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A double blind randomised controlled trial comparing standard dose of iron supplementation for pregnant women with two screen-and-treat approaches using hepcidin as a biomarker for ready and safe to receive iron

Brief title: Hepcidin and anaemia in pregnancy (HAPn)

Amat Bah

Thesis submitted in accordance with the requirements for the degree of

Doctor of Philosophy (PhD)

Of the

University of London

October, 2019

Department of Population Health

Faculty of Epidemiology and Population Health

LONDON SCHOOL OF HYGIENE & TROPICAL MEDICINE

Funded by the UK Medical Research Council (MRC) and the UK Department for International Development (DFID), under the MRC/DFID Concordat agreement to the MRC International Nutrition Group, grant MC-A760-5QX00.

Research group affiliation: Medical Research Council Unit, The Gambia at the London School of Hygiene & Tropical Medicine (MRCG at LSHTM).

Declaration



London School of Hygiene & Tropical Medicine
Keppel Street, London WC1E 7HT

T: +44 (0)20 7299 4646
F: +44 (0)20 7299 4656
www.lshtm.ac.uk

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Student ID Number	025190	Title	Mr
First Name(s)	AMAT		
Surname/Family Name	BAH		
Programme of Study	EPIDEMIOLOGY AND POPULATION HEALTH (EPH)		
LSHTM Email (if this is no longer active, please provide an alternative)	amat.bah@lshtm.ac.uk		

2. TITLE OF THESIS

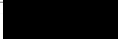
Title of Thesis	A DOUBLE BLIND RANDOMISED CONTROLLED TRIAL COMPARING STANDARD DOSE OF IRON SUPPLEMENTATION FOR PREGNANT WOMEN WITH TWO SCREEN-AND-TREAT APPROACHES USING HEPcidIN AS A BIOMARKER FOR READY AND SAFE TO RECEIVE IRON.
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Dedication

To my mum; Rohey Njie and my late dad, Pa Geran Bah.

Acknowledgement

I am sincerely grateful to Prof Andrew M. Prentice my supervisor and Dr Sophie E. Moore my associate supervisor for not only having confidence in me to pursue this PhD but also obtaining the financial support needed. I am so thankful to them and to Dr Rita Wegmuller, Dr Hans Verhoef for all the support in the design and development of the PhD.

Thank you to my Advisory Committee members of Dr Hans Verhoef, Dr Kalifa Bojang and Dr Rita Wegmuller for all the input throughout the PhD. I would also want to thank all the administrative staff of the Nutrition Theme (Ms Jenny Howard, Ms Myriam Benyahia, Ms Isatou Dibba, Mr Buba Jabang, Ms Joanne Tapper, Mr Amulai Touray), and Ms Jenny Flemming and Ms Lauren Dalton of LSHTM who made my work and stay both in The Gambia and UK pleasant.

My sincere gratitude is also extended to Prof Jay Berkley, Dr Momodou Jasseh and Dr Stefan Unger for serving as my data safety and monitoring board members and the valuable advises they have always volunteered. Many thanks also go to Dr Momodou K. Darboe, Mr Mohammed Ngum, Ms Jenny Muller, Mr Bai Lamin Dondoh, Mr Abdoulie Peters and wife Mrs Isatou Peters, Mr Baba Darboe, Mr Kabiru Ceesay, Mr Musa Jarjou, Ms Helen Nabwera, Mr Schadrac Agbla, Mr Modou Cheyassin Phall and the team at the National Nutrition Agency [NaNA], Dr Adama Demba, the then Director of Health Services, Mr Modou Lamin Manneh of the Lower River Regional Health Team and staff of the health facilities of Kaiak and Soma for their encouragement and feedback. I am grateful to Mr Muhammed Khalie Abdul and Ms Lindsay Kendall for the statistical support and analysis.

I wish to acknowledge the immense contribution of people of Jarra West and Kiang East, especially the pregnant women and their husbands for being part of the trial. This work would not have been successful without their commitment and participation.

I am extremely grateful to MRCG at LSHTM for the PHD studentship; Bill and Melinda Gates Foundation for the financial support and the Dutch State Mines (DSM) for the provision of the supplements.

I would want to appreciate the enormous contribution of the MRCG at LSHTM (Keneba research station) laboratory, data management, clinic, and transport staff for the immense support accorded to my team and me. I am highly indebted to the study team for the two years we spent in the field conducting the trial.

Finally, let me thank my family (Maimuna, Isatou, Ida, Hujaijah, Fatou and Awa) for the support, prayers, and always being there for Daddy.

