Associations of environmental characteristics and lifestyle behaviours with donor blood parameters

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- CHAPTER FIVE -

Lifestyle behaviours are not associated with haemolysis: results from Donor InSight

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Abstract

Background

Lifestyle behaviours such as physical activity, sedentary behaviour and dietary habits have been shown to influence blood lipid levels, and both lifestyle and blood lipids may be associated with haemolysis during storage of blood products. We aimed to investigate whether lifestyle behaviours are associated with degree of haemolysis in red cell concentrates (RCC), and if such associations are mediated by LDL cholesterol and triglyceride levels.

Material and methods

Cross-sectional analyses were performed in data from 760 Dutch blood donors participating in Donor InSight; an observational cohort study. Linear regression analyses were conducted to assess associations of lifestyle behaviours with haemolysis levels in RCC 28 days after blood sampling. Lifestyle behaviours included moderate-to-vigorous physical activity and sedentary behaviour measured by accelerometry, and self-reported intake of a selection of foods potentially related to blood lipids i.e. consumption of eggs, meat, nuts and fish. Separately, potential mediating roles of both LDL cholesterol and triglyceride levels were investigated. All analyses were adjusted for relevant confounders.

Results

No statistically significant nor substantial associations of any of the lifestyle behaviours with haemolysis in RCC were found, nor were there any associations between lifestyle behaviours and blood lipids. We did find consistent positive associations of LDL cholesterol and triglyceride levels with haemolysis in RCC during storage.

Discussion

In this large cohort, blood lipid levels were consistently associated with haemolysis in RCC. Nonetheless, there was no evidence for an association between lifestyle behaviours and haemolysis in RCC, or for mediating effects by blood lipid levels.

Introduction

Blood supply organizations depend on donors who can give blood or plasma of sufficient quality. An important blood quality criterion is that plasma must have a clear to slightly turbid appearance before freezing, where high blood lipid levels primarily are the cause of non-conformance to this criterion[1,2]. Another quality feature of blood products relates to haemolysis levels. Different manufacturing methods of blood components lead to differences in free haemoglobin levels in plasma units and red cell concentrates (RCC) immediately after processing. For RCCs the degree of haemolysis increases gradually during cold storage. For red blood cells, at the end of the storage period, a maximum of 0.8 percent haemolysis is allowed according to European guidelines[2]. Haemolysis leads to free haemoglobin, which might be toxic for recipients[3,4]. Further, the haemolysed cells must also be cleared from circulation which is not desirable in critically ill patients. Debate continues about whether longer-stored blood negatively affect clinical outcomes[5-8]. Whole blood as well as plasma donations that are classified as lipaemic or haemolytic are discarded. Visits of donors whose blood products are rejected or are of low quality bring a high burden for blood supply organizations. Knowledge on factors underlying these undesirable outcomes is the first step towards diminishing them.

Donor characteristics including sex, age, the presence of subclinical diseases and lifestyle behaviours such as smoking have been associated with increased levels of haemolysis during storage in RCC[9-12]. Exercise-induced intravascular haemolysis has been reported in literature, but mainly in athletes and in particular in endurance athletes such as long distance runners[13,14]. Additionally, lipaemic plasma is associated with increased haemolysis in RCC[1], even when RCC are resuspended in an additive solution with a low remaining plasma content. Plasma lipid levels are partly influenced by lifestyle behaviours. Moderate-to-vigorous physical activity (MVPA) is associated with higher high-density lipoprotein (HDL) cholesterol and declines in levels of low-density lipoprotein (LDL) cholesterol and triglycerides[15,16]. Sedentary behaviour –i.e. any waking activity characterized by an energy expenditure ≤ 1.5 metabolic equivalents (MET) and a sitting or reclining posture–[17] is associated with higher LDL cholesterol and triglyceride levels and lower HDL cholesterol levels[18,19]. High dietary intakes of saturated fat and cholesterol are associated with more unfavourable blood lipid levels, whereas foods high in unsaturated fat and especially omega-3 fatty acids are associated with more favourable blood lipid levels[20-23]. Hence, lifestyle behaviours of donors may be relevant determinants of blood product rejection due to high blood lipid contents or high levels of haemolysis during storage.

As lifestyle behaviours are associated with blood lipid levels and blood lipid levels with haemolysis, we aimed to test the hypothesis that healthy lifestyle behaviours –i.e. higher levels of physical activity, lower levels of sedentary behaviour, more consumption of foods high in unsaturated and omega-3 fatty acids, and less consumption of foods high in saturated fat– are inversely associated with haemolysis in RCC 28 days after blood sampling. We further tested the hypothesis that the associations between lifestyle behaviours and haemolysis in RCC are partly mediated by blood lipids.

