevant confounders. Detailed in "Supplemental Methods" and "Supplementary Table 1".

Results

As shown in Table 1, a total of 1,074 males and 1,249 females were included with a mean (SD) age of 51.1 (13.0) and 47.0 (13.0) respectively. In total, 1,016 males and 1,171 females provided information on haem and non-haem iron intake, 795 males and 962 females provided information on self-reported MVPA and 313 males and 357 females had information on accelerometry-derived MVPA. Men had higher median ferritin and mean haemoglobin levels than women: 56.8 versus 35.9 μg/L and 9.3 versus 8.4 mmol/L, respectively. Median haem and non-haem iron intake were higher in men than in women (haem: 1.1 versus 0.9 mg/day; non-haem: 9.7 versus 7.9 mg/day). Higher medians were seen for questionnaire-based compared with accelerometry-derived MVPA and these medians were higher in men compared with women (questionnaire: 64.3 versus 51.4 minutes/day; accelerometer: 32.4 versus 26.7 minutes/day)[38,39]. In total, 232 males and 433 females had a stable haemoglobin trajectory and 468 males and 438 females had a declining haemoglobin trajectory.

Associations of lifestyle behaviours with hb and ferritin levels

Associations of haem and non-haem iron intake, and MVPA with haemoglobin levels are presented in Table 2. Age was found not to be an effect modifier. Adjustments were made for (1) age, smoking, menstruation (in women only), (2) number of donations in the previous two years, time since last donation, (3) sedentary behaviour, haem and non-haem iron intake or MVPA and (4) initial haemoglobin levels. A higher intake of one mg of haem iron per day was associated with 0.160 and 0.065 mmol/L higher haemoglobin levels in men and women, respectively. Higher intake of non-haem iron, however, was associated with slightly lower haemoglobin levels in men and women (-0.014 and -0.017 mmol/L respectively). Table 2 also shows that spending more time per day on MVPA was associated with lower haemoglobin levels, but these results were only statistically significant in men (β (95% CI): -0.005 (-0.008 to -0.001)).

Both, haem and non-haem iron intake, showed positive associations with ferritin levels. Table 3 (*a* path) shows log transformed results, as ferritin levels were not normally distributed. Back-transformation of these results showed 1.334 and 1.249 mmol/L higher ferritin levels per mg higher haem iron intake in men and women respectively. For non-haem iron intake these values

Table 1: Characteristics of the study population.

	Males (n=1,074)	Females (n=1,249)
Age at DIS-III, years	51.1 ± 13.0	47.0 ± 13.0
haemoglobin level, mmol/L	9.3 ± 0.6	8.4 ± 0.6
RBC, x104	497.1 ± 36.2	452.8 ± 34.1
Hct, %	44.9 ± 2.7	41.2 ± 2.9
MCV, fL	90.5 ± 4.6	91.2 ± 4.8
MCH, amol	1875.9 ± 106.5	1862.7 ± 252.2
MCHC, mmol/L	20.7 ± 0.6	20.4 ± 2.0
RDW, %	13.6 (13.1 – 14.2)	13.6 (13.1 – 14.4)
Subgroup		
Stable haemoglobin trajectory	232 (22%)	433 (35%)
Declining haemoglobin trajectory	468 (44%)	438 (35%)
Random sample	374 (35%)	378 (30%)
Ferritin level, µg/L	56.8 (31.2 – 95.5)	35.9 (19.3 – 59.3)
Haem iron intake, mg/day	1.1 (0.8 – 1.5)	0.9 (0.6 – 1.2)
Non-haem iron intake, mg/day	9.7 (7.9 – 11.7)	7.9 (6.4 – 9.5)
MVPA (questionnaire), min/day	64.3 (31.8 – 139.6)	51.4 (26.1 – 107.7)
MVPA (accelerometer), min/day	32.4 (19.9 – 49.3)	26.7 (17.3 – 40.3)
Sedentary behaviour (questionnaire), min/day	480.0 (300.0 – 720.0)	420.0 (265.0 – 615.0)
Sedentary behaviour (accelerometer), min/day	575.3 (509.6 – 632.3)	532.7 (485.9 – 580.6)
Initial haemoglobin level*, mmol/L	9.5 ± 0.6	8.5 ± 0.6
Number of donations in 2 years before DIS-III	4 (0 – 7)	2 (0 – 4)
Donation interval, months	6 (3 – 25)	9 (5 – 35)
Current smoker		
Yes	86 (8%)	94 (8%)
No	917 (85%)	1,060 (85%)
Menstruation in past 6 months		
Yes	NA	563 (45%)
No	NA	671 (54%)

