Title: Minimal impact of an iron-fortified lipid-based nutrient supplement on haemoglobin and iron status: a randomised controlled trial in malnourished HIV-positive African adults starting antiretroviral therapy.

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Abstract

Anaemia, redistribution of iron, malnutrition and heightened systemic inflammation during HIV-infection confer an increased risk of morbidity and mortality in HIV patients. We analysed information on iron status and inflammation from a randomised, double blind, controlled phase-III clinical trial in Lusaka, Zambia and Mwanza, Tanzania. Malnourished patients (n=1815) were recruited at referral to antiretroviral therapy (ART) into a two-stage nutritional rehabilitation programme, randomised to receive a lipid-based nutrient supplement with or without added micronutrients. Iron was included in the intervention arm during the second stage, given from 2-6 weeks post-ART. Haemoglobin (Hb), serum C-reactive protein (CRP), serum ferritin and soluble transferrin receptor (sTfR) were measured at recruitment and 6 weeks post-ART. Multivariable linear regression models were used to assess the impact of the intervention, and the effect of reducing inflammation from recruitment to week 6, on Hb and iron status. There was no effect of the intervention on Hb, serum ferritin, sTfR or serum CRP. A one-log decrease of serum CRP from recruitment to week 6 was associated with a 1.81g/L increase in Hb (95% CI: 0.85, 2.76; p<0.001) and a 0.11 log decrease in serum ferritin (95% CI: -0.22, 0.03; p=0.012) from recruitment to week 6. There was no association between the change in serum CRP and the change in sTfR over the same time period (p=0.78). In malnourished, HIV-infected adults receiving dietary iron, a reduction in inflammation in the early ART treatment period appears to be a precondition for recovery from anaemia.

Introduction

Independent risk factors for mortality amongst African patients starting antiretroviral therapy (ART) include anaemia[1], a failure to increase haemoglobin (Hb) within the first few months of ART[2], and malnutrition, represented by body mass index (BMI) < 18.5 kg/m^2[3–5]. Heightened systemic inflammation is a hallmark of both untreated and treated HIV infection[6], and higher levels of persistent inflammation despite treatment with ART[7] confer an increased risk of morbidity and mortality in HIV patients[8,9]. Redistribution of iron during HIV infection can lead to increased iron sequestration in macrophages with an accompanying decline in iron available for tissue supply and erythropoiesis[7,10]. This disordered iron metabolism has been associated with rapid progression of HIV[11–13], exacerbation of co-infections,[14] especially tuberculosis[15,16], and early death[17,18]. Although ART is increasingly available, initiation of ART is associated with a high mortality: 17% of patients starting ART in sub-Saharan Africa die within one year and the majority within the first 3 months[19]. Given the detrimental effects of anaemia, iron redistribution, malnutrition, and inflammation on early ART mortality, together with the fact that in Sub-Saharan

Africa the HIV disease burden remains vast[20] with a third of adults starting ART being malnourished in some African countries[3,21], the control of anaemia and normalisation of iron metabolism within this population remains a critical strategy for improving patient survival.

Markers other than Hb are required to assess iron status. Serum ferritin can be used as a marker of body stores of iron[22] and soluble transferrin receptor (sTfR) to estimate tissue iron demand[23]. The determination of iron status in the presence of inflammation is notoriously challenging[24], with as yet no internationally agreed methodology[25]. It is well known that inflammation alters many markers of iron status, including increasing serum ferritin as an acute phase protein[25]. sTfR is less affected by the inflammatory response and can therefore be used to distinguish anaemia of inflammation (with elevated serum ferritin and normal-to-elevated sTfR) from iron deficiency anaemia (with low serum ferritin and high sTfR)[25,26]. However, iron deficiency and inflammation often co-exist, complicating assessment of iron status and needs. Measurement of the acute phase proteins C-reactive protein (CRP) and α_1-acid glycoprotein (AGP) can assist interpretation of the iron biomarkers in order to separate those patients with inflammation-induced iron sequestration from those who are both sequestering iron and iron-deficient[27]. Hepcidin, the peptide hormone regulating iron metabolism through influencing the absorption of dietary iron and how iron is distributed among different cell types, can also be measured to help elucidate the complex interplay between anaemia, iron status and immunity[28].

There are currently many unanswered questions about the range and characteristics of disordered iron metabolism among malnourished, HIV-infected adults, the preferred iron-related biomarkers for assessing health risks, and the effect of oral iron supplementation on iron status and health. The common assumption that low Hb requires therapeutic correction through iron supplementation may be erroneous in HIV since supplementation can exacerbate the risk of co-infections and hasten disease progression. Paradoxically it is high serum ferritin that predicts a worse outcome despite the association of anaemia and mortality[18]. Controversy therefore remains as to what extent iron supplementation amongst HIV-patients affects infection rates and mortality[29]. Some interventions supplementing iron to HIV-positive adults have reduced anaemia without increasing viral load[30,31]; longer-term outcomes were not assessed. This creates a therapeutic dilemma for the clinician as to how HIV-infected patients with anaemia should be treated. Given the clear link between anaemia and early ART mortality, the existing knowledge gaps jeopardise the health and survival of thousands of malnourished HIV/AIDS patients.

Our study uses a clinical trial amongst malnourished adults starting ART to assess three main research questions. Firstly, what effect does a nutritional intervention including iron have on iron status? Secondly, does any impact depend on the baseline iron status of patients? Thirdly, does inflammation have an independent effect on changes in iron status? We hypothesised that the

nutritional intervention would improve iron status, indicated by an increase in haemoglobin accompanied by no change or a slight decrease in sTfR; effects on sTfR would depend on whether the anaemia was due primarily to chronic disease which has little effect on sTfR or to iron deficiency which results in increased sTfR[25]. We expected overall serum ferritin results to be harder to predict: decreasing in the correction of anaemia of inflammation but increasing for the correction of iron deficiency. We speculated that failing to normalise systemic inflammation after starting ART would attenuate any improvements.