Abstract

WHO recommends daily iron supplementation (60mg) for all pregnant women where anaemia prevalence exceeds 40%. However, recent evidence suggests that iron supplementation may be harmful as it increases the risks of hypertension and of infection. Iron absorption is regulated by hepcidin, a key iron regulatory hormone with the potential to be a useful marker to determine if oral iron can be absorbed effectively and safely. We aimed to identify a hepcidin threshold to define 'safe and ready' to receive iron and then test whether a hepcidin-guided screen-and-treat approach to iron supplementation is non-inferior to the WHO-recommended universal daily supplementation.

Method: We established our screening threshold by measuring haemoglobin and serum hepcidin, ferritin, iron, soluble transferrin receptor (sTfR), and C-reactive protein (CRP) at 14, 20 and 30 week of gestation among 395 pregnant rural Gambian women using archived maternal blood samples (2010-2013), and analysed hepcidin's diagnostic test accuracy [area under the receiver operating characteristic curve (AUC^{ROC}), sensitivity, specificity, cut offs] for iron deficiency at each time point. We established a threshold of 2.5 μ g/L. We then conducted a 3-arm randomised-controlled proof-of-concept trial in rural Gambia from June 2014 to March 2016. We recruited 498 pregnant women aged 18-45 years with 14-22 weeks gestation to receive either: (A) UNU/UNICEF/WHO international multiple micronutrient preparation (UNIMMAP) containing iron 60mg/d; (B) UNIMMAP containing iron 60mg/d but based on a weekly hepcidin screening <2.5 μ g/L indicating if iron can be given for the next 7 days or not; or (C): as in (B), but with iron 30mg/d. We report the per protocol analysis for primary and secondary outcomes at Day 84. We assessed non-inferiority with the primary endpoint being haemoglobin concentration at Day 84 with a non-inferiority margin of -5.0g/L.

Results: The evidence for non-inferiority for screen-and-treat approaches using either 60mg iron (mean haemoglobin difference relative to Reference arm: -2.2g/L; 95% CI: -4.6, 0.1g/L) or 30mg iron (-2.7g/L; 95% CI: -5.0, -0.5g/L) was marginal. Anaemia (haemoglobin <110g/L) at the end of intervention was less prevalent in the Daily iron supplementation (Reference) arm than both Screen-and-treat arms. Among those without inflammation at the end of intervention, the prevalence of iron deficiency (ferritin <15 μ g/L) was less in the Reference arm compared to the two Screen-and-treat arms; corresponding prevalence values for transferrin saturation <16%; soluble transferrin receptor >4.4mg/L and hepcidin <2.5 μ g/L were lower in the Reference arm. The Screen-and-treat approaches had no added

advantage than universal daily iron supplementation in terms of adherence, side effects or safety outcomes.

Conclusion: The daily 60mg iron supplementation arm performed better than both screen-and-treat arms for anaemia and other iron markers (hepcidin, ferritin, soluble transferrin receptor, transferrin saturation). We therefore found no support for a screen-and-treat iron supplementation based on hepcidin concentration $<2.5\mu\text{g/L}$ in pregnant Gambian women.

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Acronyms:

AGP	Alpha-1 acid Glycoprotein
AOR	Adjusted Odds Ratio
CRP	C-reactive protein
DSS	Demographic Surveillance
System	
ID	Iron Deficiency
IDA	Iron Deficiency Anaemia
LBW	Low Birth Weight
LSHTM	London School of Hygiene and Tropical Medicine
MRC- ING	Medical Research Council International Nutrition Group
MRC	Medical Research Council
PI	Principal Investigator
PVE	Plasma Volume Expansion
RCT	Randomised Control Trial
SCC	Scientific Coordinating Committee
SF	Serum Ferritin
sTfR	soluble transferrin receptors
TBI	Total Body Iron
TIBC	Total Iron Binding Capacity
TSAT	Transferrin Saturation
WHO	World Health Organisation
WIMM	Weatherall Institute of Molecular Medicine
ZnPP	Zinc Protoporphyrin

Chapter 1: Introduction

1.1 Candidate's involvement

After working for over two decades in many areas of nutrition policy and programme implementation including heading the micronutrient deficiency control programme in The Gambia, I developed a special interest in the control of anaemia among pregnant women and children. Therefore, when this opportunity to do a PhD arose, I undertook it. The concept of this study was conceived by my supervisor, Prof Andrew M. Prentice and myself with input from Dr Sophie E. Moore. I did all the background literature review; wrote the design; developed the study proposal and the trial protocols; and presented the proposal to both the MRC Scientific Coordinating Committee and the Gambia Government/MRC Ethics Committee for approval. The proposal was also submitted to the LSHTM Ethics Committee for approval. I was responsible for the identification of the study site and coordinated all the fieldwork which included the training and management of all field staff. I assisted in the laboratory analysis of some of the samples and with the assistance of the data team in Keneba, I undertook all of the data cleaning and some basic statistical analysis. I wrote this thesis and for the papers incorporated, I wrote the manuscripts and have the co-authors comments and contributions included. All the authors approved the manuscripts before submission for publication.