Continuous variables: mean ± SD or median (interquartile range) if skewed; Dichotomous variables: n (%). NA = not applicable. DIS-III = Donor InSight-III. RBC = red blood cell count; Hct = haematocrit; MCV = mean cell volume; MCH = mean cell haemoglobin; MCHC = mean cell haemoglobin concentration; RDW = red cell distribution width; MVPA = moderate-to-vigorous physical activity. Note: due to missing data numbers might not add to total for dichotomous variables. Percentages might not sum to 100 because of rounding. *First capillary haemoglobin measurement available in the blood bank information system.

were 0.988 and 0.972 for males and females respectively. MVPA showed no statistically significant association with ferritin levels in either men (β (95% CI): 1.000 (0.995 to 1.004)) nor women (β (95% CI): 1.001 (0.996

Table 2: Associations between lifestyle behaviours (haem and non-haem iron intake and MVPA) and haemoglobin levels.

	Lifestyle behaviour	Crude model β (95% CI)	Crude model 1 β (95% CI)	Crude model 2 β (95% CI)	Crude model 3 β (95% CI)	Crude model 4 β (95% CI)
Male	Haem intake (mg/day)	0.125 (0.057 to 0.193)	0.123 (0.055 to 0.191)	0.126 (0.058 to 0.194)	0.188 (0.103 to 0.272)	0.160 (0.083 to 0.238)
	Non-haem intake (mg/day)	-0.016 (-0.026 to -0.007)	-0.017 (-0.026 to -0.008)	-0.017 (-0.026 to -0.008)	-0.021 (-0.033 to -0.008)	-0.014 (-0.025 to -0.003)
	MVPA (10 minutes/day)	-0.005 (-0.009 to -0.002)	-0.005 (-0.009 to -0.002)	-0.005 (-0.009 to -0.001)	-0.006 (-0.009 to -0.002)	-0.005 (-0.008 to -0.001)
Female	Haem intake (mg/day)	0.106 (0.033 to 0.178)	0.102 (0.030 to 0.174)	0.106 (0.034 to 0.178)	0.093 (0.005 to 0.181)	0.065 (-0.018 to 0.148)
	Non-haem intake (mg/day)	-0.020 (-0.032 to -0.008)	-0.021 (-0.033 to -0.010)	-0.022 (-0.033 to -0.010)	-0.022 (-0.036 to -0.007)	-0.017 (-0.031 to -0.003)
	MVPA (10 minutes/day)	-0.000 (-0.004 to 0.004)	-0.002 (-0.006 to 0.002)	-0.002 (-0.006 to 0.002)	-0.003 (-0.007 to 0.001)	-0.003 (-0.007 to 0.001)

β=regression coefficient, 95% CI=95% confidence interval. MVPA=moderate-to-vigorous physical activity. Model 1: adjusted for age, smoking, and menstruation (women only). Model 2: additionally adjusted for number of donations in the 2 years before DIS-III and donation interval. Model 3: additionally adjusted for sedentary behaviour and MVPA in models with haem and non-haem iron intake as lifestyle behaviour or sedentary behaviour, haem and non-haem iron intake in models with MVPA as lifestyle behaviour. Model 4: additionally adjusted for initial haemoglobin level. More than 10% of participants excluded due to missing data in males model 3-4 for haem and non-haem iron intake and models 1-4 for MVPA and in females model 1-4 for haem and non-haem iron intake and MVPA.

Table 3: Associations between lifestyle behaviours (haem and non-haem iron intake and MVPA) and haemoglobin levels and mediation by ferritin levels.

	Lifestyle behaviour	Lifestyle behaviour - haemoglobin *total effect* (c-path)† β (95%CI)	Lifestyle behaviour - ferritin (a-path) LN(β (95% CI))	Ferritin - haemoglobin (b-path) β (95% CI)	Lifestyle behaviour - haemoglobin *direct effect* (c'-path) β (95% CI)	Lifestyle behaviour - haemoglobin *indirect effect* (a path * b path) β (95% BCI)
Male	Haem intake (mg/day)	0.160 (0.083 to 0.238)	0.288 (0.192 to 0.383)	0.256 (0.198 to 0.314)	0.090 (0.014 to 0.165)	0.074 (0.045 to 0.111)
	Non-haem intake (mg/day)	-0.014 (-0.025 to -0.003)	-0.012 (-0.027 to 0.002)		-0.011 (-0.022 to -0.000)	-0.003 (-0.008 to 0.001)
	MVPA (10 minutes/day)	-0.005 (-0.008 to -0.001)	-0.000 (-0.005 to 0.004)		-0.005 (-0.009 to -0.002)	-0.000 (-0.001 to 0.001)
Female	Haem intake (mg/day)	0.065 (-0.018 to 0.148)	0.222 (0.115 to 0.328)	0.276 (0.225 to 0.327)	0.002 (-0.077 to 0.080)	0.061 (0.030 to 0.096)
	Non-haem intake (mg/day)	-0.017 (-0.031 to -0.003)	-0.028 (-0.046 to -0.010)		-0.010 (-0.023 to 0.004)	-0.008 (-0.013 to -0.003)
	MVPA (10 minutes/day)	-0.003 (-0.007 to 0.001)	0.001 (-0.004 to 0.006)		-0.003 (-0.007 to 0.000)	0.000 (-0.001 to 0.002)

