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Original article

Iron loading, alcohol and mortality: A prospective study

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SUMMARY

Background & aims: The relationship between total body iron and cardiovascular disease remains controversial and information absent in black sub-Saharan Africans in whom alcohol consumption tends to be high. The level of total body iron is tightly regulated, however this regulation is compromised by high alcohol intake causing iron loading. The aim of this study is to investigate total body iron, as represented by serum ferritin, and its interaction with measures of alcohol intake in predicting all-cause and cardiovascular mortality.

Methods: We followed health outcomes for a median of 9.22 years in 877 randomly selected HIV negative African women (mean age: 50.4 years).

Results: One hundred and five deaths occurred of which 40 were cardiovascular related. Ferritin averaged 84.0 (5th to 95th percentile interval, 7.5–533.3) ng/ml and due to the augmenting effect of inflammation, lowered to 75.3 (6.9–523.2) ng/ml after excluding 271 participants with high-sensitivity C-reactive protein (CRP) levels (above 8 mg/l). CRP increased by quartiles of ferritin in the total group (P trend = 0.002), but this relationship was absent after excluding the 271 participants with high CRP values (P trend = 0.10). Ferritin, gamma-glutamyl transferase and carbohydrate deficient transferrin (all $P < 0.0001$) were higher in drinkers compared to non-drinkers, but CRP was similar (P = 0.77). In multivariable-adjusted analyses, ferritin predicted both all-cause (hazard ratio, 2.08; 95% confidence interval, 1.62–2.68; $P < 0.0001$) and cardiovascular (1.94; 1.29–2.92; $P = 0.002$) mortality. In participants with CRP levels below or equal to 8 mg/l, the significant relationship remained between ferritin and all-cause (2.51; 1.81–3.49; $P < 0.0001$) and cardiovascular mortality (2.34; 1.45–3.76; $P = 0.0005$). In fully adjusted models, interactions existed between ferritin and gamma-glutamyl transferase, self-reported alcohol use and carbohydrate deficient transferrin in predicting all-cause ($P \leq 0.012$) and cardiovascular mortality ($P \leq 0.003$).

Conclusions: Iron loading in African women predicted all-cause and cardiovascular mortality and the intake of alcohol seems mechanistically implicated.

1. Introduction

People living in sub-Saharan Africa have the lowest life expectancy [1], burdened by high rates of infectious and cardiovascular disease [2]. Even coronary artery disease is on the increase [2] which was previously an uncommon finding in black populations generally known to have a favorable lipid profile [3]. The prevalence of micronutrient deficiencies in sub-Saharan Africans is high [4] and the fortification of maize meal and wheat flour with especially iron [4,5] is common since iron deficiency anemia is the most prevalent hematological disorder [6,7]. This anemia burden tends to overshadow the hypothesis involving iron loading and disease which may also contribute to the high morbidity and mortality burden in black sub-Saharan Africans [8].

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The level of body iron is tightly regulated by the hepcidin-ferroportin axis [9]. However, iron loading ensues when this regulatory axis is compromised with high alcohol intake causing down-regulation of hepatic hepcidin transcription [10]. The result is uncontrolled iron absorption through enterocytes and release by macrophages and hepatocytes via the iron exporter ferroportin [9–12]. Through the generation of reactive oxygen species, notably hydroxyl radicals [13,14], iron loading has many pathological effects [15], especially on the cardiovascular system [16–22]. However, the relationship between total body iron and cardiovascular disease remains controversial [23], and information absent in black sub-Saharan Africans in whom alcohol consumption tends to be high [24]. As part of the South African leg of the Prospective Urban Rural Epidemiology (PURE) study, we investigated in 877 randomly selected African women whether total body iron predicts all-cause and cardiovascular mortality and whether interactions exist with measures of alcohol intake.

2. Materials and methods

2.1. Study population

The Health Research Ethics Committee of the North-West University approved this sub-study as part of the multinational PURE study [25]. At baseline, the South African cohort included 2010 randomly selected black South Africans from both urban (n = 1004) and rural (n = 1006) areas from the North West Province. Participants invited were older than 35 years and reported the absence of any known diseases. Each participant gave informed consent after an introduction to the research setup and explanation of the procedures. Participants could withdraw from the study at any stage. The protocol complied with the Declaration of Helsinki of 1975 (as revised in 1983). All the women were given feedback on their general health. If health problems were identified, the women were referred to the local clinic or hospital.