1.2 Scope and composition of the thesis

The thesis is born out of the desire to contribute towards the appropriate management of anaemia and iron deficiency in pregnancy, as they are a global public health burden affecting both developed and developing countries. Since the most significant contributor to the onset of anaemia worldwide is iron deficiency, WHO recommends universal iron supplementation for pregnant women. However, recent studies and reviews show that not all pregnant women may need to receive universal iron supplementation. This, coupled with the fact that studies have shown that higher haemoglobin levels may not necessarily lead to favourable pregnancy outcomes led to our idea of testing the hypothesis that a screen-and-treat approach to iron supplementation using a predetermined hepcidin value of $<2.5\mu\text{g/L}$ will be non-inferior to the reference universal daily iron supplementation level of 60mg iron

recommended by WHO, and would represent a safer approach to iron supplementation by reducing the amount of iron administered. This PhD thesis is by a research paper style, where the research papers are incorporated in to the thesis as chapters:

Chapter 1: The preface to the thesis summarises: my supervisory team and collaborators; the candidate's involvement and declaration; the scope and composition of the thesis together with details of the source of funding and study timeline.

Chapter 2: Summarises the background and literature review on maternal anaemia and pregnancy outcome; maternal anaemia and iron status of the foetus and of the infant; maternal anaemia and birth outcomes focusing on low birth weight; current methods of assessing iron deficiency and iron deficiency anaemia and their problems; the potential of hepcidin as an improved index for safe-and-ready to receive iron and the need for a better point-of-care (PoC) diagnostic for iron deficiency.

Chapter 3: Is the published protocol paper. The paper describes the background to the main study of this thesis with its design and methods; study location; ethical permission and safety monitoring: informed consent and confidentiality as well as the sample size determination and the statistical analyses to be undertaken. Published in BMC Pregnancy and Childbirth (DOI:[10.1186/s12884-016-0934-8](https://doi.org/10.1186/s12884-016-0934-8)).

Chapter 4: Is a research paper co-authored by the student exploring the potential of hepcidin, entitled: *Serum hepcidin declines in concentration during pregnancy and may identify iron deficiency: Analysis of a longitudinal pregnancy cohort in The Gambia*. The paper characterised the changes in hepcidin and indices of iron stores, erythropoiesis and inflammation in pregnancy and the assessment of hepcidin's diagnostic potential as an index of iron deficiency. Published in the Journal of Nutrition (<http://dx.doi.org/10.3945/jn.116.245373>).

Chapter 5: is the main research paper that examines the hypothesis that a screen-and-treat approach to iron supplementation is non-inferior to universal (daily) iron supplementation. The paper summarises the study outcome of the 498 pregnant women (gestational age 14 – 22 weeks) recruited and randomised in to the 3 arms described in the protocol paper. Our data show a marginal non-inferiority for the screen-and-treat approaches (using 60mg or 30mg iron) to the universal iron supplementation. The hepcidin-guided screen-and-treat

approaches were less efficacious in combatting iron deficiency and iron deficiency anaemia than universal daily iron supplementation and had no advantages in terms of adherence, side effects or safety outcomes.

Chapter 6: Presents a summary discussion and conclusions including the study limitations, the public health implication and policy recommendation, and future research needs.

1.3 Supervisory team and collaborators:

Supervisor:

Prof Andrew M. Prentice
LSHTM, MRCG at LSHTM, UK.

Associate Supervisor:

Dr Sophie E. Moore
King's College London, UK

Advisory Committee Members:

Dr Hans Verhoef,
Senior Lecturer, LSHTM, UK.

Dr Kalifa Bojang
MRCG at LSHTM, The Gambia.

Dr Rita Wegmuller
MRCG at LSHTM, The Gambia.

Collaborators and collaborating institutions:

Dr Hal Drakesmith, Dr Sant-Rayn Pasricha, Dr Andrew E. Armitage and team, WIMM, University of Oxford, United kingdom
Dr Lorna Cox and team, MRC Elsie Widdowson Laboratory (MRC-EWL), Cambridge, UK
Dr Carla Cerami, MRCG at LSHTM, The Gambia

1.4 Funding

The PhD is funded by the UK Medical Research Council (MRC) and the UK Department for International Development (DFID), under the MRC/DFID Concordat agreement to the MRC International Nutrition Group (MRC-ING), grant MC-A760-5QX00 and the research by the Bill & Melinda Gates Foundation (OPP 1055865) awarded to MRC-ING.