β=regression coefficient; 95% CI=95% confidence interval; BCI=bootstrapped confidence interval; MVPA=moderate-to-vigorous physical activity; ferritin levels in µg/L; haemoglobin level in mmol/L. *Residuals of ferritin levels were not normally distributed and therefore log transformed, this table presents log transformed data. †These results are identical to the results of model 4 in Table 2. Adjusted for age, smoking, menstruation (in models with women only), number of donations, donation interval, sedentary behaviour, MVPA in models with haem and non-haem iron intake as lifestyle behaviour or haem and non-haem iron intake in models with MVPA as lifestyle behaviour, and initial haemoglobin level.

to 1.006)). As shown in Supplementary Table 2, lifestyle behaviours and haemoglobin trajectories showed similar, but less pronounced, associations as those with haemoglobin levels as outcome.

Mediation by ferritin levels

As shown in Table 3, associations between dietary iron intake and haemoglobin levels were mediated by ferritin levels. In both men and women, higher intake of haem iron was significantly associated with higher ferritin levels, and higher ferritin levels with higher haemoglobin levels (*b* path: 0.256 (0.198 to 0.314) in men and 0.276 (0.225 to 0.327) in women). The association between haem iron intake was largely mediated by ferritin, showing indirect effects of 0.074 (0.0458 to 0.111) in men and 0.061 (0.030 to 0.096) in women. The direct, non-mediated effect of haem iron intake on haemoglobin levels was only statistically significant in men. Higher intake of non-haem iron was associated with lower ferritin and haemoglobin levels. The association between non-haem iron intake and haemoglobin levels was also mediated by ferritin, but only significantly in women (indirect effect: -0.003 (-0.008 to 0.001) in men and -0.008 (-0.013 to -0.003) in women).

Ferritin levels did not mediate the association between MVPA and haemoglobin levels (Table 3). As shown in Table 3, MVPA was not associated with ferritin levels (*a* path) and accordingly, the indirect effects were close to zero. The direct effect (*c*' path) of MVPA on haemoglobin level was statistically significant in men only (β (95% CI): -0.005 (-0.009 to -0.002)). With regard to haemoglobin trajectories, a significant indirect effect was found for haem and non-haem iron intake in women only (0.094 (0.013 to 0.203) for haem iron and -0.011 (-0.026 to -0.002) for non-haem iron). Ferritin did not mediate the other associations between lifestyle behaviours and haemoglobin trajectories (Supplementary Table 3).

Sensitivity and post-hoc analyses

Table 4 shows the results of post-hoc analyses on associations between lifestyle behaviours and erythrocyte parameters. Haem iron intake, non-haem iron intake and MVPA were mainly associated with haematocrit. In men, associations were also found between haem iron intake and MCV and MCH, and between MVPA and RBC.

In sensitivity analyses in a subset of the study population with accelerometry-derived MVPA the direct effect of MVPA on haemoglobin levels in men was not statistically significant anymore in any model (Supplementary Table 4).

Table 4: Associations between erythrocyte parameters and lifestyle behaviours.