Materials and methods

Study-design and study population

The current study was a cross-sectional analysis of data from Donor InSight (DIS) -III, the second follow-up on an observational cohort study of whole blood and plasma donors in the Netherlands, DIS-I started in 2007-2009[24]. The donor eligibility criteria at Sanquin –the only organization authorized to collect and supply blood products in the Netherlands– include a minimum age of 18 years and eligibility to donate according to several criteria as assessed using a donor health questionnaire (DHQ) before each donation. A total of 6140 donors who participated in DIS-I and/or DIS-II were invited to participate in DIS-III between April 2015 and December 2016. Of those, 2,551 participants completed the general questionnaire and provided a blood sample. To objectively measure physical activity and sedentary behaviour, 1,944 DIS-III participants were invited to additionally wear an accelerometer for seven consecutive days of whom 760 provided complete accelerometer data. The present study on lifestyle behaviours and haemolysis included participants with accelerometer data only. The Medical Ethical Committee in the Amsterdam Academic Medical Center approved DIS-III and all participants gave their written informed consent.

Measures

Haemolysis and blood lipid levels

Non-fasting whole blood samples were collected in a 2 mL Ethylene-Diamine-Tetra-Acetic acid (EDTA) and a 3 mL lithium heparin tube from the diversion pouch. This pouch collects the first 20-30 mL of a donation and is routinely used for screening and blood typing purposes. For DIS-III, a venepuncture was performed if no donation was provided.

The primary outcome variable was haemolysis level 28 days after collection of the blood sample, expressed as the percentage of free haemoglobin of the total haemoglobin present in the red blood cells after correction for haematocrit. Potentially mediating variables were LDL cholesterol and triglyceride levels.

Preparation of miniature red cell concentrates and plasma samples
We used a model system to study haemolysis in miniature RCC, reflecting the degree of haemolysis in the RCC prepared by standard Dutch blood bank procedures from the corresponding whole blood unit. As our standard RCCs are resuspended in SAGM (saline adenine glucose mannitol) and have only a low amount of residual plasma, the conditions to produce miniature RCC were selected to reflect the composition of our standard RCCs. The miniature RCC differ with respect to being leukoreduced and not leukodepleted as only buffy coat was removed, without additional filtration step and the storage container, not being a polyvinylchloride di(2-ethylhexyl) phthalate (PVC-DEHP) container, but a Eppendorf cup (VWR International, Amsterdam, the Netherlands). Increased haemolysis has previously been found in blood bags without phthalate-based plasticizers, and the Eppendorf cups used in this study have no plasticizers at all[25-27]. Leucocyte reduction in stored RCCs has a favourable effect on haemolysis due to less accumulation of cytokines and diminished release of enzymes by leucocyte[28,29]. Due to these negative storage effects haemolysis was measured after 28 days of storage instead of after the conventional 35 days. Post-hoc analyses were conducted to gain better insight in the actual agreement of haemolysis between these two storage methods. The miniature RCC haemolysis levels exceeded that of standard blood units 3.42 times. Further, exponentially plotting the standard blood unit measurements indicated haemolyses as measured in 28 days stored miniature RCCs resembled that of a 63 days stored standard whole blood unit (see Appendix A)[30]. Total haemoglobin was measured from the 2 ml EDTA tube with a haematology analyser (Sysmex XT 2000T, Kobe, Japan) after homogenization. To produce the miniaturized RCC the sample was centrifuged for five minutes at 2000 x g. 600 microliter of the lower erythrocyte pellet was diluted with 400 microliter SAGM to a haematocrit of about 60% and stored in 1.5 mL Eppendorf cups at 2-6°C for 28 days (under similar conditions as standard RCC). At day 28 of storage, full blood count was repeated, followed by centrifuging the Eppendorf cups for 5 minutes at 18,000 x g. The supernatant was transferred to a clean Eppendorf cup and centrifuged again (18,000 x g, 5 min). For measurement of free haemoglobin, 50 microliters were pipetted into a 96 wells plate and supplemented with 200 microliter of distilled water and homogenized. Free haemoglobin of the RCC was determined at 415 nm by a spectrophotometer (Biotek EON plate reader, Winooski, U.S.A.).

Total cholesterol (TC), HDL cholesterol and triglycerides were determined by enzymatic colorimetric methods using the plasma from the lithium heparin tubes (Roche/Hitache Cobas C, Basel, Switzerland). LDL cholesterol levels were calculated using the Friedewald formula: TC cholesterol – HDL

cholesterol– (TG / 2.2)[31].

Lifestyle behaviours
Physical activity was operationalized as mean minutes per day of MVPA (≥3 MET). Sedentary behaviour (≤ 1.5 MET) was also expressed in mean minutes per day. Both MVPA and sedentary behaviour were measured by means of accelerometers (wGT3X-BT and GT3X Actigraph, Pensacola, U.S.A.). Troiano Adult (2008) cut-off points for MVPA and sedentary behaviour were used[32]. To calculate mean minutes per day per category, the total number of minutes were divided by the number of valid days. A day was considered valid if the wear time was at least ten hours with a minimum of four valid days[33]. A date as close to the blood donation date as possible was pursued. Dietary behaviour was estimated using items of a short food frequency questionnaire (FFQ). The FFQ used for this study was originally designed to assess dietary iron intake, but covered a number of foods high in saturated fat and/or cholesterol or high in unsaturated and omega-3 fatty acids for which a relation with blood lipids has been established in earlier research. These foods were fish and nuts –rich sources of unsaturated and omega-3 fatty acids, for which an inverse association with LDL cholesterol and triglycerides has been found[34,35]; and meat and eggs –rich sources of saturated fat (meat) and dietary cholesterol (both meat and eggs) for which a positive association with LDL cholesterol and triglycerides has been found in earlier research[36]. All food items, questions and answer categories are presented in Appendix B.