1.5 Study timeline

The timeline for the PhD work is shown in **Table 1**. The PhD started as a full time programme but after the recruitment of the study participants for the first cohort, we realised that we could not complete the required sample size within the anticipated timeframe. Therefore, my supervisor and Faculty Research Degree Director recommended that I resort to part-time to enable me finish the field work and the PhD on time.

Table 1: Timeline for the PhD

Activity	2012				2013				2014				2015				2016				2017				2018			
	J-M	A-J	J-S	O-D	J-M	A-J	J-S	O-D	J-M	A-J	J-S	O-D	J-M	A-J	J-S	O-D	J-M	A-J	J-S	O-D	J-M	A-J	J-S	O-D	J-M	A-J	J-S	O-D
<ul style="list-style-type: none"> Develop research proposal Officially register and attend relevant courses Conduct literature search 	█	█																										
<ul style="list-style-type: none"> Conduct systematic literature review Refine research proposal Prepare Upgrading report 			█	█																								
<ul style="list-style-type: none"> Upgrading seminar Attend courses and workshops Apply for ethics approval Refine proposal 			█	█	█																							
<ul style="list-style-type: none"> Conduct analysis of stored aliquots for hepcidin cut-off point Design data collection tools 					█	█	█	█	█	█																		
<ul style="list-style-type: none"> Recruit study participants Data collection, entry cleaning & locking 													█	█	█	█	█	█	█	█	█	█	█	█				
<ul style="list-style-type: none"> Data analysis Thesis writing Thesis submission 																									█	█	█	█
<ul style="list-style-type: none"> Completion process (Viva and corrections) 																									█	█	█	█

Chapter 2: Background and literature review

Anaemia is a global public health problem affecting both developing and developed countries with major consequences for human health, social and economic development [1]. Anaemia affects all population groups but those at greatest risk are pregnant women and children [2]. For women, consequences of anaemia include poor pregnancy and birth outcomes including premature delivery, low birth weight and increased perinatal mortality [3].

2.1 Maternal anaemia and pregnancy outcome (maternal mortality):

The impact of anaemia on maternal mortality has been demonstrated in many studies and reviews that found a strong association between severe anaemia and maternal mortality [4, 5]. In a review of anaemia in pregnancy in developing countries, van den Broek [6] concluded that estimates of maternal mortality from anaemia range from 34 per 100,000 live births in Nigeria to as high as 194 per 100,000 in Pakistan and that in combination with obstetric haemorrhage, anaemia is estimated to be responsible for 17 - 46% of cases of maternal deaths. During pregnancy, low haemoglobin levels, indicative of moderate (between 7.0 and 9.0 g/dL) or severe (less than 7.0 g/dL) anaemia, are associated with increased risk of maternal and child mortality and infectious diseases [7].

Cham et al [8] in determining the causes and contributing factors to maternal deaths in rural Gambia showed that anaemia was the leading cause of death followed by haemorrhage. In a retrospective study of maternal deaths in a referral hospital in The Gambia, a four-fold increase in the proportion of maternal deaths due to anaemia was recorded between 1991 - 1992 and 2001 - 2002 (8%-32% respectively) and a six fold increase of maternal mortality ratio due to anaemia ($P = 0.000003$) between 2001 - 2002 [9].

However, according to Greenwood et al [10], although maternal mortality was found to be very high (22 per 1000 live births), the major contributing factors to maternal mortality in The Gambia were post-partum haemorrhage and infections, early or late pregnancies (under 20 or over 40 years), multiple pregnancies and obstructed labour. Other indirect contributing factors included anaemia, low standard of health care for obstetric referrals, delayed decision making for referral and lack of transport [10, 11]. Post-partum haemorrhage being the major cause of maternal deaths has also been shown in many studies together with obstructed labour, post-partum sepsis, eclampsia and unsafe abortion. [5, 12-14].

The contribution of anaemia as one of the major indirect causes of maternal mortality has been demonstrated in many studies [11, 13, 14]. Whereas a strong association has been shown between severe anaemia and maternal mortality, the same cannot be said for mild or moderate anaemia where the relative risk associated with moderate anaemia (Hb 40-80 g/L) is 1.35 [95% CI: 0.92-2.00] and that for severe anaemia (< 47 g/L) is 3.51 [95% CI: 2.05-6.00] [4].

Urging on the side of caution in the interpretation of the data on maternal anaemia and maternal mortality, Allen [15] is quoted thus, *“some data show an association between a higher risk of maternal mortality and severe anemia. Such data were predominantly retrospective observations of an association between maternal hemoglobin concentrations at, or close to, delivery and subsequent mortality. Such data do not prove that maternal anaemia causes higher mortality because both the anaemia and subsequent mortality could be caused by some other condition. No prospective studies have proven that anemia per-se increases the risk of maternal mortality, and there is inadequate information on an established hemoglobin concentration below which the risk of mortality increases”*.