	Lifestyle behaviour	RBC β (95% CI)	Hct β (95% CI)	MCV β (95% CI)	MCH β (95% CI)	MCHC β (95% CI)	RDW* LNβ (95% CI)
Male	Haem intake (mg/day)	4.242 (-0.397 to 8.881)	0.681 (0.330 to 1.033)	0.612 (0.005 to 1.220)	16.284 (1.833 to 30.736)	0.043 (-0.036 to 0.122)	-0.004 (-0.014 to 0.006)
	Non-haem intake (mg/day)	-0.484 (-1.166 to 0.198)	-0.076 (-0.128 to -0.025)	-0.072 (-0.162 to 0.017)	-1.137 (-3.260 to 0.987)	0.004 (-0.008 to 0.015)	-0.001 (-0.002 to 0.001)
	MVPA (10 minutes/day)	-0.248 (-0.457 to -0.040)	-0.023 (-0.039 to -0.007)	-0.002 (-0.029 to 0.025)	-0.094 (-0.744 to 0.556)	0.000 (-0.004 to 0.003)	0.000 (-0.001 to 0.000)
Female	Haem intake (mg/day)	4.600 (-0.791 to 9.991)	0.465 (0.022 to 0.908)	0.099 (-0.624 to 0.822)	1.786 (-14.487 to 18.058)†	-0.019 (-0.109 to 0.071)†	0.003 (-0.010 to 0.016)
	Non-haem intake (mg/day)	-0.573 (-1.477 to 0.330)	-0.082 (-0.156 to -0.008)	-0.073 (-0.194 to 0.048)	-2.076 (-4.799 to 0.647)†	-0.005 (-0.020 to 0.010)†	-0.001 (-0.003 to 0.001)
	MVPA (10 minutes/day)	-0.083 (-0.329 to 0.163)	-0.016 (-0.037 to 0.004)	-0.021 (-0.054 to 0.012)	-0.236 (-0.977 to 0.506)†	0.002 (-0.002 to 0.006)†	0.000 (-0.001 to 0.000)

β: regression coefficient; 95% CI= 95% confidence interval; MVPA=moderate-to-vigorous physical activity; RBC=red blood cell count in $x10^4$; Hct=haematocrit in %; MCV=mean cell volume in fL; MCH=mean cell haemoglobin in mmol; MCHC=mean cell haemoglobin concentration in mmol/L; RDW=red cell distribution width in %; ferritin levels in µg/L; *Residuals of ferritin levels and RDW were not normally distributed and therefore log transformed, this table presents log transformed data. Adjusted for age, smoking, menstruation (in models with women only), number of donations, donation interval, sedentary behaviour, MVPA in models with haem and non-haem iron intake as lifestyle behaviour or haem and non-haem iron intake in models with MVPA as lifestyle behaviour, and initial Hb. †results with outlier removed, without outlier removed: haem (-29.812 (-77.210 to 17.585)), non-haem (-0.534 (-8.474 to 7.407)) and MVPA (-0.864 (-3.025 to 1.297)) for MCH and haem (-0.292 (-0.688 to 0.103)), non-haem (0.009 (-0.058 to 0.075)) and MVPA (-0.003 (-0.021 to 0.015)) for MCHC.

Discussion

In this study among Dutch blood donors, we found that dietary iron intake was associated with haemoglobin levels of blood donors via ferritin levels. Haem iron intake showed a positive and non-haem iron intake a negative association with haemoglobin levels. To put this amount of iron into perspective, 1 mg higher haem iron intake -equivalent to 58 grams of prepared beef or 700 grams of prepared chicken filet[40]- was associated with 0.160 mmol/L higher haemoglobin levels in men. With regard to non-haem iron, 1 mg higher non-haem iron intake -equivalent to 60 grams of cooked whole wheat pasta or 2.5 salty herring (187.5 grams)[40]- was associated with -0.014 mmol/L lower haemoglobin levels in men. A statistically significant, but rather small (-0.015 mmol/L for 30 min/day MVPA in men), negative association between questionnaire-based but not accelerometry-derived physical activity and haemoglobin levels was found in men only. This association was independent of ferritin levels. Results were independent of frequency of previous donations as we adjusted for number of donations in the two years before DIS-III and donation interval.

As hypothesized a positive association was found between haem iron intake and haemoglobin and ferritin levels. It seems that donors who consume more haem iron can restore their iron stores better, resulting in higher ferritin and haemoglobin levels. Further analyses in which lifestyle behaviours were associated with erythrocyte parameters showed that haem iron intake increases the volume of blood that is occupied by red blood cells (higher Hct) and vice versa (lower Hct) for non-haem iron intake and MVPA. In men, higher haem iron intake was also associated with higher MCV and MCH, indicating that in men in addition to a larger volume of red blood cells, these cells also contain more haemoglobin. Interestingly, higher intake of non-haem iron was associated with lower haemoglobin levels, independent of haem iron intake. An explanation might be that with higher intake of non-haem iron, more phytate-rich and polyphenol-rich foods and beverages -e.g. legumes, grains and coffee- are consumed preventing absorption of non-haem iron[41-43]. Indeed, post-hoc analyses with additional adjustments for phytate-rich and polyphenol-rich food items (i.e. legumes, bread, pasta, cereals, nuts and coffee) diminished the negative associations between non-haem iron intake and haemoglobin levels. More precise measurements of total phytate and polyphenol intake, rather than the consumption of food items, would enable more accurate adjustments for these substances. With regard to MVPA, the negative association with haemoglobin levels may be due to exercise-induced haemolysis, however this has mainly been found in studies investigating endurance athletes[17,19,44]. In the additional analyses of MVPA with erythrocyte parameters, we did find that more MVPA was associated