Covariates
The following self-reported variables were tested as potential confounders in the analyses: smoking status (yes/no), sex, age and the use of lipid-modifying medication. Medication was classified according to the World Health Organization recommended Anatomical Therapeutic Chemical classification (ATC) system. All drugs with ATC code *C10 Lipid modifying agents* were considered lipid-modifying medication.

Statistical analyses
Descriptive statistics are presented as mean ± standard deviation (SD), or in the case of a skewed distribution as median and interquartile range (IQR).

Missing data
Missing data were assumed to be missing at random. Item non-response ranged from 0.1% (lipid-modifying medication) to 6.2% (smoking) with 91% of the participants with complete data, therefore multiple imputation was performed on an item-score level using Predictive Mean Matching[37]. All missing data in variables that were used for analyses were imputed. A total of

ten imputed datasets were generated as recommended by White et al., 2011[37]. Alcohol use was not included in the analysis and therefore not imputed, but did serve as a predictor of the missing data. Time spent in sedentary, light, moderate and vigorous activity had no missing data, but were also used in the imputation model to predict the missing values.

Mediation analyses

To assess whether lifestyle behaviours were associated with haemolysis four weeks after blood sampling, and whether blood lipid levels mediated these associations, mediation analyses (with linear regression) were done using the framework of Preacher and Hayes and the work of Baron and Kenny[38,39]. A graphical representation to illustrate the mediation design is provided in Figure 1. Panel A of Figure 1 shows the association of X (lifestyle behaviours) with Y (haemolysis) and is marked with the letter *c*. From Panel B of Figure 1 three pathways can be distinguished, the *c'*-pathway indicates the association of X on Y after adjusting for M (blood lipid levels). Consistent with the definition of Baron and Kenny (1986), M is considered to be a mediator if X significantly predicts Y (c-pathway), and X significantly predicts M (a-pathway), while M significantly predicts Y adjusting for X (b-pathway). It was hypothesized that regression coefficients of the lifestyle behaviours and haemolysis (X – Y) would diminish after M was added to the model. In the present analyses we did mediation analyses with three lifestyle behaviours (physical activity, sedentary behaviour and food items; X) and haemolysis (Y) separately of each other mediated by LDL cholesterol and triglycerides (M). Because of collinearity between LDL cholesterol and triglyceride levels, LDL cholesterol and triglycerides have been analysed separately[40]. Finally, a mediation analysis with all lifestyle behaviours in one model was done to test whether the lifestyle behaviours were independently associated with haemolysis.

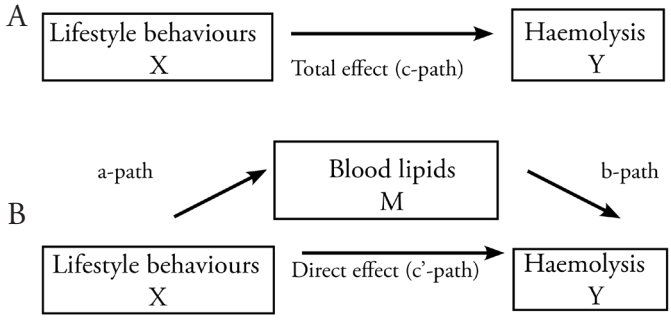

Figure 1: Mediation analysis framework.

(A) Association of X on Y. (B) X is associated by Y through M.

To increase readability of the analyses, regression coefficients for associations with haemolysis were expressed in hundredths of a percent. A covariate was considered a confounder if the regression coefficient of the determinant changed with >10%. P-values below 0.05 were considered statistically significant. Statistical analyses were performed using SPSS version 23.0.

Results

A total of 1,269 (65%) of the donors invited to wear an accelerometer were interested. Of these, 800 randomly chosen donors were contacted to participate and received an accelerometer by post. Complete accelerometer data was provided by 760 participants, an overview of reasons for not providing data is shown in Figure 2. Table 1 provides characteristics of the study sample. The majority of the participants were female (n=411, 54%) and active as donors, meaning that they were registered as available for invitations to give a donation (n=571, 75%). Median haemolysis level in miniature RCC 28 days after blood sampling was 1.26% (IQR: 1.00 – 1.62%).

Table 1: Participant characteristics.