Subjects and Methods

Study design

The study analyses information on iron status and inflammation from a randomised, double blind, controlled phase-III clinical trial in Lusaka, Zambia and Mwanza, Tanzania: the Nutritional Support for Africans Starting Antiretroviral Therapy (NUSTART) Trial (registered on the Pan-African Clinical Trials Register as PACTR201106000300631). Details of the trial are described in full elsewhere[32,33]. In brief, the NUSTART trial was conducted between August 2011 and December 2013 to assess the effect of a fortified lipid-based nutrient supplement (LNS; prepared by Nutriset, Malauney, France) on survival of malnourished patients starting ART. This paper focuses on two secondary outcomes: markers of iron status and inflammation. A total of 1815 patients were recruited at the two sites, using inclusion criteria of >18 years, BMI<18.5 kg/m^2, CD4 count<350 cells/μl or stage 3 or 4 AIDS, ART-naïve apart from those who received ART during standard prevention of mother-to-child transmission regimens, and informed consent. Self-reported pregnancy was an exclusion criterion.

The trial intervention was based on established protocols for managing severe malnutrition in young children involving two phases aimed at stabilisation and then rehabilitation[34]. Figure 1 summarizes the NUSTART design. The first phase took place between referral and 2 weeks post-ART initiation. Participants were randomized to receive vitamins and minerals, without iron as is done for malnourished children, in low calorie (30 g containing ~150 kcal/day) LNS (low dose LNS-VM) in the intervention group versus LNS without the vitamins and minerals (low dose control LNS) in the control group. This phase aimed to stabilise metabolism before trying to promote weight gain during the second phase. The second phase involved a 4-week intervention, starting 2 weeks after ART initiation and continuing to 6 weeks post-ART. Participants in the intervention group received a higher calorie (250g containing ~1400kcal/day) LNS containing the same added vitamins and minerals as in phase 1 plus iron as sulphate (high dose LNS-VM). The control group received the high dose LNS without the added vitamins, minerals, or iron (high dose

control LNS). Vitamin and mineral levels in both the high and low dose LNS-VM were mostly set at 3 times the UK recommended nutrient intakes (RNI) for adult women[35] with the exception of iron which was only in the second stage, high dose LNS and only at one RNI (14.7 mg/day) (nutritional composition details in Supplementary Material Table 1).

The interval between referral for ART and starting ART was based on the individual patient's readiness to start life-long drug treatment and practices of the different clinics from which the study recruited patients. Study personnel were not involved in deciding when to initiate ART and the duration of phase 1 reflects routine practice in these populations at the time. The median interval between referral for ART and starting ART for both arms was 21 days, (interquartile range [IQR] 15, 30). 55.4% of patients went on to take the ART regime Tenofovir (TDF)/ Emtricitabine (FTC)/ Efavirenz (EVF), 16.0% took Zidovudine (AZT)/ Lamivudine (3TC)/ Nevirapine (NVP), 9.2% took AZT/ 3TC/ EVF, 4.3% took TDF/ FTC/ NVP, 3.6% were on another regime and 11.5% had no ART regime information[32].

The Data Safety and Monitoring Board (DSMB) statistician conducted the randomisation using 16 computer-generated blocks stratified by site. The contents of the LNS packets were assigned an allocation code (letters A to H), known only to the DSMB statistician and Nutriset, which were linked to study ID numbers using a randomisation code. This randomisation code was only known to the DSMB statistician and site-based pharmacists, none of whom had direct patient contact. The LNS and LNS-VM packets were delivered by Nutriset in lots assigned by allocation code. Clinic pharmacists labelled packets with study ID numbers as packets were dispensed. Clinic nurses (with no access to the allocation or randomisation code) then recruited eligible participants to the study using sequential IDs.

Blood collection

Patients were seen weekly from referral for ART until the ART initiation visit, then at 2, 6, 8, and 12 weeks after starting ART. They were asked at each follow-up whether they were taking iron supplements in addition to the study supplement. Haemoglobin (Hb) and serum CRP were measured for all patients, whilst serum ferritin and sTfR were analysed on one fifth of the patients, referred to as the iron marker subsample and chosen systematically for every patient ID divisible by 5. Haemoglobin was measured at recruitment and at 6 weeks post-ART. Iron markers and CRP were measured in serum from the recruitment and 6 weeks post-ART samples which were stored at -80ºC until batched analysis. The flow of participants included in the iron marker subsample from identification to analysis at baseline and week 6 is shown in Figure 2.

Iron and inflammatory marker analysis

Hb levels were analysed by a portable haemoglobinometer (Hemocue®; Angelholm, Sweden) on fingerstick capillary blood samples from all patients. Anaemia severity cut-offs followed standard World Health Organisation categorisations[36]. Mild anaemia was defined as Hb <120 g/L for women and <130 g/L for men. Moderate and severe anaemia categories used the same cut-offs for both sexes, defined as <110 g/L and <80 g/L respectively.

Serum ferritin was measured by ELISA (AssayPro Human Ferritin ELISA Kit, Catalogue No. EF2003-1; St. Charles, MO, USA). The intra-assay and inter-assay coefficients of variability (CVs), respectively, were 2% and 7% in Mwanza and 5% and 23% in Lusaka. sTfR was measured by ELISA (Quantikine® IVD® Human sTfR Immunoassay, Ref DTFR1, R&D Systems, Inc. Minneapolis, USA). The intra-assay and inter-assay CVs were 2% and 4% in Mwanza and 3% and 13% in Lusaka. Serum CRP was analysed by ELISA (AssayPro, St. Charles, MO, USA). The intra-assay and inter-assay CVs were 3% and 37% in Mwanza and 6% and 32% in Lusaka. For all analytes values over the upper range of the standard curve were set to the top standard multiplied by the dilution factor. For all assays plates with poor precision were re-run.

