SUPPLEMENTARY APPENDIX

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

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SUPPLEMENTS

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 Hb trajectories (n=2,071), (2) declining Hb trajectories (n=2,548), and (3) a randomly selected group (1,521). Stable and declining Hb trajectories were identified by fitting growth mixture models on routinely measured capillary Hb 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 Hb trajectories to one group and captured donors who were most alike with regard to Hb 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

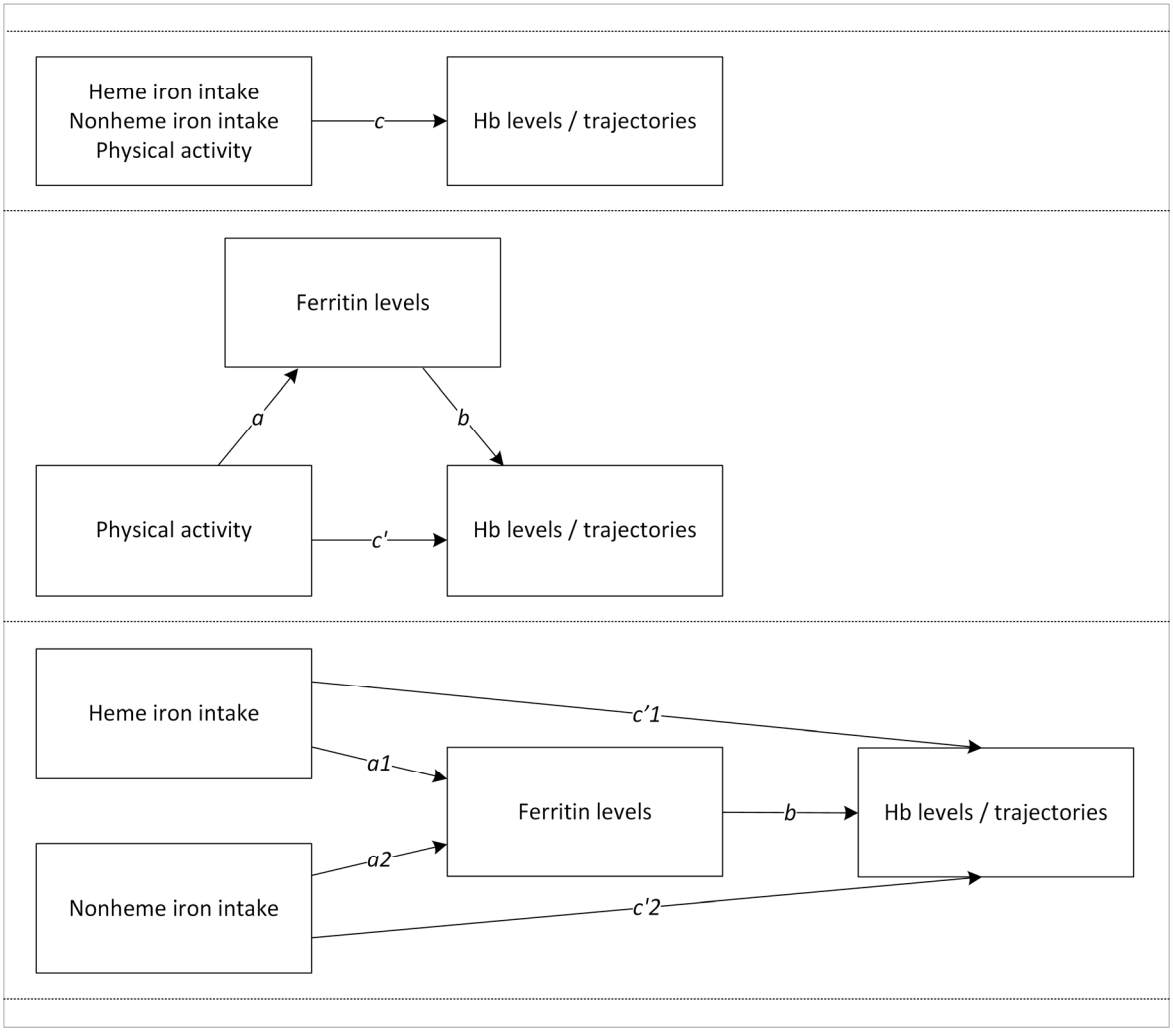
Sex, age, smoking, use of iron supplements/-medication, and menstruation were assessed using the general questionnaire. Use of (prescribed) iron supplements/-medication was classified according to the WHO Anatomical Therapeutic Classification (ATC) code system and codes starting with B03A (i.e. iron 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 Hb 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 Hb level comprised screening Hb 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 Hb 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 Hb 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 Hb 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 Hb 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 Hb 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 Hb levels were already incorporated in the Hb trajectories and showed high collinearity, models with Hb trajectories were not adjusted for initial Hb 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 (heme and non-heme iron intake or physical activity) on the outcome variable (Hb level or Hb trajectory) is represented by *c*. The effect of the exposure variable (heme and non-heme iron intake or physical activity) on the outcome variable (Hb level or Hb trajectory) is represented by *a, a1 and a2*. The direct, non-mediated, effect of the exposure variable (heme and non-heme iron intake or physical activity) on the outcome variable (Hb level or Hb 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 (Hb level or Hb 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 (women only), yes/no	Yes	Yes	Yes	Yes
Donations in the two years before DIS-III, number		Yes	Yes	Yes
Donation interval, days		Yes	Yes	Yes
Sedentary behavior, minutes/day			Yes	Yes
MVPA (in models with heme and non-heme iron intake as determinants), minutes/day			Yes	Yes
Heme iron intake (in models with MVPA as determinant), mg/day			Yes	Yes
Non-heme iron intake (in models with MVPA as determinant), mg/day			Yes	Yes
Initial Hb level, mmol/L				Yes