2.2 Maternal anaemia and iron status of the foetus and of infants

There has been mounting evidence to indicate that maternal iron deficiency in pregnancy reduces foetal (and hence infant) iron store perhaps well into the first year of life and that this deserves further exploration because of the tendency of infants developing iron deficiency anaemia coupled with the documented adverse consequences of this condition on infant development [15].

Several studies have demonstrated that haemoglobin, serum iron, transferrin saturation and ferritin are significantly lower in the cord blood of anaemic women suggesting that iron supply to the foetus is reduced in maternal anaemia. This further suggests that maternal anaemia adversely affects the iron status including iron stores of the newborn [16-24].

Even moderate or mild iron deficiency in mothers has been shown to contribute to lower iron reserves, if not frank iron depletion in the foetus [25-27] and that even non-anaemic iron deficient mothers may affect the iron status in their babies and predispose them to iron deficiency [28].

In a study of 617 pregnant women and their children in Benin, the relationship of newborn anaemia to maternal anaemia had an odds ratio equivalent to 1.8 (CI = 1.2-2.5) [29] and Faber et al [30] found that the child of an anaemic mother had a relative risk of 1.63 of also being anaemic. Meinzen-Derr et al [31] also found that maternal anaemia was independently associated with a 3 fold increased risk of infant anaemia. In a study in Malawi, Brabin et al [32] found that where foetal anaemia occurred in 23.4% of babies, the factors associated with foetal anaemia were among other things; maternal Hb at delivery < 8 g/dL (AOR 1.61, 1.10-2.42) or <11 g/dl (AOR 1.60, 1.10-2.31) (AOR= adjusted odds ratio). They also noted that up to 60% of cases of foetal anaemia were directly attributable to maternal anaemia, **Figure 1**.