We excluded all men due to lacking data on ferritin (n = 746), 218 women infected with the human immunodeficiency virus, and 169 who had missing information, leaving 877 women for statistical analysis.

2.2. Measurements at baseline

Trained fieldworkers completed the demographic and lifestyle questionnaires in the participants' home language, obtaining information on self-reported current smoking and alcohol use, medical history, and menopausal status.

Anthropometrists measured body height with a stadiometer (SECA, Germany) and body weight with a portable electronic scale (A&D Medical, UK) while participants wore light indoor clothing and no shoes. We assessed physical activity using a modified Baecke's physical activity questionnaire giving a continuous physical activity score. This questionnaire is deemed reliable and valid for South African adults when compared with 24-h activity recall [26].

Systolic- and diastolic blood pressure were measured with the validated OMRON HEM-757 device (OMRON HEM-757, Omron Healthcare, Kyoto, Japan). After a 10-min rest period, brachial BP measurements were performed in duplicate (five minutes apart) on the right arm, while the participants were seated upright and relaxed with the right arm supported at heart level.

Fasting serum and plasma were prepared according to appropriate methods. Irrespective of collection site, all samples were snap frozen on dry ice. Where samples were obtained in rural areas, serum was stored at −18 °C (for a maximum of five days) until transported to the laboratory for storage at −80 °C until further analysis. Ferritin is stable in serum or plasma for seven days when stored at 2–8 °C [27] and may be frozen at −20 °C to −70 °C for years before analysis [28].

Quantitative determination of serum total cholesterol, glucose, the liver enzymes and high-sensitivity C-reactive protein (CRP) were analyzed using the Sequential Multiple Analyzer Computer (Konelab 20i™ auto-analyzer, Thermo Fisher Scientific Oy, Vantaa, Finland). Serum ferritin concentrations were determined quantitatively using an enzyme immunoassay procedure (Ramco Laboratories, Inc., Stafford Texas). Serum % carbohydrate deficient transferrin analyses were performed using an *in vitro* heterogeneous immunoassay with column separation followed by a turbidimetric measurement (Axis-Shield %CDT kit, Oslo, Norway). The coefficient of variation for all assays was less than 10%.

2.3. Assessment of outcome

To retain participants and ascertain their vital status, fieldworkers under supervision of a senior researcher performed three-monthly follow-up visits. The cause of death was obtained from the family's death certificate and verbal autopsy and coded by a physician according to the International Classification of Disease codes (10th edition) for the underlying causes of death.

Cardiovascular mortality included all fatal cardiac and stroke events. Death due to cardiac reasons included heart failure, congestive heart failure, myocardial infarction, or any other cardiac-related reason. Death due to stroke included any stroke or cerebral vascular incident.

2.4. Statistical analysis

We used SAS software, version 9.4 (SAS Institute Inc., Cary, NC) for database management and statistical analysis. Means and proportions were compared by the standard normal z-test and the χ^2 statistic, respectively, and survival curves by Kaplan–Meier survival function estimates and the log-rank test. Statistical significance was set at a level of 0.05 on 2-tailed tests.

We used Cox proportional hazard regression to calculate standardized relative hazard ratios expressing the risk for a 1-standard deviation increase in the independent variables, while allowing for covariables and potential confounders. Using the forward stepwise procedure, we included baseline age, body mass index, aspartate transaminase-to-alanine transaminase ratio, CRP, glucose, physical activity, systolic blood pressure, self-reported smoking and alcohol use, and menopausal status as covariables. Due to the augmenting effect of inflammation on ferritin levels, irrespective of iron status [29], we repeated our analysis after excluding 271 participants with CRP levels above 8 mg/l [30]. We used the Kolmogorov-type supremum test to check the proportional hazards assumption. Finally, we plotted the 10-year risk of all-cause and cardiovascular mortality in relation to ferritin and CRP.

3. Results

3.1. Baseline characteristics

The baseline characteristics of the participants by quartiles of ferritin are presented in Table 1.