Sample size

The original trial sample size was powered on the primary outcome (mortality). Our one in five subsample for the iron markers was sufficient to detect an inter-group difference of 0.35 standard deviations using 90% power.

Data analysis

Continuous variables were assessed for normality using normal probability plots and visual inspection of histograms. Hb and sTfR approximated a normal distribution and remained on the linear scale. Serum CRP and serum ferritin were skewed to the right and natural log-transformed. We considered using correction factors for ferritin derived from the methodology suggested by Thurnham et al.[27]; however, since our population was extremely malnourished, and exhibited high levels of inflammation with very deranged iron metabolism, it was unclear whether correction factors derived from less ill populations were appropriate. We decided instead to simply adjust for CRP in regression analyses as has been done elsewhere[37].

We compared baseline characteristics of those in the smaller sub-sample containing data on sTfR and serum ferritin (n=353) with those not in the sub-sample (n=1462) to assess the generalizability to the whole sample. The chi-squared test was used to compare proportions, independent t-tests to compare means of normally distributed data, and the Wilcoxon-Mann-Whitney test to compare medians of non-parametric data. Sample sizes of all further analyses were set by the number of available samples at 6 weeks post-ART.

A variable was created to summarise the frequency of taking iron supplements in addition to the study supplement over the follow-up period; this was categorised as never consumed (62%), reported consumed at one follow-up (19%) and reported consumed at two or more follow-up visits (19%). We assessed the within-subject changes in markers of iron status and inflammation between baseline and week 6 by intervention arm using paired t-tests.

For our first objective assessing the effect of the intervention on iron marker status at week 6 we used multivariable linear regression. The first model adjusted only for the baseline value of the iron marker being assessed, the second model additionally adjusted for serum CRP at week 6 given our hypothesis that inflammation would affect iron markers, and the third model further adjusted for sex, site and being on TB treatment at recruitment as binary variables; taking iron supplements in addition to the study supplement as a categorical variable; and baseline BMI, age, CD4 count and length of time taken from recruitment to starting ART as continuous variables.

For our second objective we repeated the third (fully adjusted) model analysis stratified by baseline values of the iron markers to determine whether these modified the effect of the intervention. We used a binary Hb category: normal Hb and mild anaemia vs. those with moderate and severe anaemia. Due to lack of internationally agreed cut-offs for serum ferritin and sTfR, as well as the specific context of our malnourished sample with heightened systemic inflammation, we divided these variables into two groups using the median value to create binary categories. We chose binary categories rather than continuous measures since we felt this would provide a more accessible way of interpreting overall trends that may have physiological significance. The test for interaction between the baseline iron marker category and intervention arm used a likelihood ratio test between the multivariable linear regression models with and without the interaction term.

For our third objective we assessed to what extent inflammation was driving the changes in our iron markers independently of the intervention. We investigated interrelations among the iron markers and serum CRP using Pearson correlation matrices. We then created a multiple linear regression model exploring the association between change in iron marker from baseline to week 6 with change in serum CRP over the same timeframe, adjusting for trial arm, sex, site and being on TB treatment at recruitment as binary variables; taking iron supplements in addition to the study supplement as a categorical variable; and baseline BMI, age, CD4 count and length of time taken from recruitment to starting ART as continuous variables.

Stata version 13.1 (StataCorp, College Station, TX, USA) was used for all analyses.

Ethical considerations. This study was conducted according to the guidelines laid down in the Declaration of Helsinki. All NUSTART trial procedures, including the collection and analysis of the iron markers, were approved by the ethics committee of the London School of Hygiene and

Tropical Medicine, the University of Zambia Biomedical Research Ethics Committee (reference number 009-01-11), and the National Institute for Medical Research, Tanzania. Written informed consent or thumbprint was obtained from all patients before enrolment.

Results

Detailed baseline characteristics of the 1815 patients recruited are published elsewhere[32]. In summary, one-third had BMI <16 kg/m^2 and mean (SD) age was 35.8 (9.4) years. Only 10% of the patients were without anaemia at baseline, with two-thirds categorised as either moderately or severely anaemic. Table 1 shows the baseline characteristics for the subsample assessed for iron markers (n=353). Mean (SD) baseline Hb was lower amongst those in the iron marker sub-sample compared to those not included (93 (23) g/L vs. 96 (23) g/L, p=0.012). Median (IQR) serum CRP was higher amongst those in the sub-sample compared to those not included (71 (18,160) mg/L vs. 57 (13,155) mg/L, p=0.004). Patient baseline characteristics in the iron subsample were very similar in the two treatment arms (Table 1), as was the case for the whole sample[32].

In the control group from baseline to week 6 post-ART, patients gained a mean of 3 g/L Hb (p=0.029, n=369), decreased their serum ferritin by 100 μg/L (p=0.021, n=89), increased their sTfR by 4 nmol/L (p=0.045, n=101), but experienced no overall change in CRP levels (p=0.08, n=407) (Table 2). The intervention group displayed similar trends: patients gained a mean of 6g/L Hb (p=<0.001, n=383), decreased their serum ferritin by 141 μg/L (p=0.004, n=76), increased their sTfR by 4 nmol/L (p=0.030, n=85), and also experienced no overall change in CRP levels (p=0.36, n=431). There was no effect of the vitamins and minerals added to the intervention LNS on Hb, serum ferritin, sTfR or serum CRP in any of the three statistical models (Table 3). Note that sample sizes in Table 3, which used various adjusted models, were restricted to the patients who had no missing data in all the variables we adjusted for and therefore differ to those seen in Table 2, which used unadjusted data.