Participants	760
Male	349 (46)
Age (years)	50.6 ± 13.1
Donor status (activea)	571 (75)
Physical activity – light (minutes per day)	309 (259 - 369)
Physical activity – moderate (minutes per day)	27 (18 - 41)
Physical activity – vigorous (minutes per day)	0 (0 - 2)
MVPA (minutes per day)	29 (19 - 45)
Sedentary time (minutes per day)	550 (491 - 600)
Current smoker	62 (9)
Alcohol consumption	
None	89 (12.6)
< once a week	166 (23.5)
1-2 days a week	188 (26.6)
3-5 days a week	153 (21.6)
almost every day	111 (15.7)
Haemolysisb (%)	1.26 (1.00 – 1.62)
LDL cholesterol (mmol/l)	2.92 ± 0.84
Triglycerides (mmol/l)	1.27 (0.93 – 1.74)

Values are N(%), mean±SD, or median (interquartile range).[a]Donors who could be invited to donate according to the blood bank information system, eProgesa. [b]Haemolysis in miniature RCC 28 days after blood sampling. MVPA: moderate to vigorous physical activity.

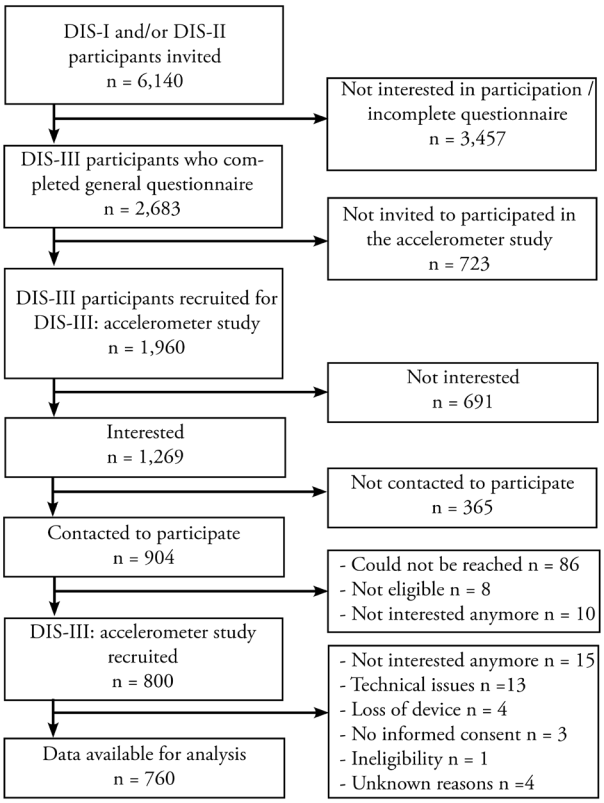

Figure 2: Flow chart of accelerometer study DIS: Donor InSight Study.

Lifestyle behaviours and haemolysis in RCC

The results obtained with linear regression analyses are presented in Table 2 for the model with LDL cholesterol and triglycerides as potential mediators. Analyses were adjusted for the following confounders: age, sex, smoking and lipid-modifying medication. The hypothesized inverse associations of lifestyle behaviours and haemolysis in RCC were found for MVPA, fish and nut consumption, however these associations were not statistically significant. Meat consumption was positively, but not statistically significantly associated with haemolysis in RCC. The expected positive associations of the other lifestyle behaviours considered unhealthy and haemolysis in RCC were not found, and these were also not statistically significant. The results of the models with all lifestyle behaviours showed similar results.

Lifestyle behaviours and haemolysis with potential mediators

The expected diminishment of the regression coefficients for associations of lifestyle behaviours and haemolysis in RCC after incorporating LDL cholesterol as potential mediator was only found for the unhealthy food items

Table 2: Mediation analysis[a] - Associations of lifestyle behaviours with haemolysis in RCC.

	LDL mediation model			
	c-pathway lifestyle behaviour - haemolysis[a] Beta (95%CI)	c'pathway lifestyle behaviour – haemolysis[a] Beta (95%CI)	a-pathway lifestyle behaviour - LDL Beta (95%CI)	b-pathway LDL – haemolysis[a] Beta (95%CI)
MVPA	-1.78 (-3.66 to 0.10)	-1.77 (-3.65 to 0.11)	0.02 (0.00 to 0.04)	7.95 (2.90 to 13.00)
Sedentary behaviour	-0.1 (-0.61 to 0.41)	-0.13 (-0.64 to 0.37)	0.01 (0.00 to 0.01)	7.78 (2.58 to 2.90)
Meat and egg consumption				
Eggs	-16.5 (-43.74 to 10.74)	-14.2 (-41.44 to 13.04)	-0.17 (-0.56 to 0.22)	7.53 (2.46 to 12.60)
Meat	2.60 (-5.63 to 10.83)	1.8 (-6.43 to 10.05)	0.11 (0.00 to 0.22)	7.53 (2.46 to 12.60)
Fish and nuts consumption				
Fish	-24.79 (-54.41 to 4.83)	-24.19 (-53.43 to 5.05)	0.07 (-0.33 to 0.47)	7.79 (2.74 to 12.84)
Nuts	-10.33 (-38.24 to 17.58)	-8.62 (-36.43 to 19.19)	-0.03 (-0.42 to 0.36)	7.79 (2.74 to 12.84)
	TG mediation model			
	c-pathway lifestyle behaviour - haemolysis[a] Beta (95%CI)	c'pathway lifestyle behaviour – haemolysis[a] Beta (95%CI)	a-pathway lifestyle behaviour - TG Beta (95%CI)	b-pathway TG – haemolysis[a] Beta (95%CI)
MVPA	-1.78 (-3.66 to 0.10)	-1.77 (-3.65 to 0.11)	0.02 (0.00 to 0.04)	7.95 (2.90 to 13.00)
Sedentary behaviour	-0.1 (-0.61 to 0.41)	-0.13 (-0.64 to 0.37)	0.01 (0.00 to 0.01)	7.78 (2.58 to 2.90)
Meat and egg consumption				
Eggs	-16.5 (-43.74 to 10.74)	-14.2 (-41.44 to 13.04)	-0.17 (-0.56 to 0.22)	7.53 (2.46 to 12.60)
Meat	2.60 (-5.63 to 10.83)	1.8 (-6.43 to 10.05)	0.11 (0.00 to 0.22)	7.53 (2.46 to 12.60)
Fish and nuts consumption				
Fish	-24.79 (-54.41 to 4.83)	-24.19 (-53.43 to 5.05)	0.07 (-0.33 to 0.47)	7.79 (2.74 to 12.84)
Nuts	-10.33 (-38.24 to 17.58)	-8.62 (-36.43 to 19.19)	-0.03 (-0.42 to 0.36)	7.79 (2.74 to 12.84)