The major cardiovascular risk factors increased with ferritin, i.e. age (P trend<0.0001), blood pressure (systolic- and diastolic blood pressure, both P trend<0.0001), total cholesterol (P trend = 0.044), glucose (P trend = 0.002), and the prevalence of self-reported smoking (P trend < 0.0001). C-reactive protein also increased by quartiles of ferritin (P trend = 0.002), but significance was lost (P trend = 0.13) after excluding 271 women with CRP levels above

Table 1
Characteristics of African women by quartiles of ferritin.

Characteristic	Quartiles of ferritin (ng/ml)				*P* trend
	Low (n = 218)	Medium low (n = 220)	Medium high (n = 216)	High (n = 223)	
Means (5th–95th)	14.2 (2.1–36.8)	65.5 (42.5–98.8)	140.9 (100.9–204.3)	377.7 (217.4–1060.5)	
Age (years)	46.1 ± 8.5	50.1 ± 9.7	51.6 ± 10.3	53.7 ± 9.7	<0.0001
BMI (kg/m^2)	26.9 ± 6.8	27.9 ± 7.1	29.0 ± 7.4	2.3 ± 7.4	0.10
Systolic BP (mmHg)	127.6 ± 22.6	131.1 ± 23.4	136.1 ± 25.4	138.8 ± 24.0	<0.0001
Diastolic BP (mmHg)	85.3 ± 14.5	87.5 ± 13.8	89.7 ± 14.1	92.0 ± 14.0	<0.0001
Total cholesterol (mmol/l)	5.15 ± 1.38	5.23 ± 1.40	5.25 ± 1.34	5.42 ± 1.35	0.044
Blood glucose (mmol/l)	4.71 (3.30–6.00)	4.96 (3.50–7.30)	4.94 (3.50–7.00)	5.07 (3.10–7.30)	0.002
CRP (mg/l)	2.39 (0.21–24.40)	3.73 (0.28–35.41)	4.24 (0.38–37.41)	3.68 (0.27–44.56)	0.002
GGT (U/l)	32.3 (16.0–81.0)	37.6 (18.3–118.0)	47.7 (19.0–167.9)	92.9 (23.1–516.1)	<0.0001
CDT (%)	2.73 ± 1.03	2.48 ± 1.06	2.58 ± 1.11	2.74 ± 1.10	0.74
AST/ALT	1.62 (0.80–3.38)	1.45 (0.58–2.89)	1.42 (0.69–2.83)	1.66 (0.72–3.75)	0.72
Physical activity score	2.84 (1.95–3.72)	2.91 (2.15–3.70)	2.89 (2.13–3.68)	2.82 (2.04–3.74)	0.56
Current drinking n (%)	29 (13.3)	42 (19.1)	59 (27.3)	116 (52.0)	<0.0001
Current smoking n (%)	80 (36.7)	101 (45.9)	92 (42.6)	140 (62.8)	<0.0001
Postmenopausal n (%)	70 (32.1)	117 (53.2)	131 (60.6)	160 (71.7)	<0.0001

Values are arithmetic mean ± SD, geometric mean (5th to 95th percentile interval), or number of subjects (%). BMI, body mass index; BP, blood pressure; CRP, C-reactive protein; GGT, gamma-glutamyl transferase, AST/ALT, aspartate transaminase-to-alanine transaminase ratio.

8 mg/l [30] (data not shown). Gamma-glutamyl transferase (*P* trend < 0.0001) and the prevalence of self-reported current drinking (*P* trend < 0.0001) increased with increasing ferritin, but not carbohydrate deficient transferrin (*P* trend = 0.74).

A frequency histogram of log ferritin appear in Supplemental Fig. 1. In the total group, ferritin averaged 84.0 (5th to 95th percentile interval; 7.5–533.3) ng/ml and lowered to 75.3 (6.9–523.2) ng/ml after excluding the 271 women with CRP levels above 8 mg/l. Ferritin ($P < 0.0001$), gamma-glutamyl transferase ($P < 0.0001$) and carbohydrate deficient transferrin ($P < 0.0001$) were higher in drinkers compared to non-drinkers (Fig. 1), whereas CRP was similar (3.50, 0.27–35.4 vs. 3.38, 0.25–28.9 mg/l; $P = 0.77$).