Table 4 shows to what extent the intervention effect differed for patients based on their baseline iron marker category. The coefficient shows the change in week 6 iron marker associated with the intervention in comparison to the control within the baseline iron marker category strata. There was no evidence that the impact of the intervention on Hb at week 6 was affected by baseline iron marker category (p values >0.18 for interaction tests). Amongst those with moderate and severe anaemia at baseline, the intervention was associated with a decrease in 0.40 of log serum ferritin at week 6 (p=0.023). However, evidence for an overall interaction between the intervention and baseline Hb on log serum ferritin was weak (p=0.12). There was no evidence of any interaction

between the intervention and baseline iron marker categories on sTfR at week 6 (p values >0.52 for interaction tests).

At both baseline and week 6, Hb was negatively correlated with serum CRP, serum ferritin was positively associated with serum CRP and there was no correlation between sTfR with serum CRP (Table 5). At both time points sTfR was negatively correlated with Hb and serum ferritin. Serum ferritin was not correlated with Hb at baseline, but showed a weak positive correlation at week 6.

Table 6 shows the associations between changes in serum CRP and changes in iron markers. A decrease in one-log of serum CRP from baseline to week 6 was associated with an increase of 1.81g/L of Hb (95% CI: 0.85, 2.76; p<0.001) and a decrease of 0.11 log of serum ferritin (95% CI: -0.20, 0.03; p=0.012) from baseline to week 6. There was no association between the change in serum CRP and the change in sTfR over the same time period (p=0.78).

Discussion

We hypothesised that the two-stage nutritional intervention involving a stabilisation phase followed by the provision of iron together with other micronutrients would help reverse anaemia of chronic disease and improve iron deficiency anaemia among malnourished, HIV-infected adults in sub-Saharan Africa. Contrary to expectations, our results show the intervention with fortified LNS-VM made no overall difference to Hb or any iron markers. Furthermore, there was no obvious sub-group, defined by baseline anaemia, serum ferritin or sTfR, which demonstrated any clinically meaningful improvement from the intervention. Although there was weak evidence to suggest that the effect of the intervention on serum ferritin at week 6 was dependent upon baseline levels of Hb, the reduction in log serum ferritin was small and there was no concomitant improvement in Hb or reduction in sTfR in this sub group, suggesting this finding was of no clinical significance.

In unadjusted correlation analyses between the iron markers and CRP it was not surprising that serum ferritin, being a positive acute phase protein, was positively correlated with CRP at both time points. The negative correlation of sTfR with Hb and serum ferritin was also to be expected, due to sTfR being a marker of tissue iron deficiency and, more specifically, the requirement of iron for erythropoiesis[23]. The linear regression model exploring the relationship between serum CRP and Hb suggested that reducing systemic inflammation between baseline and week 6 was associated with an increase of Hb over that time period. Iron metabolism involves a series of complex, tightly regulated mechanisms to ensure homeostasis, especially during infection or inflammation. Chief amongst these is the need to maintain iron tightly chaperoned in order to avoid oxidative damage and to limit its availability to pathogens[28]. During HIV infection the chronic inflammation causes a

hepcidin-mediated redistribution of iron within the body, a process that becomes more pronounced as the HIV stage progresses[1,38]. Up-regulated hepcidin inactivates ferroportin (the only iron-efflux channel in cells) causing decreased intestinal iron absorption as well as sequestration of iron in macrophages[28] thus blocking erythropoiesis. This leads to anaemia and possibly creates a niche for intra-cellular pathogens such as mycobacteria[14,39]. Our results suggest that to reverse anaemia and normalise iron redistribution, the source of the innate immune activation first needs to be identified and addressed, and then only after systemic inflammation has been brought under control will an iron-containing nutritional intervention be likely to have an impact.

Irrespective of whether the LNS was fortified with vitamins and minerals, it appeared that ART plus LNS improved haemoglobin levels and reduced serum ferritin. ART has been associated with a reduction in prevalence of anaemia in other studies[40–42], although some ART drugs, e.g. zidovudine[43] which was prescribed to 26% of NUSTART patients[33], have increased anaemia in some patients. However, in the NUSTART context the overall mean improvement of Hb and reduction of serum ferritin was modest. For there to have been enough of a functional improvement in the distribution and use of iron in the body we would have expected sTfR to at least remain stable if not drop, and yet in this context sTfR levels increased slightly. Irrespective of whether LNS was fortified with vitamins and minerals or not, the combination of LNS and ART for 6 weeks does not appear to sufficiently improve the iron profile of our patients or reduce their systemic inflammation.

Our study carries several limitations. Patients in the sub-sample had lower baseline Hb and were more inflamed compared to those not in the sub-sample. This suggests the sub-sample patients were slightly sicker than those not included and may restrict our ability to extrapolate the results to the whole sample. Budget limitations precluded analysis of iron markers in the full cohort and analysis of results at other time points, for example, at the end of phase 1, as well as assessment of other potentially interesting markers such as hepcidin or AGP. Since there was no control group not receiving LNS (for ethical reasons) we are unable to separate the overall impact of ART and LNS on our outcomes.

The level of iron fortification of the LNS during stage two was modest (1 RNI) in comparison to higher levels (usually 3 RNIs) of other micronutrients. This was a conservative approach to avoid potentially increasing the risks associated with higher serum ferritin stores. It would appear that the level of iron included in the fortified LNS was safe in this regard, since there was no overall increase in serum ferritin from the intervention. That said, we would recommend that iron dosage within fortified LNS not be increased in future research amongst similar populations before investigating the impact this modest fortification level has once inflammation has been successfully controlled. Further research is required: firstly, to determine whether non-nutritional interventions designed to reduce systemic inflammation are sufficient to correct anaemia of inflammation in HIV;

secondly, to assess whether a product with a different nutrient composition may also assist this process; and thirdly, to quantify the level of improvement in inflammation necessary before a nutritional intervention will improve iron deficiency anaemia.