with lower numbers of erythrocytes. Another potential explanation could be haemodilution, caused by exercise-induced plasma volume expansion[20]. This phenomenon is often seen in athletes and is also known as sports anemia[20,21]. However, based on the results of this study no firm conclusions on mechanisms behind the association of MVPA with haemoglobin levels can be drawn. In contrast to our findings on associations with haemoglobin levels, there was only one statistically significant association between lifestyle behaviours and haemoglobin trajectories (Supplementary Table 2). An explanation could be the loss of power to detect an association due to the dichotomization of the haemoglobin level measurements into haemoglobin trajectories (stable/declining) and the lower number of participants with a known haemoglobin trajectory[45].

Our finding that iron intake was associated with haemoglobin and ferritin levels is in contrast with previous studies conducted among blood donors[16,17]. However, these previous studies have assessed consumption of iron-containing food items. Since the majority of iron in food is consumed as non-haem iron, and we found this to be negatively associated with haemoglobin levels, distinguishing between haem and non-haem iron is needed to recognize the positive effect on haemoglobin levels of haem iron intake. Research among a general population of Dutch adults supports our findings; they also found that haem iron intake was positively, and non-haem iron intake negatively associated with iron status[46]. With regard to physical activity, our results are in agreement with another study among Danish blood donors that also found a negative association for questionnaire-based physical activity (hours/week) with haemoglobin levels in men only (β (95% CI) of -0.09 (-0.11 to -0.06) for non-smokers and -0.11 (-0.18 to -0.05) for smokers)[17].

Strengths of this study include the large study population and the detailed assessment of lifestyle behaviours, erythrocyte parameters and ferritin levels. As the FFQ enabled us to calculate both haem and non-haem iron intake rather than assessing total iron intake, we were able to show that the direction and magnitude of the associations between haem and non-haem iron intake and haemoglobin levels differ importantly. A limitation of the FFQ however is that it does not measure when and in which combination food items are consumed[47]. Another limitation of this study is the use of questionnaire-based MVPA, which is prone to social desirability and recall bias, and the validity is known to differ across respondents from different socio-economic strata, but it is also the most cost-effective way to measure physical activity in a large sample and enables differentiation between types of activity[39,48,49]. We did however used a validated questionnaire[35,36], and were able to perform sensitivity analyses with accelerometry-derived data in a subgroup of the

participants. Results were similar but not significant in the accelerometry sub-group, probably due to the smaller study population. Next, it could be argued that including menstruating women in the analyses could have altered associations between haem and non-haem iron intake and haemoglobin levels in this subpopulation. However, analyses showed that menstrual status was not an effect modifier of the association between iron intake and haemoglobin levels, indicating that the association between iron intake and haemoglobin levels is similar for menstruating versus non-menstruating women. Last, analyses of this study were cross-sectional, and we are therefore unable to infer causation.

The results from this observational study do not implicate that blood donors will benefit from dietary advices. A review regarding solutions to iron deficiency in young women living in industrialized countries showed that dietary advice was not associated with an increase in haemoglobin levels in two studies, but these studies showed conflicting results with regard to the effect of dietary advice on ferritin levels[50]. A study among Swiss donors found that iron supplementation, extension of the donation interval, and/or suggestions of dietary changes resulted in a decrease in the prevalence of anaemia and iron deficiency[30]. However, this study was limited by the fact that donors were not randomized to one of the three interventions and no standardized information was handed out to the donor[30]. Lastly, a study among blood donors conducted at our research group found that dietary advice did not reduce the risk of low-haemoglobin deferral[51]. Tailored donation intervals might be useful in preventing low haemoglobin levels in blood donors[3,6,7], however the usefulness of dietary information in tailoring these intervals should first be investigated further.

In conclusion, blood donors with a higher intake of haem iron and lower intake of non-haem iron generally had higher haemoglobin levels, and this was mediated by higher ferritin levels. In men more time spent in moderate-to-vigorous physical activity was associated with lower haemoglobin levels, independent of ferritin. Taking the haem iron intake, separated from non-haem iron intake, of blood donors into account may be useful in the prevention of low haemoglobin levels in blood donors.

Conflict-of-interest disclosure.
The authors declare no competing financial interests.

Author contributions
K.v.d.H. and W.L.A.M.d.K. designed the project. T.C.T. and R.d.G. developed the theory. T.C.T. took the lead in writing the manuscript, performed the analyses and made the tables and figures. J.J.M.R. verified the analytical methods. K.v.d.H. supervised the findings of this work. All authors contributed to the interpretation of the results, provided critical feedback and helped shape the research, analyses and manuscript.