3.2. Risk prediction by ferritin

Median follow-up was 9.22 (interquartile range, 2.11 to 9.87) years. There were 105 deaths due to any cause of which 40 (38.1%) were cardiovascular related. There were 56 women with a ferritin level below 10 ng/ml (low), 577 women with a level between 10 and 200 ng/ml (normal), and 244 women with a level above 200 ng/ml (high) [30]. Of these women, there were respectively, 2 (3.57%), 48 (8.32%) and 55 (22.54%) deaths due to any cause ($P < 0.0001$) and 0 (0%), 18 (3.12%) and 22 (9.02%) deaths due to cardiovascular causes ($P = 0.0003$).

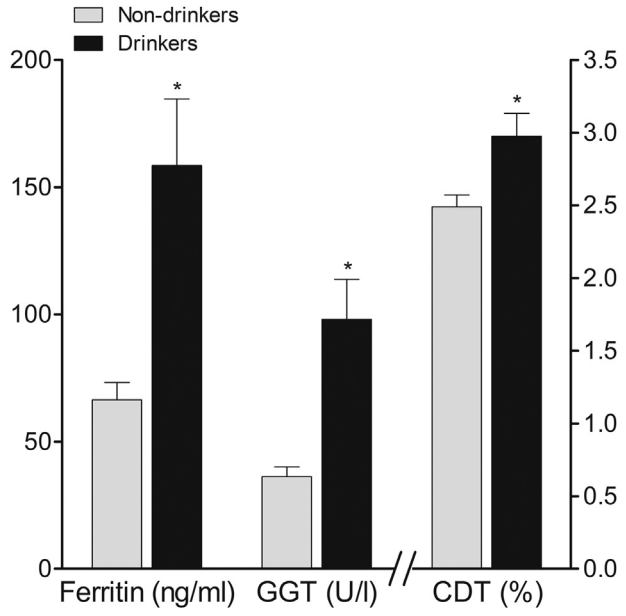

Fig. 1. Comparison of ferritin, gamma-glutamyl transferase (GGT) and carbohydrate deficient transferrin (CDT) by drinking status. * denotes $P \leq 0.0001$ for non-drinkers versus drinkers.

In analyses of Kaplan–Meier estimates by quartiles of ferritin, the log-rank test was significant for both all-cause mortality (Fig. 2A; $P < 0.0001$) and cardiovascular mortality (Fig. 2B; $P = 0.0004$). Ferritin fulfilled the proportional hazard assumption for both all-cause ($P = 0.40$) and cardiovascular mortality ($P = 0.51$). The univariate standardized hazard ratios for mortality of the variables included in the Cox models are presented in Supplemental Table 1. In Table 2, ferritin predicted both all-cause and cardiovascular mortality during (1) univariate analysis (all-cause mortality, $P < 0.0001$; cardiovascular mortality, $P < 0.0001$), (2) minimally adjusted for age and body mass index (all-cause mortality, $P < 0.0001$; cardiovascular mortality, $P = 0.0001$), and (3) multivariate analyses (all-cause mortality, $P < 0.0001$; cardiovascular mortality, $P = 0.002$).

In fully adjusted models (Table 2) including both ferritin and gamma-glutamyl transferase, or both ferritin and carbohydrate deficient transferase, ferritin interacted with gamma-glutamyl transferase and carbohydrate deficient transferase in predicting both all-cause (both interactions, $P < 0.0001$) and cardiovascular (both interactions $P \leq 0.003$) mortality. An interaction also existed with self-reported current alcohol use in predicting all-cause ($P = 0.012$) and cardiovascular mortality ($P < 0.0001$). No interaction existed between ferritin and menopausal status ($P \geq 0.21$).

3.3. Sensitivity analyses

Inflammation augments ferritin levels irrespective of iron status [29] and CRP increased by quartiles of ferritin as shown in Table 1. Therefore, in addition to our findings being independent of CRP by inclusion in the Cox models (Table 2) and illustrated in Fig. 3, we excluded the 271 participants with CRP levels above 8 mg/l. By doing so, in fully adjusted models, the predictive value remained for both all-cause (hazard ratio 2.51; 95% CI, 1.81–3.49; $P < 0.0001$) and cardiovascular mortality (2.34, 1.45–3.76; $P = 0.0005$).