DIS-III=Donor InSight-III. MVPA=Moderate-to-vigorous physical activity.Hb=Hemoglobin.

Supplementary Table 2. Associations between lifestyle behaviors (heme and non-heme iron intake and MVPA) and Hb trajectories (stable/declining).

	Independent variable	Crude model OR (95% CI)	Model 1 OR (95% CI)	Model 2 OR (95% CI)	Model 3 OR (95% CI)
♂	Heme	1.222 (0.912; 1.649)	1.164 (0.862; 1.580)	1.145 (0.846; 1.557)	1.222 (0.835; 1.805)
	Non-heme	0.971 (0.933; 1.011)	0.966 (0.926; 1.006)	0.966 (0.926; 1.005)	0.943 (0.890; 0.996)
	MVPA	0.995 (0.981; 1.010)	0.992 (0.976; 1.008)	0.992 (0.976; 1.008)	0.991 (0.976; 1.008)
♀	Heme	0.929 (0.682; 1.265)	0.950 (0.692; 1.302)	0.931 (0.677; 1.277)	1.054 (0.720; 1.545)
	Non-heme	0.993 (0.943; 1.046)	0.992 (0.941; 1.045)	0.993 (0.942; 1.046)	0.959 (0.897; 1.025)
	MVPA	0.999 (0.984; 1.015)	0.998 (0.982; 1.015)	0.998 (0.982; 1.014)	0.991 (0.973; 1.009)

♂=males. ♀=females. OR=odds ratio, 95% CI=95% confidence interval. MVPA=moderate-to-vigorous physical activity in 10 minutes/day. Hb trajectory: 0=stable, 1=declining. Heme and non-heme iron in mg/day. 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 behavior and MVPA in models with heme and non-heme iron intake as independent variables or sedentary behavior, heme and non-heme iron intake in models with MVPA as independent variable. More than 10% of participants excluded due to missing data in males for heme and non-heme iron intake model 3 and for MVPA model 1-3 and in females for heme and non-heme iron intake and MVPA model 1-3.

Supplementary Table 3. Associations between lifestyle behaviors (heme and non-heme iron intake and MVPA) and Hb trajectories (stable/declining) and mediation by ferritin levels adjusted for confounders.

	Lifestyle behaviors	Mediator	Dependent variable	Total effect (*c* path)*	Effect of lifestyle behaviors on ferritin levels (*a* path)	Effect of ferritin levels on Hb trajectories (*b* path)	Direct effect (*c'* path)	Indirect effect (*a* path * *b* path)
				LN(OR (95% CI))	LN(β (95% CI))	LN(OR (95% CI))	LN(OR (95% CI))	LN(OR (95% BCI))
♂	Heme	Ferritin	Hb trajectories	0.201 (-0.180; 0.591)	0.285 (0.165; 0.406)	-0.244 (-0.542; 0.049)	0.299 (-0.095; 0.705)	-0.070 (-0.171; 0.014)
	Non-heme			-0.059 (-0.116; -0.004)	-0.019 (-0.037; -0.001)		-0.068 (-0.126; -0.012)	0.005 (-0.001; 0.014)
	MVPA			-0.009 (-0.025; 0.008)	-0.001 (-0.006; 0.004)		-0.008 (-0.024; 0.009)	0.000 (-0.002; 0.002)
♀	Heme	Ferritin	Hb trajectories	0.053 (-0.328; 0.435)	0.308 (0.183; 0.433)	0.305 (0.049; 0.565)	-0.050 (-0.442; 0.342)	0.094 (0.013, 0.203)
	Non-heme			-0.041 (-0.109; 0.025)	-0.035 (-0.056; -0.013)		-0.030 (-0.097; 0.037)	-0.011 (-0.026; -0.002)
	MVPA			-0.009 (-0.027; 0.009)	0.001 (-0.005; 0.007)		-0.009 (-0.028; 0.009)	0.000 (-0.002; 0.003)