Conclusion

Our large clinical trial of iron supplementation as part of a nutritional intervention showed no appreciable effect on Hb and iron metabolism, even when the majority of patients were anaemic at baseline. HIV-related inflammation resulting in disordered iron metabolism appears to severely attenuate the potential impact of receiving dietary iron in an intervention. Given the clear associations between anaemia, disordered iron metabolism and mortality amongst HIV-positive patients starting ART, it is of critical importance that strategies to reduce the level of systemic inflammation (going beyond the provision of ART) are investigated. Without the ability to control inflammation it would appear the impact of a nutritional intervention of this kind is likely to remain severely restricted.

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Conflict of Interest

None.

Authorship

SF, PK & HF designed the study. PJ performed the data analyses with input from AMR and SW. PJ wrote the first draft of the manuscript. All authors were involved in the interpretation of results and the editing of the manuscript final version.

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Figure 1: Overview of the NUSTART trial design

Abbreviations: ART, Antiretroviral therapy; LNS, lipid-based nutrient supplement; LNS-VM, LNS with added vitamin and mineral mix.

Figure 2: Flow of subsample participants from identification to analysis of iron markers at baseline and week 6 post-ART.

Abbreviations: ART, antiretroviral therapy; LNS, lipid-based nutritional supplement without added vitamins and minerals; LNS-VM, lipid-based nutritional supplement with added vitamins and minerals; BMI, body mass index; sTfR, soluble transferrin receptor.

*Inclusion criteria: >18 years, BMI<18.5 kg/m^2, CD4 count<350/µl or stage 3 or 4 AIDS, ART-naïve apart from those who received ART during standard prevention of mother-to-child transmission regimes, and informed consent. Self-reported pregnancy was an exclusion criterion.

† Note haemoglobin (Hb) and C-reactive protein (CRP) were collected from all patients at recruitment and 6 weeks post-ART. The flow diagram for the whole sample is published elsewhere[32] . Available samples at baseline: CRP, n=1762; Hb, n=1670. Available samples at week 6: CRP, n=863; Hb, n=826.

Table 1: Baseline characteristics of the iron marker subsample by trial arm and overall summaries of those included in and excluded from the iron marker sub-sample

Variable		Level	Iron marker sub-sample (overall)	Iron marker sub-sample (LNS-VM)	Iron marker sub-sample (LNS control)	Not included in iron marker sub-sample (overall)	P value Sub-sample vs. not in sub-sample*
N (%)			353 (100)	175 (49.6)	178 (50.4)	1462 (100)	
Site (Lusaka), n (%)			218 (62)	107 (61)	111 (62)	893 (61)	0.82
Age (years), mean (SD)			36.0 (9.1)	36.4 (9.2)	35.7 (9.1)	35.8 (9.5)	0.67
Female, n (%)			181 (51)	91 (52)	90 (51)	728 (50)	0.72
BMI (kg/m^2), mean (SD)		All	16.4 (1.4)	16.5 (1.3)	16.4 (1.5)	16.5 (1.3)	0.58
	n (%)	BMI <16 kg/m^2	111 (31)	51 (29)	60 (33)	495 (34)	0.26
	n (%)	BMI 16-16.9 kg/m^2	103 (29)	56 (32)	47 (26)	365 (25)	
	n (%)	BMI 17-18.5 kg/m^2	139 (39)	68 (39)	71 (40)	602 (41)	
Oedema, n (%)			12 (3)	6 (3.4)	6 (3.3)	54 (4)	0.79
CD4 count (cells/µl), mean (sd)		All	138 (100)	134 (99)	141 (101)	137 (100)	0.86
Hb (g/L), mean (sd)		All	93 (23)	93 (23)	93 (22)	96 (23)	0.012
Hb group†	n (%)	Severe anaemia	98 (28)	51 (29)	47 (26)	300 (21)	0.011
	n (%)	Moderate anaemia	141 (40)	68 (39)	73 (41)	669 (46)	
	n (%)	Mild anaemia	54 (15)	23 (13)	31 (17)	231 (16)	
	n (%)	Normal	26 (7)	15 (9)	11 (6)	151 (10)	
	n (%)	Missing	34 (10)	18 (10)	16 (9)	111 (7)	
Serum CRP (mg/L), median (IQR)			71 (18, 160)	69 (20, 160)	83 (16, 160)	57 (13,155)	0.014
TB treatment pre-ART, n (%)			99 (28)	49 (28)	50 (28)	352 (24)	0.12
Using Co-trimoxazole, n(%)			294 (84)	141 (81)	153 (86)	1192 (82)	0.43
sTfR (nmol/L), mean (SD)			44 (18)	43.2 (17.6)	43.7 (19.0)	N/A	N/A
Serum ferritin (µg/L), median (IQR)			752 (288, 1246)	722 (325, 1287)	754 (281, 1200)	N/A	N/A

Abbreviations: LNS-VM, lipid-based nutritional supplement with added vitamins and minerals; LNS, lipid-based nutritional supplement without added vitamins and minerals; SD, standard deviation; BMI, body mass index; Hb, haemoglobin; CRP, C-reactive protein; TB, tuberculosis; ART, antiretroviral therapy; sTfR, soluble transferrin receptor.

* Chi-squared test to compare proportions, independent t-tests to compare means of normally distributed data, and the Wilcoxon-Mann-Whitney test to compare medians of non-parametric data.

† Normal defined as ≥120g/L for women and ≥130g/L for men. Mild anaemia defined as Hb <120g/L for women and <130g/L for men. Moderate and severe anaemia categories defined as <110g/L and <80g/L respectively for both sexes.