4. Discussion

We investigated in African women whether total body iron, as reflected by serum ferritin, relates to mortality in sub-Saharan

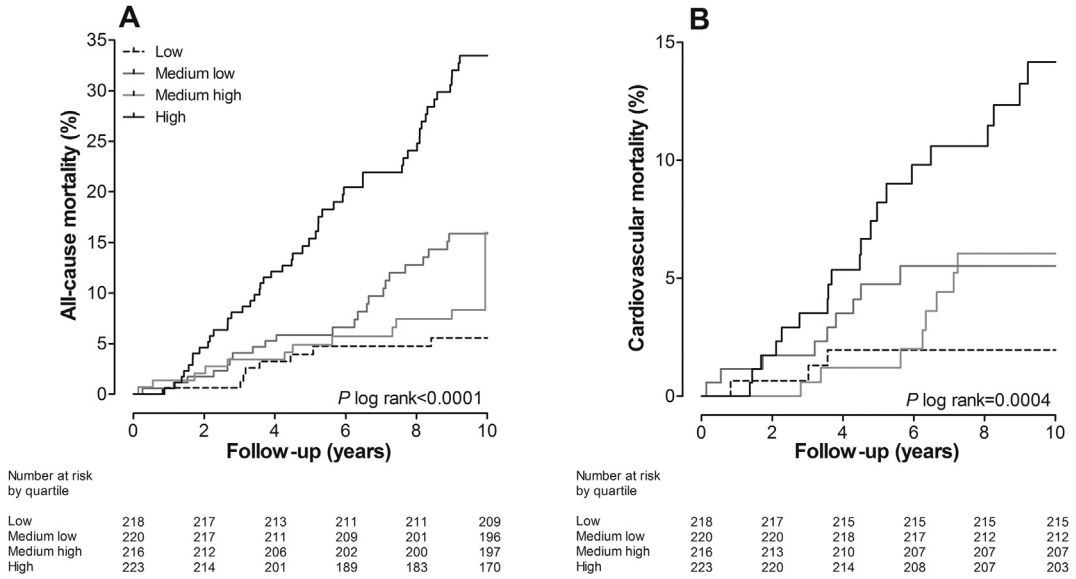

Fig. 2. Kaplan–Meier survival function estimates for (**A**) all-cause mortality and (**B**) cardiovascular mortality by quartiles of ferritin. *P* values refer to the significance of the log-rank test.

Table 2
Unadjusted and adjusted standardized hazard ratios for end points in relation to ferritin and interactions.

Analysis	Ferritin (log ng/ml)			
	All-cause mortality		Cardiovascular mortality	
	HR (95% CI)	*P*	HR (95% CI)	*P*
Model 1: Univariate	2.43 (1.91–3.09)	<0.0001	2.47 (1.48–4.14)	0.0005
Model 2: Adjusted for age and BMI	2.26 (1.76–2.89)	<0.0001	2.14 (1.22–3.74)	0.008
Model 3: Primary model	2.05 (1.60–2.62)	<0.0001	1.82 (1.02–3.09)	0.026
Model 4: Model 3 + GGT	1.52 (1.13–2.04)	0.006	1.82 (1.02–3.09)	0.026
	Interactions			
Ferritin*GGT (+Model 3)	2.05 (1.70–2.48)	<0.0001	1.72 (1.23–2.40)	0.001
Ferritin*self-reported alcohol use (+Model 3)	1.24 (1.05–1.47)	0.012	1.28 (1.05–1.56)	0.016
Ferritin*CDT (+Model 3)	1.96 (1.55–2.47)	<0.0001	1.75 (1.21–2.53)	0.003

The primary Cox models included baseline age, body mass index, aspartate transaminase-to alanine transaminase ratio, high sensitivity C-reactive protein (CRP), blood glucose, physical activity, systolic blood pressure, self-reported smoking and alcohol use and menopausal status as covariates. Hazard ratios (HR) are given with 95% confidence intervals (CI). BMI, body mass index; GGT, gamma-glutamyl transferase; CDT, carbohydrate deficient transferrin.