[a]All models are adjusted for age, sex, smoking and lipid modifying medication. To increase readability of the analyses regression coefficients for associations with haemolysis were expressed in hundredths of a percent. MVPA: moderate-to-vigorous physical activity, LDL: low density lipoprotein cholesterol, TG: triglycerides. Food items are presented per 100 grams per day, MVPA and sedentary behaviour are presented per 10 minutes.

and fish consumption (Table 2). The regression coefficient of egg consumption decreased marginally from β= -16.50 (95% CI -43.74 – 10.74) to β= -14.20 (95% CI -41.44 – 13.04). All associations were statistically insignificant. A similar pattern was found with triglycerides as potential mediator as can be seen in Table 2. The model with all lifestyle behaviours showed the same results, regardless of the potential mediator (Table 3).

Lifestyle behaviours and blood lipids

We found meat consumption being statistically significantly associated with LDL cholesterol and triglyceride levels, β= 0.11 (95% CI 0.00 – 0.22) and β=0.21 (95% CI 0.10 – 0.31) respectively, indicating that an increase of 100 gram meat intake is associated with an increase of 0.11 mmol/L LDL cholesterol. The expected inverse association of healthy lifestyle behaviours and blood lipid levels was found for fish consumption only, however without statistical significance. Non-significant positive associations were found for haemolysis in RCC with time spent in sedentary behaviour and with meat consumption.

Blood lipids and haemolysis

Higher LDL cholesterol levels were significantly associated with higher levels of haemolysis in RCC after adjusting for sedentary behaviour (β=7.78, 95% CI 2.58– 2.90). The regression coefficients for the association of LDL cholesterol and haemolysis in RCC changed slightly when adjustments for lifestyle behaviours were made, but remained statistically significant. Effect sizes found for the association of triglyceride levels and haemolysis in RCC (β=17.75 95% CI 12.50 – 23.14) were higher than the effect sizes for the association of LDL cholesterol and haemolysis in RCC. This indicates that a 1 mmol/L higher triglyceride level is associated with 1.78 percent point higher haemolysis (as previously stated, analyses of associations with haemolysis were expressed as hundredths of a percent to increase the readability). Table 3 shows that in the model with all lifestyle behaviours and haemolysis LDL cholesterol was also significantly associated with haemolysis in RCC (β=7.93 95% CI 2.86 – 13.01).

Discussion

No evidence was found for associations between measured lifestyle behaviours and haemolysis levels in RCC during storage. Neither were lifestyle behaviours associated with blood lipid levels nor did we find statistically significant associations of lifestyle behaviours with haemolysis in RCC with the blood lipids as mediators. We did find that both LDL cholesterol and triglycerides were significantly associated with haemolysis levels in RCC during storage. As significance of all previously mentioned associations is required (according

Table 3: Mediation analysis[a] - Associations of all lifestyle behaviours with haemolysis in RCC.