Table 2: Overview of changes in iron and inflammatory markers from baseline to week 6 by trial arm, unadjusted.

	LNS-VM (intervention)				LNS Control			
Variable	N*	Baseline	Week 6	P value†	N*	Baseline	Week 6	P value†
Hb (g/L), *mean (95% CI)*	383	98 (96, 100)	104 (102, 106)	<0.001	369	100 (97, 102)	103 (100, 105)	0.029
Serum ferritin (µg/l), *geometric mean (95% CI*)	76	425 (329, 547)	284 (219, 368)	0.004	89	466 (373, 581)	366 (289, 463)	0.021
sTfR (nmol/L), *mean (95% CI)*	85	41 (38, 45)	46 (43, 50)	0.030	101	41 (38, 45)	45 (42, 48)	0.045
Serum CRP (mg/L), *geometric mean (95% CI)*	431	35 (30, 40)	32 (28, 37)	0.36	407	28 (24, 33)	33 (28, 38)	0.08

Abbreviations: LNS, lipid-based nutrient supplement; LNS-VM, LNS with added vitamins and minerals; Hb, haemoglobin; CI, confidence interval; sTfR, soluble transferrin receptor; CRP, C-reactive protein

*Only patients with week 6 data included therefore lower sample size than Table 1.

† Paired t-test

Table 3: Linear regression showing the effect of the intervention on haemoglobin, iron and inflammatory markers at week 6 with regression coefficients (B), 95% CIs and the corresponding *P* values, using three models of adjustment.

Variable (week 6)	N§	Model 1*			Model 2†			Model 3‡		
		B‖	95% CI	P value¶	B‖	95% CI	P value¶	B‖	95% CI	P value¶
Hb (g/L)	705	2.16	-0.91, 5.23	0.17	1.88	-1.13, 4.90	0.22	1.60	-1.30, 4.49	0.28
Log serum ferritin	165	-0.20	-0.50, 0.09	0.18	-0.20	-0.49, 0.09	0.18	-0.19	-0.46, 0.07	0.14
sTfR (nmol/L)	164	0.99	-3.57, 5.55	0.67	1.02	-3.55, 5.59	0.66	1.68	-2.95, 6.33	0.47
Log serum CRP	838	-0.10	-0.29, 0.10	0.33				-0.11	-0.29, 0.08	0.26

Abbreviations: B, regression coefficient; CI, confidence interval; Hb, haemoglobin; sTfR, soluble transferrin receptor; CRP, C-reactive protein

*Adjusted for baseline value of the same dependent variable

†Adjusted for the baseline value of the same dependent variable and log-CRP at week 6 for the iron markers.

‡Adjusted for the baseline value of the same dependent variable, log-CRP at week 6 for the iron markers, sex, site, age, baseline CD4 count, being on TB medicine at recruitment, taking iron supplements in addition to the study supplement, length of time from recruitment to ART and baseline BMI.

§Number restricted to the same sample as in the fully adjusted Model 3.

‖The coefficient shows the effect associated with the intervention on week 6 outcomes in comparison to the control.

¶Two sample t-test

Table 4: Linear regression models showing the effect of the intervention on iron markers at week 6, stratified by baseline iron marker category[*]

Dependent Variable (week 6)	N	Baseline iron marker category stratification[†]	Coefficient[‡]	95% CI	P value§	P value (test for interaction)‖
Hb (g/L)	705	Normal & mild anaemia	3.31	-1.82, 8.44	0.21	0.38
		Moderate & severe anaemia	0.58	-2.95, 4.10	0.75	
	138	Ferritin below median	0.70	-7.56, 8.97	0.87	0.18
		Ferritin above median	8.73	-0.83, 18.28	0.07	
	138	sTfR below median	3.59	-5.00, 12.19	0.41	0.61
		sTfR above median	6.64	-2.75, 16.04	0.16	
Log Serum ferritin	148	Normal & mild anaemia	0.05	-0.43, 0.54	0.83	0.12
		Moderate & severe anaemia	-0.40	-0.74, -0.06	0.023	
	165	Ferritin below median	-0.08	-0.42, 0.26	0.63	0.30
		Ferritin above median	-0.35	-0.76, -0.06	0.10	
	165	sTfR below median	-0.27	-0.63, 0.09	0.14	0.52
		sTfR above median	-0.11	-0.48, 0.26	0.55	
sTfR (nmol/L)	147	Normal & mild anaemia	1.77	-6.77, 10.31	0.68	0.60
		Moderate & severe anaemia	-0.91	-6.98, 5.17	0.77	
	164	Ferritin below median	2.77	-3.05, 8.59	0.35	0.52
		Ferritin above median	-0.06	-7.09, 6.97	0.99	
	164	sTfR below median	2.74	-3.71, 9.19	0.40	0.59
		sTfR above median	0.32	-6.24, 6.88	0.92	

Abbreviations: CI, confidence interval; Hb, haemoglobin; sTfR, soluble transferrin receptor.

[*] Adjusted for the baseline value of the same dependent variable, log-CRP at week 6 for the iron markers, sex, site, age, baseline CD4 count, being on TB medicine at recruitment, taking iron supplements in addition to the study supplement, length of time from recruitment to ART and baseline BMI.

[†]Hb categories defined as normal ≥120g/L for women and ≥130g/L for men, mild anaemia <120g/L for women and <130g/L for men, moderate and severe anaemia <110g/L and <80g/L respectively for both sexes. Serum ferritin median = 752µg/l. sTfR median = 45 nmol/L.

[‡]The coefficient shows the change in week 6 iron marker associated with the intervention in comparison to the control within the baseline iron marker category strata.

§Two sample t-test

||Likelihood ratio test comparing models with and without the interaction between trial arm and baseline iron marker category.