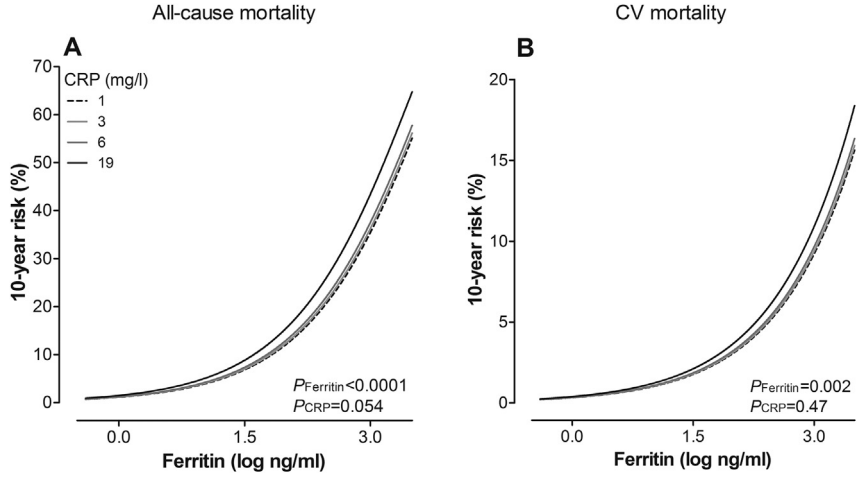

Fig. 3. Absolute 10-year risk of all-cause mortality (**A**) or a cardiovascular (CV) event (**B**) in relation to log ferritin at different levels of C-reactive protein (CRP). Mean log ferritin along the *x-axis* covers the 5th to 95th percentile interval. The high-sensitivity C-reactive protein is presented by 4 risk functions corresponding with 1, 3, 6, and 19 mg/l (approximate quartile midpoints). The risk functions were standardized to the distributions (mean or ratio) of baseline age, body mass index, aspartate transaminase-to-alanine transaminase ratio, high sensitivity C-reactive protein, blood glucose, physical activity, systolic blood pressure, smoking and alcohol use as covariates. Among 877 participants, 105 all-cause and 40 cardiovascular deaths occurred.

Africans. The main finding was that ferritin, independent of inflammation, predicted all-cause and cardiovascular mortality with a potential mechanistic involvement of alcohol. To our knowledge, this is the first study to investigate the prognostic significance of iron loading and its interaction with measures of alcohol intake in African women.

The controversy surrounding the iron hypothesis has been ongoing for more than three decades [31]. Iron's involvement in cardiovascular morbidity and mortality is, at least in part, through its role in the atherosclerosis process by causing oxidative stress [16,32], and relates with both coronary heart disease [17,33,34] and peripheral arterial disease [18]. Despite the above evidence, a recent systematic review and meta-analysis of prospective studies could not confirm the link between coronary heart disease and increased body iron stores in the general population [35]. Similarly, a review on the link with cardiovascular disease involving 55 studies of various designs, could not find a high level of evidence to support the iron hypothesis [23]. The latest evidence comes from the Atherosclerosis Risk in Communities (ARIC) study [21] and the Prevention of Renal and Vascular End-Stage (PREVEND) trial [22]. In the ARIC study [21] involving 1063 men and women followed for 21 years, ferritin predicted incident heart failure, but this relationship was lost when adjusting for a time-varying covariate for coronary heart disease. In addition, no relationship existed between ferritin and risk of death. In the PREVEND trial, Klip et al. [22] also found a relationship with incident heart failure, but only in women, and ferritin was again not predictive of all-cause or cardiovascular mortality in men or women. Both studies were vulnerable to selection bias in addressing this research question, i.e., with the over selection of African-Americans in the ARIC study, and participants with high urinary albumin excretion (above 10 mg/l) in the PREVEND trial. Surprisingly, as it is known that alcohol consumption causes iron loading [36,37], most of the above studies did not include alcohol or merely adjusted for alcohol intake in the statistical models. In addition, no studies included gamma-glutamyl transferase as marker of alcohol intake and/or oxidative stress, which is mechanistically linked with iron in the generation of reactive oxygen species as described below [38]. The descriptive data of Kiechl et al. [39] showed a higher prevalence of drinkers (drinking above 51 g/day) in those who progressed to atherosclerosis over five years compared to those with no change in vascular status. Kim et al. [40] reported higher alcohol intake by quintiles of ferritin, while Friedrich et al. [41]. found higher ferritin in participants who consumed more alcohol captured as glasses per week.