	LDL mediation model			
	c-pathway lifestyle behaviour - haemolysis[a] Beta (95%CI)	c'pathway lifestyle behaviour – haemolysis[a] Beta (95%CI)	a-pathway lifestyle behaviour - LDL Beta (95%CI)	b-pathway LDL – haemolysis[a] Beta (95%CI)
MVPA	-1.93 (-3.91 to 0.05)	-1.97 (-3.95 to 0.00)	0.02 (-0.01 to 0.05)	7.93 (2.86 to 13.01)
Sedentary behaviour	-0.25 (-0.78 to 0.29)	-0.29 (-0.82 to 0.25)	0.01 (0.00 to 0.01)	7.93 (2.86 to 13.01)
Meat and egg consumption				
Eggs	-12.27 (-40.65 to 16.11)	-1.02 (-38.52 to 18.08)	-0.18 (-0.57 to 0.20	7.93 (2.86 to 13.01)
Meat	1.29 (-7.05 to 9.62)	0.04 (-7.93 to 8.67)	0.12 (0.01 to 0.23)	7.93 (2.86 to 13.01)
Fish and nuts consumption				
Fish	-20.02 (-50.68 to 10.63)	-2.03 (-50.68 to 10.00)	0.16 (-0.25 to 0.58)	7.93 (2.86 to 13.01)
Nuts	-8.47 (-36.57 to 19.62)	-0.65 (-34.54 to 21.44)	-0.07 (-0.46 to 0.32)	7.93 (2.86 to 13.01)
	TG mediation model			
	c-pathway lifestyle behaviour - haemolysis[a] Beta (95%CI)	c'pathway lifestyle behaviour – haemolysis[a] Beta (95%CI)	a-pathway lifestyle behaviour - TG Beta (95%CI)	b-pathway TG – haemolysis[a] Beta (95%CI)
MVPA	-1.93 (-3.91 to 0.05)	-1.63 (-3.56 to 0.31)	0.00 (-0.03 to 0.02)	18.59 (13.41 to 23.76)
Sedentary behaviour	-0.25 (-0.78 to 0.29)	-0.25 (-0.77 to 0.28)	0.00 (0.00 to 0.01)	18.59 (13.41 to 23.76)
Meat and egg consumption				
Eggs	-12.27 (-40.65 to 16.11)	-10.63(-38.25 to 17.00)	-0.07 (-0.43 to 0.30)	18.59 (13.41 to 23.76)
Meat	1.29 (-7.05 to 9.62)	-1.10 (-9.21 to 7.09)	0.21 (0.10 to 0.31)	18.59 (13.41 to 23.76)
Fish and nuts consumption				
Fish	-20.02 (-50.68 to 10.63)	-20.34 (-50.26 to 9.59)	0.13 (-0.26 to 0.52)	18.59 (13.41 to 23.76)
Nuts	-8.47 (-36.57 to 19.62)	-1.45 (-28.99 to 26.09)	-0.34 (-0.70 to 0.03)	18.59 (13.41 to 23.76)

[a]All models are adjusted for age, sex, smoking and lipid modifying medication. To increase readability of the analyses regression coefficients for associations with haemolysis were expressed in hundredths of a percent. MVPA: moderate-to-vigorous physical activity, SB: sedentary behaviour, LDL: low density lipoprotein cholesterol, TG: triglycerides. Food items are presented per 100 grams per day, MVPA and SB are presented per 10 minutes.

to the criteria of Baron and Kenny[39]) for a variable to be a mediator, we did not find evidence that LDL cholesterol or triglycerides were mediators in the association of lifestyle behaviours on haemolysis in RCC during storage.

We hypothesized that physically active donors would provide red blood cells with lower levels of haemolysis during storage, mainly focusing on the indirect effect of physical activity on haemolysis in RCC through blood lipid levels. In contrast to earlier findings on physical activity and blood lipids in men in a systematic review of randomized controlled trials on exercise and blood lipids by Kelley and Kelley (2006), we did not find lower levels of LDL cholesterol and triglycerides in donors who spent more minutes in MVPA; associations were inconsistent and non-significant (-0.01 and 0.02 mmol/L respectively for triglycerides and LDL cholesterol)[15]. This discrepancy between the present study and the systematic review might be due to differences between study populations. Mean triglycerides and LDL cholesterol at baseline were 1.5 and 3.7 mmol/L, respectively, in studies reported in the systematic review. In the present study, blood lipid levels were lower (median triglycerides 1.3 mmol/L, mean LDL cholesterol 2.9 mmol/L); even the donors who were less physically active had relatively low blood lipid levels. The target populations of the randomized controlled trials included in the review of Kelley and Kelley (2006) more often consisted of participants with an already increased (cardiovascular) disease risk or even diagnosed diseases. Due to eligibility screening and self-selection, donors are generally a more 'healthy' subset of the general population[41,42]. This might have caused a lack of variation in blood lipid levels as the blood lipid levels are well within the normal range. The same accounts for physical activity; an absolute increase of 10 minutes MVPA in a person who is already engaged in the recommended 150 minutes of MVPA per week probably results in less lowering of blood lipid levels as compared to 10 minutes more MVPA in a person who is not physically active[43]. The median time spent in MVPA was 203 minutes per week in our study, which is significantly higher than the (updated) recommendation of 150 minutes per week[44]. Another Dutch population-based cohort study reported similar levels of objectively measured physical activity, namely an average of 202 minutes MVPA per week[45].

Both triglycerides and LDL cholesterol were significantly associated with haemolysis levels in RCC. As residual plasma in RCC can have a high impact on haemolysis levels, the design of the haemolysis model was set up to have comparable, low residual plasma levels as in our standard RCC. Because the amount of residual plasma differ between studies with RCC produced by different methods, different associations between donor blood lipids and haemolysis can be expected in other studies. Our findings are in line with

previous research by Bashir et al., where haemolysis levels for red cells in lipaemic and non-lipaemic plasma were compared after 24 and 48 hours in different storage conditions[46]. In the study by Bashir et al., the associations were stronger which could be explained by storage of RCCs in 100% plasma, whereas in our study the percentage plasma was about 15%. De Korte et al., also reported a major difference in haemolysis level after 35 days between lipaemic and non-lipaemic donations with more haemolysis in the lipaemic donations[47,48]. Triglyceride levels were measured and were more than three times higher in the lipaemic donations as compared to the non-lipaemic donations. Despite the limited range in blood lipids and haemolysis levels, and the restricted number of donors studied, the associations of lipids with haemolysis were strong and consistently significant.