Table 5: Pairwise correlation matrix between iron markers and CRP at baseline and week 6, unadjusted[†‡]

	Log serum CRP Baseline Coefficient	Hb Baseline Coefficient	sTfR Baseline Coefficient	Log serum ferritin Baseline Coefficient	Log serum CRP Week 6 Coefficient	Hb Week 6 Coefficient	sTfR Week 6 Coefficient	Log serum ferritin Week 6 Coefficient
Log serum CRP, baseline	1.00							
Hb, baseline	-0.31**	1.00						
sTfR, baseline	-0.10	-0.26**	1.00					
Log serum ferritin, baseline	0.34**	-0.02	-0.12*	1.00				
Log serum CRP, week 6	0.30**	-0.19**	-0.15*	0.24**	1.00			
Hb, week 6	-0.13**	0.51**	-0.10	0.11	-0.26**	1.00		
sTfR, week 6	-0.10	-0.14	0.32**	-0.31**	0.00	-0.27**	1.00	
Log serum ferritin, week 6	0.16*	0.20*	-0.07	0.54**	0.24**	0.16*	-0.22**	1.00

Abbreviations: CRP, C-reactive protein; Hb, haemoglobin; sTfR, soluble transferrin receptor

[†] Pearson's correlation.

[‡] N: serum CRP baseline (1762), Hb baseline (1670), sTfR baseline (353), serum ferritin baseline (353), serum CRP week 6 (863), Hb week 6 (826), sTfR week 6 (186), serum ferritin week 6 (165). Note that CRP and Hb were available for the whole trial sample, sTfR and ferritin only for the the subsample, and sample sizes for the individual variables are determined by the availability of completed week 6 data.

*P<0.05

**P<0.01

Table 6: Multivariable linear regression model showing the effect of a one-log decrease in CRP on change in iron markers from baseline to week 6[*]

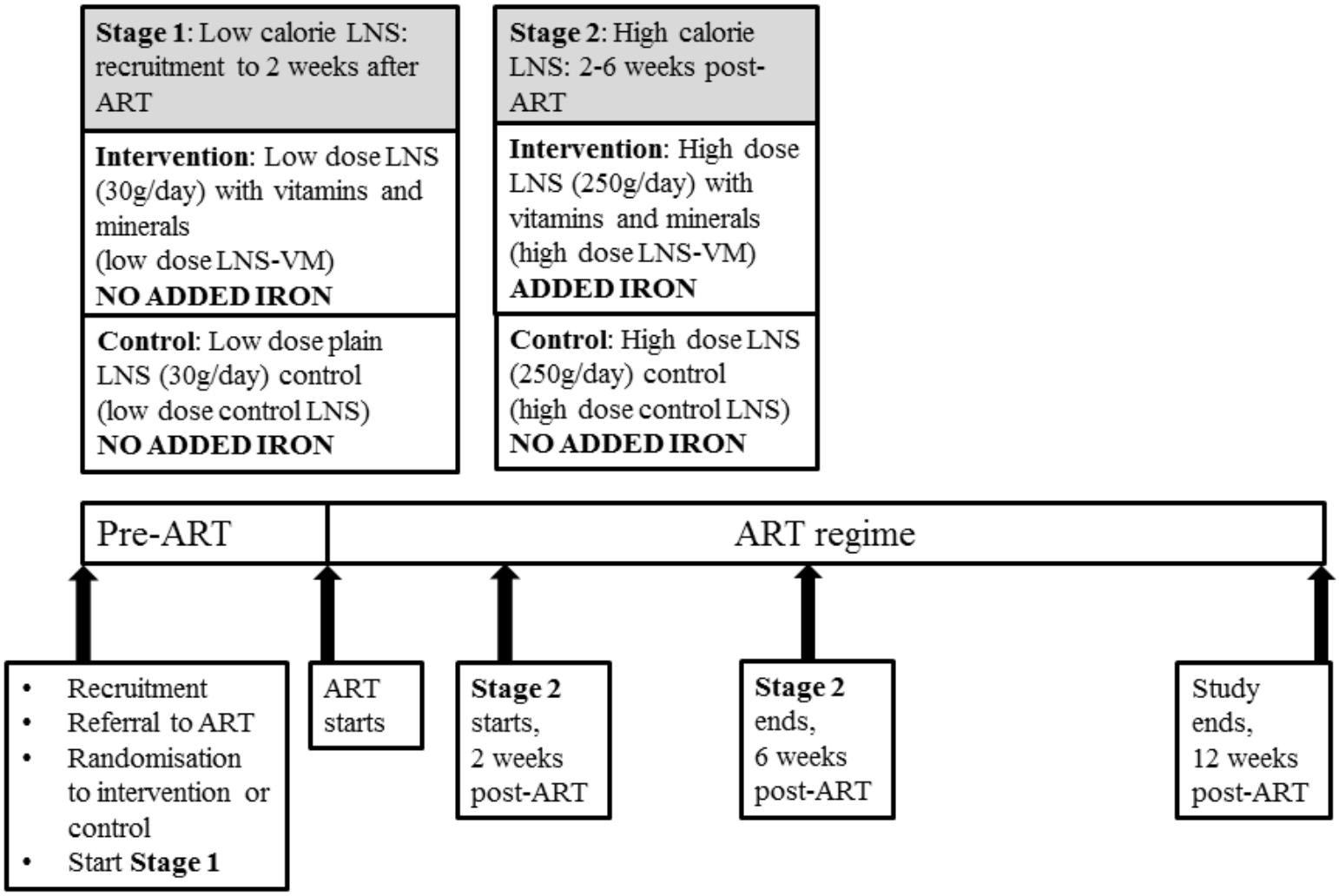
Variable (change from baseline to week 6)	N	Coefficient[†]	95% CI	P value[‡]
Hb (g/L)	687	1.81	0.85, 2.76	<0.001
Log serum ferritin	165	-0.11	-0.20, 0.03	0.012
sTfR (nmol/L)	164	-0.24	-1.89, 1.42	0.78