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Appendix

Supplemental Methods

Study population

In short, three groups of donors who had participated in earlier DIS rounds (DIS-I and/or DIS-II) were invited for DIS-III, namely a group with (1) stable haemoglobin trajectories (n=2,071), (2) declining haemoglobin trajectories (n=2,548), and (3) a randomly selected group (1,521). Stable and declining haemoglobin trajectories were identified by fitting growth mixture models on routinely measured capillary haemoglobin level data (HemoCue® AB, 201+ analyser, Ängelholm, Sweden).[1,2] Growth mixture models assigned donors who were most different from each other with regard to haemoglobin trajectories to one group and captured donors who were most alike with regard to haemoglobin trajectories in another group. Methods were the same as described by Nasserinejad et al. (2015), with the difference being that two (stable and declining trajectories) instead of four groups were defined in the same study population that was used to invite donors for DIS-III.[1,2]

Measurements

Sex, age, smoking, use of iron supplements/-medication, and menstruation were assessed using the general questionnaire. Use of (prescribed) iron supplements/-medication was classified according to the WHO Anatomical Therapeutic Classification (ATC) code system and codes starting with B03A (i.e. iron preparations), A11AA (i.e. multivitamins with minerals) and A12 (i.e. mineral supplements) were considered iron supplements/-medication. Smoking and menstrual status were dichotomized into yes/no. Information on number of whole blood donations in two years before DIS-III blood sampling, initial haemoglobin level and donation interval (i.e. time in months between DIS-III blood sampling date and previous visit) were extracted from the blood bank information system (ePROGESA, MAK-SYSTEM International Group, Paris, France). Initial haemoglobin level comprised screening haemoglobin level, which is measured with a finger stick during a donor's first visit to the blood supply organization. This first visit consists of a donor health check, during which no full donation is made. If screening haemoglobin level was unavailable due to the transition -in the past related to a merger in the Netherlands- to another blood bank information system, the first capillary haemoglobin measurement available in the blood bank information system was used.

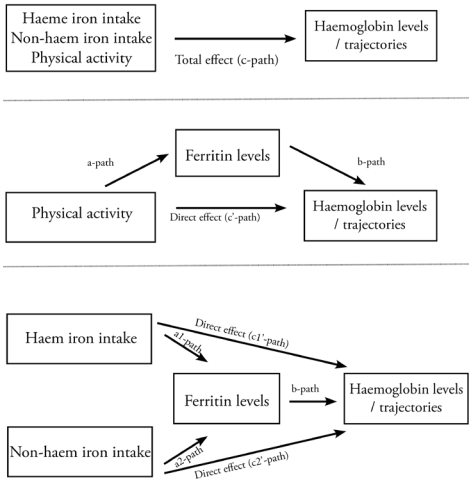
Statistical analyses

Separate models were made for questionnaire-based physical activity and dietary iron intake (i.e. heme and non-heme iron intake combined into one model). See Supplementary Figure 1 for a graphical representation of

the models. Effect modification by age and menstrual status were checked because of changes in haemoglobin levels with increasing age (i.e. an increase in women after the menopause and a decrease in men after the fourth decade of life).[3,4] Effect modification was tested by adding the variable and an interaction term with iron intake or physical activity to the model. A p-value of the interaction term <0.05 indicated effect modification. Confounding was investigated for: age , smoking, menstruation (only in women; if not an effect modifier), number of whole blood donations in two years before DIS-III, donation interval, sedentary behavior, MVPA (in models with heme and non-heme iron intake as determinants) or heme and non-heme iron intake (in models with MVPA as determinant), and initial haemoglobin level. More than 10% change in the regression coefficient of the lifestyle variable was considered confounding and the variable was then added to all models.

Mediation analysis was performed using multiple regression.[5] The amount of mediation by ferritin is assessed by calculating indirect effects (*a*b* path, Supplementary Figure 1). For these indirect effects, 95% percentile bootstrap confidence intervals were calculated based on 5,000 bootstrap resamples.[5] Further, logistic regression analyses were performed with haemoglobin trajectory as outcome variable and sensitivity analyses were performed with accelerometry-derived physical activity. In order to gain further insight into the associations found post-hoc linear regression analyses were performed on associations between lifestyle behaviors and erythrocyte parameters and also with additional adjustments for phytate-rich and polyphenol-rich food items (i.e. legumes, bread, pasta, cereals, nuts and coffee). Because initial haemoglobin levels were already incorporated in the haemoglobin trajectories and showed high collinearity, models with haemoglobin trajectories were not adjusted for initial haemoglobin levels.