♂=males. ♀=females. OR=odds ratio; β=regression coefficient; 95% CI= 95% confidence interval; BCI=bootstrapped confidence interval; MVPA=moderate-to-vigorous physical activity in 10 minutes/day; heme and non-heme iron intake in mg/day, 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. Total effect (*c* path): association between heme and non-heme iron intake or MVPA and Hb trajectory; *a* path: association between heme and non-heme iron intake or MVPA and mediating variable ferritin levels; *b* path: association between mediating variable ferritin levels and Hb trajectory; direct effect (*c'* path): association between heme and non-heme iron intake or MVPA and Hb trajectory adjusted for mediating variable ferritin levels; indirect effect: indirect effect of heme and non-heme iron intake or MVPA on Hb trajectory through mediating variable ferritin levels. Adjusted for age, smoking, menstruation (in models with women only), number of donations, donation interval, sedentary behavior, MVPA in models with heme and non-heme iron intake as independent variables or heme and non-heme iron intake in models with MVPA as independent variable.

Supplementary Table 4. Associations between MVPA measured using accelerometers and Hb level/trajectories (stable/declining) and mediation by ferritin levels adjusted for confounders.

	Lifestyle behavior	Mediator	Dependent variable	Total effect (*c* path)	Effect of MVPA on ferritin levels (*a* path)	Effect of ferritin levels on Hb levels/ trajectories (*b* path)	Direct effect (*c'* path)	Indirect effect (*a* path * *b* path)
				β (95% CI)	LN(β (95% CI))	β (95% CI)	β (95% CI)	β (95% BCI)
♂	MVPA	Ferritin*	Hb levels	-0.007 (-0.035; 0.021)	-0.020 (-0.053; 0.013)	0.337 (0.247; 0.426)	0.000 (-0.026; 0.026)	-0.007 (-0.019; 0.003)
♀				0.004 (-0.026; 0.034)	-0.007 (-0.045; 0.031)	0.219 (0.136; 0.302)	0.005 (-0.024; 0.034)	-0.002 (-0.010; 0.008)
				LN(OR (95% CI))	LN(β (95% CI))	LN(OR (95% CI))	LN(OR (95% CI))	LN(OR (95% BCI))
♂	MVPA	Ferritin*	Hb trajectories	-0.114 (-0.263; 0.033)	-0.033 (-0.082; 0.017)	-0.666 (-1.165; -0.196)	-0.149 (-0.306; 0.002)	0.022 (-0.010; 0.077)
♀				-0.041 (-0.187; 0.102)	-0.009 (-0.055; 0.038)	0.076 (-0.342; 0.493)	-0.045 (-0.193; 0.098)	-0.001 (-0.017; 0.011)

♂=males; ♀=females; 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; heme and non-heme iron intake in mg/day, ferritin levels in µg/L; Hb level in mmol/L; Hb trajectory: 0=stable, 1=declining. *Residuals of ferritin levels were not normally distributed and therefore log transformed, this table presents log transformed data. Total effect (*c* path): association between heme and non-heme iron intake or MVPA and Hb level/trajectory; *a* path: association between heme and non-heme iron intake or MVPA and mediating variable ferritin levels; *b* path: association between mediating variable ferritin levels and Hb level/trajectory; direct effect (*c'* path): association between heme and non-heme iron intake or MVPA and Hb level/trajectory adjusted for mediating variable ferritin levels; indirect effect: indirect effect of heme and non-heme iron intake or MVPA on Hb level/trajectory through mediating variable ferritin levels. Adjusted for age, smoking, menstruation (in models with women only), number of donations, and donation interval, sedentary behavior (accelerometer), MVPA in models with heme and non-heme iron intake as independent variables or heme and non-heme iron intake in models with MVPA as independent variable, and initial Hb level (in model with Hb level only).

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