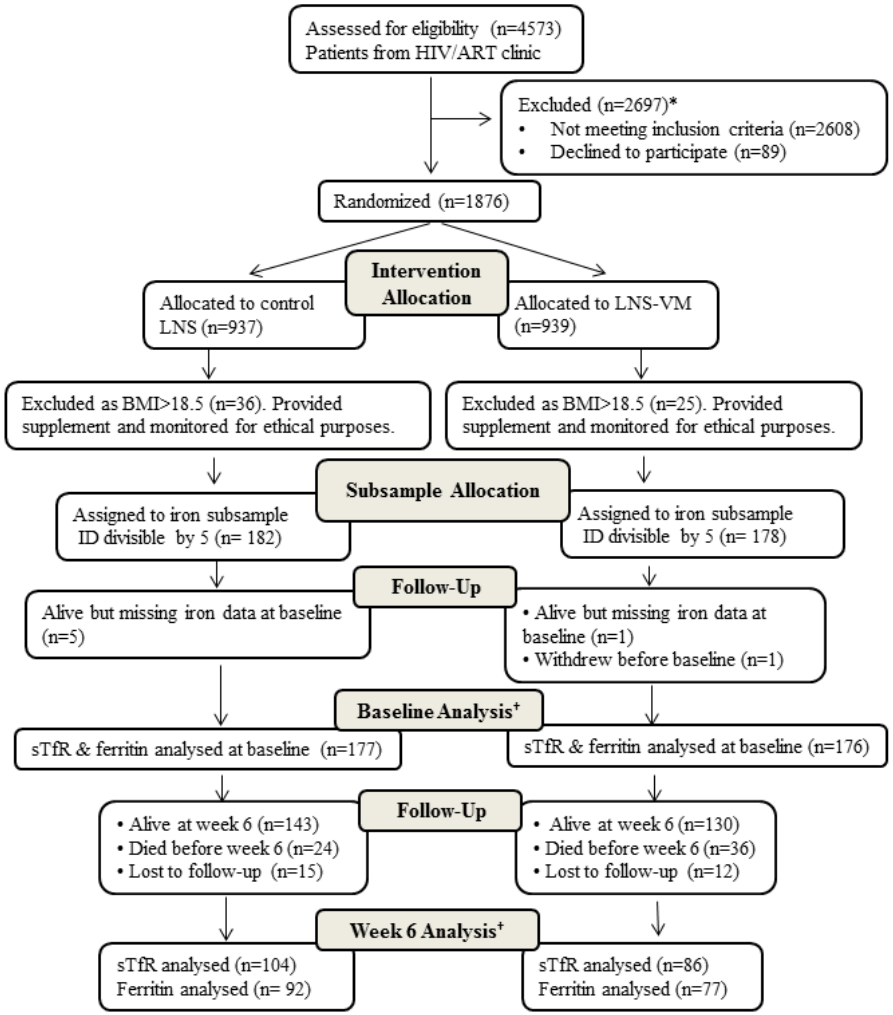
Abbreviations: CI, confidence interval; Hb, haemoglobin; sTfR, soluble transferrin receptor.

[*]Adjusted for trial arm, sex, site, age, baseline CD4 count, being on TB medicine at recruitment, taking iron supplements in addition to the study supplement, length of time from recruitment to ART and baseline BMI.

[†]The coefficient represents the change in iron marker from baseline to week 6 associated with a one-log decrease in CRP from baseline to week 6.

[‡]Two sample t-test score result.


Stage 1: Low calorie LNS: recruitment to 2 weeks after ART
Intervention: Low dose LNS (30g/day) with vitamins and minerals (low dose LNS-VM) **NO ADDED IRON**
Control: Low dose plain LNS (30g/day) control (low dose control LNS) **NO ADDED IRON**
Stage 2: High calorie LNS: 2-6 weeks post-ART
Intervention: High dose LNS (250g/day) with vitamins and minerals (high dose LNS-VM) **ADDED IRON**
Control: High dose LNS (250g/day) control (high dose control LNS) **NO ADDED IRON**
Pre-ART
ART regime
• Recruitment
• Referral to ART
• Randomisation to intervention or control
• Start **Stage 1**
ART starts
Stage 2 starts, 2 weeks post-ART
Stage 2 ends, 6 weeks post-ART
Study ends, 12 weeks post-ART


Assessed for eligibility (n=4573)
Patients from HIV/ART clinic
Excluded (n=2697)*
• Not meeting inclusion criteria (n=2608)
• Declined to participate (n=89)
Randomized (n=1876)
Intervention Allocation
Allocated to control LNS (n=937)
Allocated to LNS-VM (n=939)
Excluded as BMI>18.5 (n=36). Provided supplement and monitored for ethical purposes.
Excluded as BMI>18.5 (n=25). Provided supplement and monitored for ethical purposes.
Subsample Allocation
Assigned to iron subsample ID divisible by 5 (n= 182)
Assigned to iron subsample ID divisible by 5 (n= 178)
Follow-Up
Alive but missing iron data at baseline (n=5)
• Alive but missing iron data at baseline (n=1)
• Withdrew before baseline (n=1)
Baseline Analysis+
sTfR & ferritin analysed at baseline (n=177)
sTfR & ferritin analysed at baseline (n=176)
Follow-Up
• Alive at week 6 (n=143)
• Died before week 6 (n=24)
• Lost to follow-up (n=15)
• Alive at week 6 (n=130)
• Died before week 6 (n=36)
• Lost to follow-up (n=12)
Week 6 Analysis+
sTfR analysed (n=104)
Ferritin analysed (n= 92)
sTfR analysed (n=86)
Ferritin analysed (n=77)