Results are presented as regression coefficients with 95% confidence intervals (95% CI) for continuous outcomes and as odds ratios (OR) with 95% CI for binary outcomes. P-values <0.05 were considered statistically significant. Statistical analyses were performed using R version 3.1.2. with boot package version 1.3.18 to calculate 95% percentile bootstrap CIs for the indirect effects.



Supplementary figure 1: Path diagram of the mediation models.

The total effect of the exposure variable (haem and non-haem iron intake or physical activity) on the outcome variable (haemoglobin level or haemoglobin trajectory) is represented by *c*. The effect of the exposure variable (haem and non-haem iron intake or physical activity) on the outcome variable (haemoglobin level or haemoglobin trajectory) is represented by *a, a1 and a2*. The direct, non-mediated, effect of the exposure variable (haem and non-haem iron intake or physical activity) on the outcome variable (haemoglobin level or haemoglobin trajectory) is represented by *c', c'1* and *c'2*, and *b* represents the effect of the mediator variable (ferritin levels) on the outcome variable (haemoglobin level or haemoglobin trajectory)[5].

Supplementary table 1: Variables included in the fully adjusted model

Variable	Model 1	Model 2	Model 3	Model 4
Age (years)	Yes	Yes	Yes	Yes
Smoking (yes/no)	Yes	Yes	Yes	Yes
Menstruation[1] (yes/no)	Yes	Yes	Yes	Yes
Number of donations in the two years before Donor InSight-III		Yes	Yes	Yes
Donation interval (days)		Yes	Yes	Yes
Sedentary behaviour (minutes/day)			Yes	Yes
Moderate-to-vigorous physical activity (minutes/day)[2]			Yes	Yes
Haem iron intake (mg/day)[3]			Yes	Yes
Non-haem iron intake (mg/day)[3]			Yes	Yes
Initial haemoglobin level (mmol/L)				Yes

[1] Women only. [2]In models with haem and non-haem iron intake as lifestyle behaviour. [3]In models with MVPA as lifestyle behaviour.

Supplementary table 2: Associations between lifestyle behaviors and Hb trajectories.

	Lifestyle behaviour	Crude model OR (95% CI)	Crude model 1 OR (95% CI)	Crude model 2 OR (95% CI)	Crude model 3 OR (95% CI)
Males	Haem (mg/day)	1.222 (0.912 to 1.649)	1.164 (0.862 to 1.580)	1.145 (0.846 to 1.557)	1.222 (0.835 to 1.805)
	Non-haem (mg/day)	0.971 (0.933 to 1.011)	0.966 (0.926 to 1.006)	0.966 (0.926 to 1.005)	0.943 (0.890 to 0.996)
	MVPA (10 min/day)	0.995 (0.981 to 1.010)	0.992 (0.976 to 1.008)	0.992 (0.976 to 1.008)	0.991 (0.976 to 1.008)
Females	Haem (mg/day)	0.929 (0.682 to 1.265)	0.950 (0.692 to 1.302)	0.931 (0.677 to 1.277)	1.054 (0.720 to 1.545)
	Non-haem (mg/day)	0.993 (0.943 to 1.046)	0.992 (0.941 to 1.045)	0.993 (0.942 to 1.046)	0.959 (0.897 to 1.025)
	MVPA (10 min/day)	0.999 (0.984 to 1.015)	0.998 (0.982 to 1.015)	0.998 (0.982 to 1.014)	0.991 (0.973 to 1.009)

OR=odds ratio, 95% CI=95% confidence interval. MVPA=moderate-to-vigorous physical activity. Hb trajectory: 0=stable, 1=declining. Model 1: adjusted for age, smoking, menstruation (women only). Model 2: additionally adjusted for number of donations in the 2 years before DIS-III and donation interval. Model 3: additionally adjusted for sedentary behaviour and MVPA in models with haem and non-haem iron intake as lifestyle behaviour or sedentary behaviour, haem and non-haem iron intake in models with MVPA as lifestyle behaviour. More than 10% of participants excluded due to missing data in males for haem and non-haem iron intake model 3 and for MVPA model 1-3 and in females for haem and non-haem iron intake and MVPA model 1-3.

Supplementary table 3: Associations between lifestyle behaviors and Hb trajectories and mediation by ferritin levels adjusted for confounders.