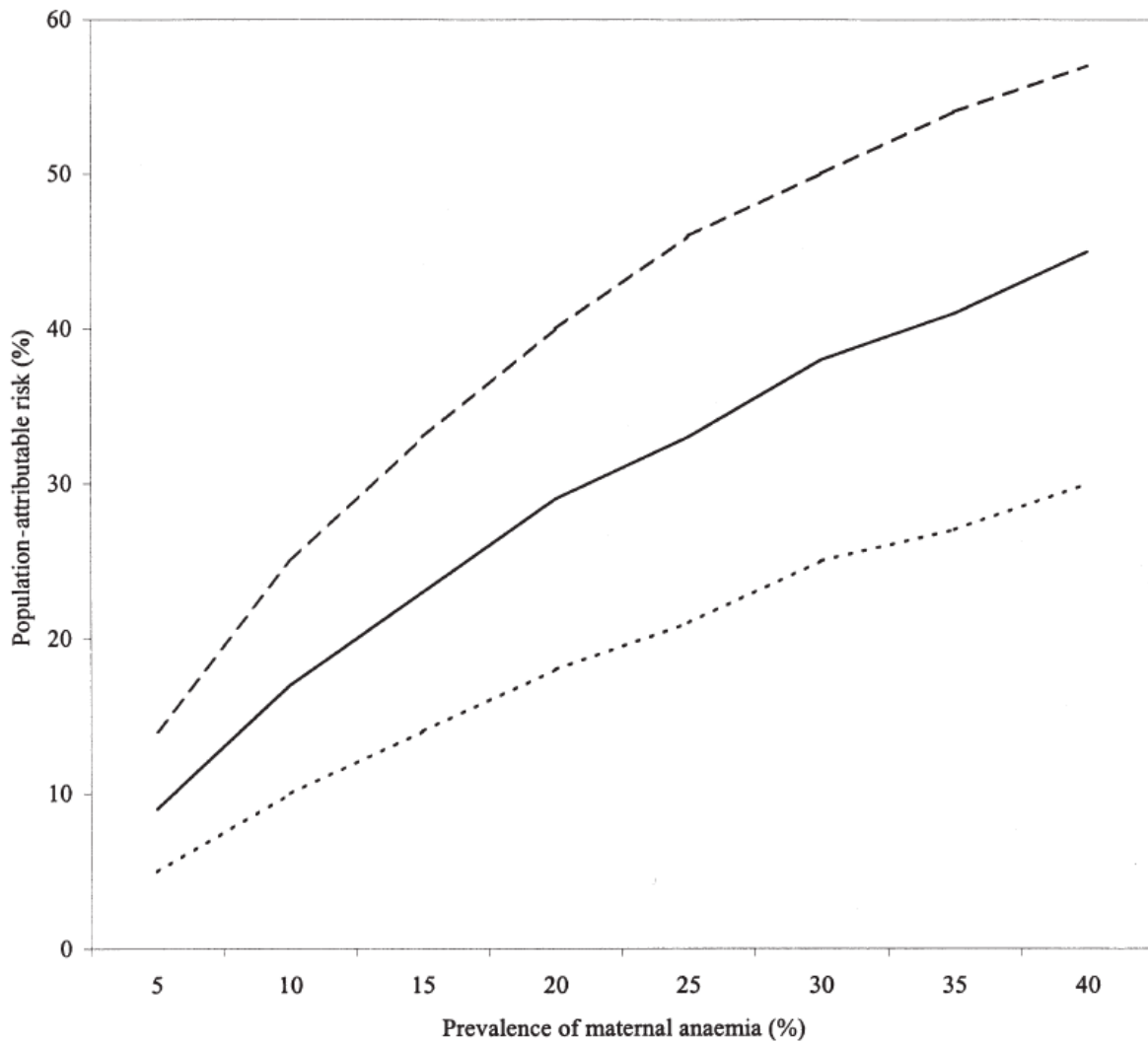


Figure 1: Estimates of population-attributable risk of fetal anaemia in a highly malarious area in relation to maternal anaemia (Hb <8g/dL; ----- upper 95% CI, lower 95% CI, — PAR for anaemia (Hb <8g/dL).

Babies born to mothers with low haemoglobin are born with less total body iron (TBI) resulting in a substantially greater risk of anaemia from 3 to 12 months of age [33]. For serum ferritin concentration of newborn babies born to mothers with low serum ferritin at

term, their serum ferritin levels have been found to be significantly lower than those born to mothers with normal ferritin concentration and the difference between the two groups of infants persisted up to 6 months of age [34]. Similar findings have also been illustrated by Savoie Rioux [27], Colomer et al [35], Kilbride et al [36] showing a relationship between iron deficiency of the mother at delivery and the development of iron deficient infants up to age 12 months which may not have been detected at birth. However, Colomer et al [35] found no association between the infants' haemoglobin at 18 months and maternal anaemia.

The linear relationship between cord iron parameters (haemoglobin, serum ferritin, transferrin saturation) and maternal haemoglobin and serum ferritin, tends to indicate that the foetus extracted iron in amounts proportional to levels available in the mother leading to mothers with moderate to severe anaemia having significantly lower cord serum ferritin levels [24] suggesting a placenta iron threshold, limiting iron acquisition by the foetuses of women with severe iron deficiency [37].

Haemoglobin concentration [38, 39] and mean corpuscular volume [38] were significantly lower in babies born to iron deficient mothers than in babies born to iron sufficient mothers. Savoie Rioux [27] found a positive association between mother's haemoglobin and haematocrit during her third trimester and her infant's haemoglobin and haematocrit levels even at 9 months of age. Sweet et al [40] found that although maternal iron depletion was associated with reduced foetal iron stores because it was associated with decreased cord blood ferritin and haemoglobin, there was no change in free iron availability.

A few studies that found a relationship of other indices such as serum ferritin have questioned the correlation between maternal haemoglobin concentration and cord venous haemoglobin levels where Altinkaynak et al [41], Erdem et al [42], Goonewardene Liyanage [43], Mowafy Youssef [44] found no such correlation. Similarly, Shyamala et al [45] found no

significant difference in mean haemoglobin concentration in neonates born to anaemic mothers compared to those born to non-anaemic mothers.

On the other hand, several investigators have found that the iron status of pregnant women with iron deficiency or mild anaemia does not seem to have a significant impact on the iron levels found in their children [46-48]. When haemoglobin, the most widely used index for assessment of anaemia [49] is used and levels assessed in maternal and cord blood, no significant relationship is found [50-53]. Redd et al [54] observed that neither the mothers haematocrit at enrolment or at delivery was associated with having a low haematocrit in infants even 3 months after birth and Ogunbode [55] even observed that babies delivered by anaemic women at term were shown to have haematocrit levels that were actually in the normally acceptable range. In fact, severe maternal anaemia has been occasionally found to be associated with higher haemoglobin and ferritin values in the foetus [56], and higher serum iron levels in cord blood [57].

Serum ferritin levels have been noted as being by far the best indicator of iron store status in the absence of acute or chronic inflammation [58] and have been widely used in the assessment of iron deficiency anaemia. However, Gebre-Medhin Birgegard [59] in looking at serum ferritin levels of Ethiopian and Swedish mothers and their newborn infants found no correlation between maternal and cord blood ferritin. On the other hand, MacPhail et al [60] only found a weak correlation between maternal and cord serum ferritin concentration in 103 pregnant women and their normal term offspring in South Africa. In the case of iron supplementation, no significant difference was observed for serum ferritin of the newborn infants whether mothers received supplementation or not [61, 62] but Preziosi et al [61] noted that 3 months after delivery, serum ferritin concentrations were higher in infants of women supplemented with iron.