The degree of haemolysis in the miniaturized RCCs was high with a median of 1.26%, as compared with the European guidelines that state a maximum haemolysis of 0.8% in blood bags. As explained previously, our miniaturized RCC samples were non-leukodepleted and stored in Eppendorf cups, which both have negative effects on storage quality conditions[25-27]. Although the storage quality in Eppendorf cups is obviously lower, there is agreement - the miniature RCC haemolysis exceed the standard whole blood unit haemolysis 3.42 times - between measurements obtained from both storage conditions (Appendix A).

To our knowledge this is the first study on lifestyle behaviours and haemolysis in RCC in a large cohort of donors. The key strength of this study lies in the thorough assessment of physical activity and sedentary behaviour of donors, using accelerometry rather than less reliable questionnaire data[49].[44] Also, the sample size with regard to the haemolysis measurements is a strong point; previous laboratory studies that investigated associations of lipid levels with haemolysis in RCC used smaller numbers[46,48]. Another asset was the high willingness (65%) of DIS-III participants to participate in the accelerometer study. There are also a number of limitations. A drawback of our study is that we analysed dietary behaviour using four items of a questionnaire that was not designed originally to estimate lipid intake and absorption. We chose these four items based on strong evidence of epidemiological studies[34]. The questionnaire aimed to assess average intake, however, it would have been informative to also know how much foods high in saturated lipids and cholesterol were consumed prior to donation[50]. The lack of significant associations might be a consequence of the way dietary behaviour was estimated in this study. Further, although haemolysis levels were quite high in this study, the variation was relatively small. The latter is likely due to the selection of participants of the observational DIS cohort. Selection for this study was not

based on previous haemolysis levels as was done in another study[12] which can be a reason for less variation but may better reflect haemolysis levels in a general donor population.

The findings of this study suggest that the investigated lifestyle behaviours of donors are not associated with haemolysis levels during storage of RCC, and these lifestyle behaviours thus do not appear to present any major concern in the present population of Dutch blood donors. However, because lipid levels were consistently associated with haemolysis in RCC, lipid levels may be helpful in the selection of donors in the future in case mean haemolysis levels rise in the blood product pool, to prevent outliers if phthalate-plasticized blood collection bags are prohibited.

Conclusion

In this population of Dutch blood donors, lifestyle factors are not associated with haemolysis levels in miniature RCC 28 days after blood collection. We also did not find evidence of mediating effects of LDL cholesterol or triglyceride levels. Nonetheless, both LDL cholesterol and triglyceride were strongly associated with haemolysis levels in RCC, which warrants replication and further exploration into potential implications.

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Conflict of interest

The Authors declare no conflicts of interest.

Author contributions

RdG, JL, JB, JWB, DdK, WLAMdK and KvdH conceived and designed the study. TH contributed to the study design. RdG and JWB analysed the data. RdG wrote the manuscript with input from all Authors. All Authors read and approved the final manuscript.

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Appendices

Appendix A - Food items and answering scales

Variables	Question	Answer possibilities
Fish	How often did you eat fish last month?	Not, one day per four weeks, 2-3 days per four weeks, and 1 to 7 days per week with intervals of 1 day.
	How many servings did you consume on such a day?	0.5 to 3.0 with intervals of 0.5.
Nuts	How often did you eat nuts last month ? (Do not count peanuts)	Not, one day per four weeks, 2-3 days per four weeks, and 1 to 7 days per week with intervals of 1 day.
	How many handfuls of nuts did you eat on such a day?	1 to 12 with intervals of 1
Meat	How often did you eat meat the last month?	Not, one day per four weeks, 2-3 days per four weeks, and 1 to 7 days per week with intervals of 1 day.
	How many servings did you eat on such a day?	0.5 to 3.0 with intervals of 0.5.
Eggs	How often did you eat eggs last month?	Not, one day per four weeks, 2-3 days per four weeks, and 1 to 7 days per week with intervals of 1 day.
	How many eggs did you eat on such a day?	1 to 12 with intervals of 1

Appendix B – Haemolysis measurements

Agreement between haemolysis measurements in miniature RCCs and standard whole blood units was assessed by using data from 12 donors who provided a whole blood unit (500 ml) and a whole blood sample (2 ml in EDTA tube). In the miniature RCCs haemolysis was measured at 28 days and in the standard whole blood unit at day 1, 35 and 42. Whole blood units were collected and processed according to the standard operating procedures of Sanquin and the whole blood samples as described in the methods section of this paper. Haemolysis during storage is not a linear process, but can better be described as exponential[51].