	Lifestyle behaviour	Lifestyle behaviour - haemoglobin trajectory *total effect* (c-path)† β (95%CI)	Lifestyle behaviour - ferritin (a-path) LN(β (95% CI))	Ferritin - haemoglobin trajectories (b-path) β (95% CI)	Lifestyle behaviour - haemoglobin trajectories *direct effect* (c'-path) β (95% CI)	Lifestyle behaviour - haemoglobin trajectories *indirect effect* (a path * b path) β (95% BCI)
Male	Haem intake (mg/day)	0.201 (-0.180 to 0.591)	0.285 (0.165 to 0.406)	-0.244 (-0.542 to 0.049)	0.299 (-0.095 to 0.705)	-0.070 (-0.171 to 0.014)
	Non-haem intake (mg/day)	-0.059 (-0.116 to -0.004)	-0.019 (-0.037 to -0.001)		-0.068 (-0.126 to -0.012)	0.005 (-0.001 to 0.014)
	MVPA (10 minutes/day)	-0.009 (-0.025 to 0.008)	-0.001 (-0.006 to 0.004)		-0.008 (-0.024 to 0.009)	0.000 (-0.002 to 0.002)
Female	Haem intake (mg/day)	0.053 (-0.328 to 0.435)	0.308 (0.183 to 0.433)	0.305 (0.049 to 0.565)	-0.050 (-0.442 to 0.342)	0.094 (0.013, 0.203)
	Non-haem intake (mg/day)	-0.041 (-0.109 to 0.025)	-0.035 (-0.056 to -0.013)		-0.030 (-0.097 to 0.037)	-0.011 (-0.026 to -0.002)
	MVPA (10 minutes/day)	-0.009 (-0.027 to 0.009)	0.001 (-0.005 to 0.007)		-0.009 (-0.028 to 0.009)	0.000 (-0.002 to 0.003)

OR=odds ratio; β=regression coefficient; 95% CI= 95% confidence interval; BCI=bootstrapped confidence interval; MVPA=moderate-to-vigorous physical activity; ferritin levels in µg/L; Hb trajectory: 0=stable, 1=declining. †These results are identical to the results of model 3 in Supplementary Table 1. Adjusted for age, smoking, menstruation (in models with women only), number of donations, donation interval, sedentary behaviour, MVPA in models with haem and non-haem iron intake as lifestyle behaviour or haem and non-haem iron intake in models with MVPA as lifestyle behaviour.

Supplementary table 4: Associations between MVPA measured using accelerometers and haemoglobin level and haemoglobin trajectories and mediation by ferritin levels adjusted for confounders.

	MVPA - haemoglobin *total effect* (c-path)† β (95%CI)	MVPA - ferritin (a-path) LN(β (95% CI))	Ferritin - haemoglobin (b-path) β (95% CI)	MVPA - haemoglobin *direct effect* (c'-path) β (95% CI)	*indirect effect* (a path * b path) β (95% BCI)
Male	-0.007 (-0.035 to 0.021)	-0.020 (-0.053 to 0.013)	0.337 (0.247 to 0.426)	0.000 (-0.026 to 0.026)	-0.007 (-0.019 to 0.003)
Female	0.004 (-0.026 to 0.034)	-0.007 (-0.045 to 0.031)	0.219 (0.136 to 0.302)	0.005 (-0.024 to 0.034)	-0.002 (-0.010 to 0.008)
	MVPA - haemoglobin trajectories *total effect* (c-path)† β (95%CI)	**MVPA - ferritin (a-path) LN(β (95% CI))**	**Ferritin - haemoglobin trajectories (b-path) β (95% CI)**	**MVPA - haemoglobin trajectories *direct effect* (c'-path) β (95% CI)**	***indirect effect* (a path * b path) β (95% BCI)**
Male	-0.114 (-0.263 to 0.033)	-0.033 (-0.082 to 0.017)	-0.666 (-1.165 to -0.196)	-0.149 (-0.306 to 0.002)	0.022 (-0.010 to 0.077)
Female	-0.041 (-0.187 to 0.102)	-0.009 (-0.055 to 0.038)	0.076 (-0.342 to 0.493)	-0.045 (-0.193 to 0.098)	-0.001 (-0.017 to 0.011)

β=regression coefficient; OR=odds ratio; 95% CI=95% confidence interval; BCI=bootstrapped confidence interval; MVPA=moderate-to-vigorous physical activity in 10 minutes/day; haem and non-haem iron intake in mg/day, ferritin levels in µg/L; haemoglobin level in mmol/L; haemoglobin trajectory: 0=stable, 1=declining. *Residuals of ferritin levels were not normally distributed and therefore log transformed, this table presents log transformed data. Adjusted for age, smoking, menstruation (in models with women only), number of donations, and donation interval, sedentary behaviour (accelerometer), and initial haemoglobin level (in model with haemoglobin level only).

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