Due to the toxicity of iron loading, the systemic level is tightly regulated by the hepatic peptide hormone hepcidin, with its expression stimulated by iron excess [42,43]. Hepcidin triggers the internalization, ubiquitination and degradation of the iron exporter ferroportin in enterocytes, hepatocytes and macrophages [44,45]. This prevents absorption of dietary iron and release of stored iron from hepatocytes and iron in macrophages from recycled red blood cells [9,46]. However, iron loading is common in alcoholic liver disease [36] and even moderate alcohol consumption (>2 drinks per day) increases iron loading [37]. *In vivo* evidence suggests this secondary iron loading is caused by the suppressive effect of alcohol on hepcidin production [10,47]. From our results, ferritin, gamma-glutamyl transferase and carbohydrate deficient transferrin were higher in drinkers compared to non-drinkers. The interaction observed between ferritin and gamma-glutamyl transferase, carbohydrate deficient transferrin and self-reported current alcohol use could be explained by this suppressive effect of alcohol on hepcidin transcription and secretion and subsequent iron loading. This leads to a state of oxidative stress where iron is responsible for the production of potent hydroxyl radicals through the Fenton reaction [32]. In addition, gamma-glutamyl transferase which maintains the physiological concentrations of cytoplasmic glutathione (one of the cellular defense mechanisms against oxidative stress) [48], is also responsible for producing superoxide, hydrogen peroxide and thiyl radicals as a result of the interaction of the glutathione metabolites, cysteinyl-glycine, with trace levels of iron present in the cell environment [38]. This pro-oxidant effect is especially heightened in conditions of excessive alcohol use when gamma-glutamyl transferase is elevated [49], resulting in even further aggravation of the oxidative stress already present from the hydroxyl radical production by iron [32]. In addition, both iron and gamma-glutamyl transferase is present in atherosclerotic plaque contributing to oxidative stress and plaque instability and ultimately cardiovascular events [20,50].

These findings may have health implications in a setting such as sub-Saharan Africa where alcohol consumption tends to be high [24] and fortification of food with iron is common [51]. Especially alarming are the recent developments in the alcohol industry aiming to target the weakly regulated, underdeveloped alcohol market in Africa to ensure growth and increase profits [52]. On balance, iron deficiency anemia is also common, and is likely due to the stimulatory effect of an inflammatory state on hepcidin production caused by infectious disease [53], preventing iron absorption. Therefore, unsurprisingly, evidence of the beneficial effects of iron fortification in this population is weak or absent [5,54]. Sub-Saharan Africans with the lowest life expectancy in the world [1], may therefore be burdened with both iron loading due to high alcohol intake, and iron deficiency anemia caused by high hepcidin levels in the presence of infectious disease. As we excluded all HIV-infected participants and accounted for inflammation, we were more likely to observe the effects of the iron-loading burden.

There are some potential limitations to consider. The analyses were limited to women. Due to reported gender differences in alcohol consumption in general [55] and iron metabolism [56], these findings need confirmation in men. We could not account for the presence of anemia in the Cox models as data on complete blood count measures were unavailable. Even though our results were multivariable adjusted and consistent in sensitivity analyses, we cannot exclude the possibility of residual confounding or the influence of some unmeasured confounders. We established death with death certificates and verbal autopsies that could have caused inaccuracies, resulting, if any, in diluting the results. We cannot necessarily extrapolate the results to other ethnicities and men as this study involved African women. Ferritin was the only marker of iron status investigated and increases when the body is in an inflammatory state [29]. However, we could show that our findings were independent of inflammation by including CRP in the Cox models, but also after excluding 271 participants with CRP levels above 8 mg/l. We did not measure hepcidin that could have provided us with more supportive information.

In conclusion, our results indicate in a sub-Saharan Africa setting where iron deficiency anemia and iron fortification programs are common, that iron loading is also present and predicts all-cause and cardiovascular mortality. The current evidence prompts further experimental investigations into iron loading and the safety of iron fortification programs, especially in a setting of high alcohol consumption.

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Statement of authorship

All authors have made substantial contributions to all of the following: (1) the conception and design of the study, or acquisition of data, or analysis and interpretation of data, (2) drafting the article or revising it critically for important intellectual content, (3) final approval of the version to be submitted.

Disclaimer

Any opinion, findings and conclusions or recommendations expressed in this material are those of the author(s) and therefore the NRF do not accept any liability in regard thereto.

Conflicts of interest

None.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.clnu.2018.05.008

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