Polat et al [63] found that although soluble transferrin receptor (sTfR) levels of mothers with iron deficiency were higher than those of mothers having no iron deficiency ($P=0.009$) there was no difference in the levels of sTfR between newborns of both groups of mothers ($P=0.790$).

Mothers with a reduced store of iron (serum ferritin) at term can still manage to provide significant iron for the foetus [64] and the foetus continues to take up iron from the mother until delivery [65]. However, Balai et al [66] suggested that even though there is a selective intake of iron by the foetus in anaemic women to a ratio of maternal versus neonatal of 1:2.02 in an anaemic group as compared to 1:1.6 in the non-anaemic group, the selective intake by the foetus cannot prevent the development of anaemia in the newborn. Recent evidence from our group also shows that even in conditions of low maternal iron and the absence of zinc supplementation, the placenta upregulates the gene expression of iron and zinc uptake proteins, presumably to meet foetal demand in the face of low maternal supply [67]

Nonetheless, Ervasti et al [68] showed that in well-nourished maternal populations, lower maternal iron status did not affect iron accumulation on the foetal side and Harthoorn-Lasthuizen et al [69] concluded that foetal iron supply is not negatively influenced by iron deficient erythropoiesis in the mother.

Breast milk iron content has been found to be significantly reduced in severely anaemic mothers but not in those with mild-to-moderate anaemia [70]. Breastmilk micronutrients including iron were observed to be significantly reduced in anaemic mothers [20]. However, mothers' haemoglobin and iron status have been found to have no relationship to breastmilk iron [71] and lactoferrin [72]. Baykan et al [73] even suggested that giving maternal iron supplementation during the first four months of lactation had no effect on serum iron and serum ferritin levels of mothers and infants, albeit the follow-up period was short in the study.

Delayed cord clamping in full term neonates for minimum of two minutes following birth is beneficial to the newborn extending in to infancy by improving iron status [74, 75]. This may lead to a clinically important reduction of the risk of anaemia in the newborn [74]. However, delayed cord clamping has been found to also lead to the increased risk of benign polycythemia [74] and jaundice requiring phototherapy. It is suggested that waiting until the umbilical cord stops pulsating is a feasible, low-cost intervention that can reduce anaemia in infants in developing countries [76].

In summary, it appears that there is a relationship between maternal anaemia and the iron status of their infants with anaemic mothers giving birth to babies with low iron stores. However, it should be noted that the measurement of haemoglobin alone may not necessarily lead to this conclusion as numerous studies found no correlation between maternal haemoglobin and cord or infant haemoglobin levels. It looks likely that where maternal iron stores are low, the newborns' iron endowment is affected. It is worth noting that different studies used different iron status markers and this makes it difficult to compare the results across studies.

2.3 Maternal anaemia and birth outcome: focus on low birth weight (LBW)

It has been shown in the West African state of Benin, that even with malaria and helminth prophylaxis, as well as iron and folic acid supplementation, prevalence of anaemia throughout pregnancy remained very high [77]. Severe anaemia was found to be associated with a higher risk of LBW. However, the adverse impact of moderate to mild anaemia was unclear [77]. There is evidence that maternal haemoglobin levels below 9.5 g/dL before or during the second trimester of gestation are associated with increased risk of giving birth to a low birthweight infant and with premature delivery [7]. However, low birth weight and preterm

delivery have been found to be related to pregnancy haemoglobin levels in a 'U' shape manner where severe anaemia and high haemoglobin concentration are both associated with increased risk of LBW and preterm deliveries [78-81].

Scanlon et al [82] noted that *"an elevated Hb level (>14.4g/dL) is an indicator of possible pregnancy complication associated with poor plasma volume expansion (PVE) and should not be mistaken for good iron status"*. Haemoglobin levels are expected to rise and fall at different stages of pregnancy due to plasma volume increase to meet the greater circulatory needs of the placenta and maternal organs with an average plasma volume increase of about 45% [83, 84]. The magnitude of the fall in haemoglobin concentration has been found to be related to birth weight and the failure of the haemoglobin concentration to fall below 10.5 g/dL indicates an increased risk of LBW and preterm delivery as seen in **Figures 2** and **3**, respectively [80, 85].