Bland and Altman plot

A Bland and Altman plot shows the difference between two measurement methods on the same subject. Differences are plotted against the mean of the two measurements[30,52]. This mean is displayed on the x-axis, on the y-axis the differences between the two measurements is shown. As the data for this plot was log transformed, the graph shows ratios rather than differences.

Figure S1 shows that the geometric mean ratio of the miniature RCC (28 days) and the standard whole blood unit (35 days) was 3.42 with 95% limits of agreement from 1.25 to 5.60[30]. Thus the miniature RCC haemolysis measurements exceeded the standard whole blood unit haemolysis measurements 3.42 times.

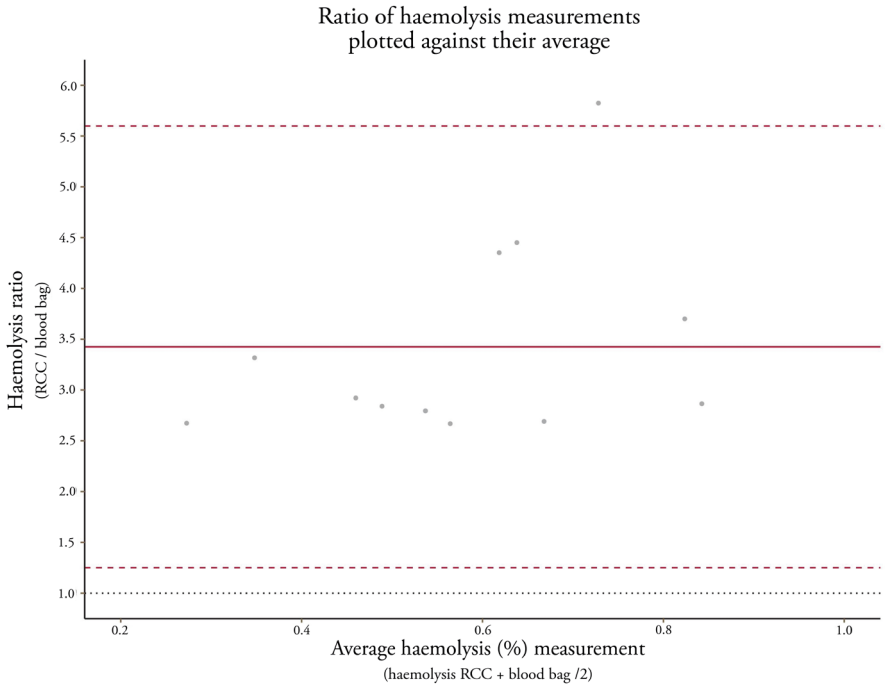

Figure S1: Bland and Altman plot.
The red solid line shows the geometric mean ratio and the red dashed lines indicate the 95% upper and lower limits of agreement.

Comparison of 28 days stored miniature red cell concentrate and whole red cell concentrates

Mean haemolysis levels of whole RCCs (n=12, ●) as measured during 1, 35 and 42 days of storage at 2-6°C are depicted in Figure S2. The haemolysis results could best be explained by an exponentially fitted curve (——, R^2 = 0.992). From this curve, it could be extrapolated that haemolysis, as observed in 28 days stored miniaturize RCCs, (○) was comparable with that of 63 days stored whole RCCs. This result corroborated well with a previous internal study where haemolysis levels after 56 days of storage amounted to 0.70-0.95% (n=4, □).

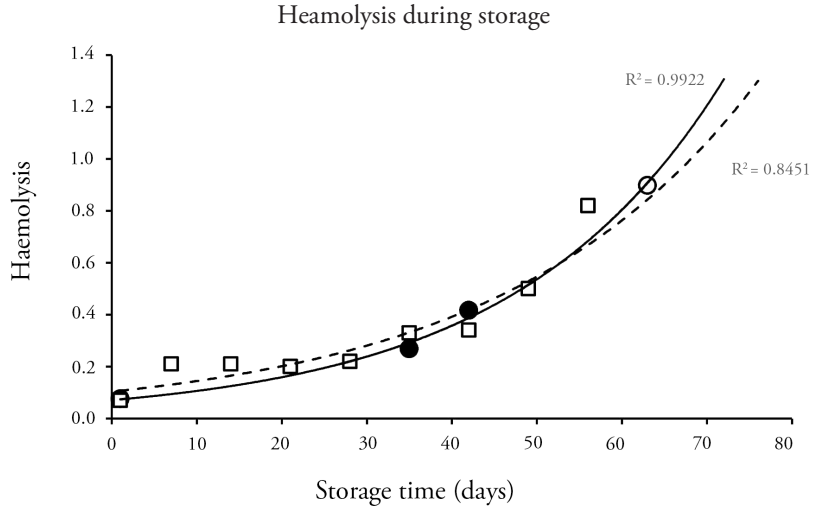

Figure S2: Haemolysis during storage.

Whole RCCs (n=12, ●) were stored at 2-6°C and were analysed for haemolysis at day 1, 35 and 42. Results were exponentially fitted (——). Miniaturized RCCs (n=12, ○) were analysed for haemolysis after 28 days of storage. □: results of an previous internal storage with weekly haemolysis measurements till day 56 (---, exponentially fit).