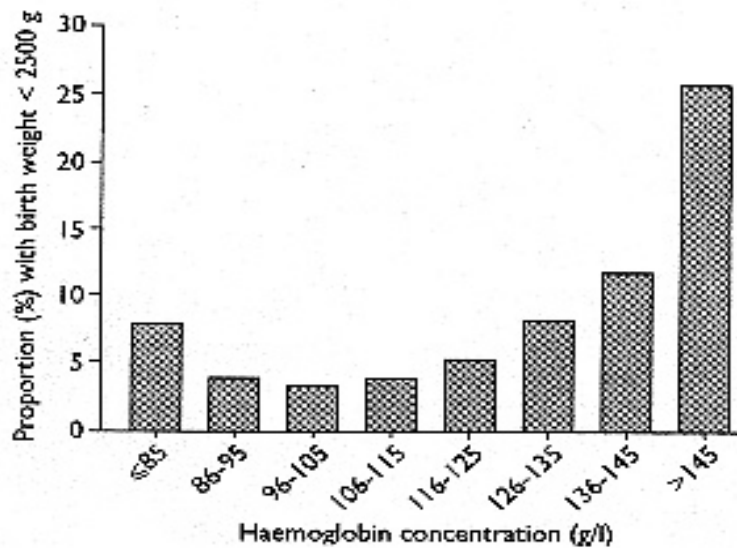


Figure 2: Incidence of low birth weight (<2500 g) by haemoglobin concentration (g/L) (data for white women only).

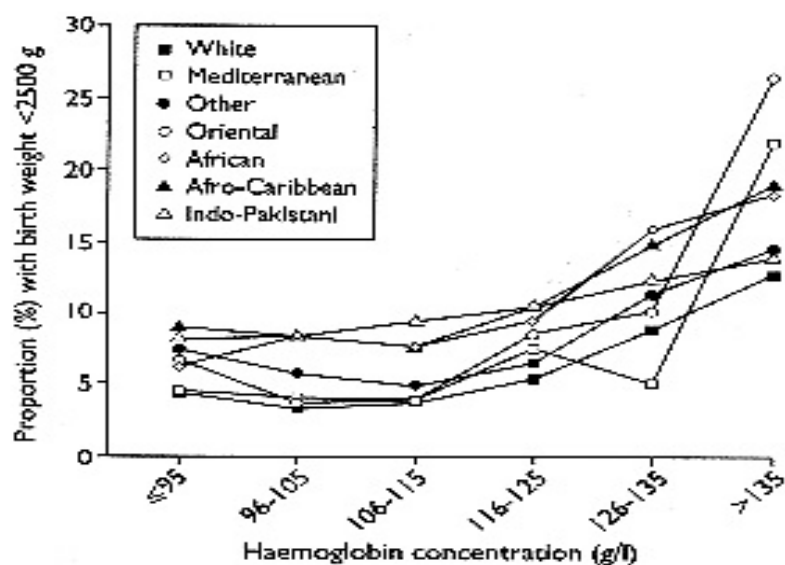


Figure 3: Adjusted odds ratios* (95% confidence intervals) for low birth weight (<2500 g) and preterm birth (<37 completed weeks).

Source: Adapted from: Steer et al [80].

Maximum mean birth weight has been observed in the haemoglobin category of 9.6-10.5 g/dl and therefore the least incidence of LBW. Mean birth weight fell with both increasing and decreasing haemoglobin values and consequently an increased incidence of LBW [80, 86].

Rasmussen [79] in a review of the literature concluded that; *“in populations with low rates of iron or folate deficiency among non-pregnant women, the primary cause of anaemia is likely to be PVE, and this anaemia is not associated with negative birth outcomes. However, maternal haemoglobin values during pregnancy are associated with birth weight and preterm birth in a ‘U’ shape relationship with higher rates of babies who are small, early or both at a low or high concentrations of maternal haemoglobin. A similar ‘U’ shape relationship is likely to be present between maternal haemoglobin concentration and neonatal and perinatal mortality but data for this remain insufficient”*.

2.4 Current methods of assessing iron deficiency (ID) and iron deficiency anaemia (IDA) and their problems

The most significant contributor to the onset of anaemia is iron deficiency and consequently iron deficiency anaemia and the two are often used synonymously [1]. Iron deficiency is defined as a condition in which there are no mobilisable iron stores and signs of a compromised supply of iron to tissues including the erythron are noted [87]. Generally, it is assumed that iron deficiency is the most frequent single cause of anaemia and that 50% of anaemia is due to iron deficiency [88-90]. WHO estimates that IDA affects almost half of the world's pre-school children and pregnant women with prevalence of over 65% in Africa and Asia, and, as discussed above, that it causes (directly or indirectly) one fourth of all maternal deaths [2]. Despite iron deficiency with or without anaemia having important consequences for human health and child development, there has been an absence of international agreement on how best to assess the iron status of populations [91].

Although accurate assessment of iron status is difficult, several well-established tests for iron status determination are available [6, 49]. However, there is no single standard test to assess iron deficiency without anaemia [49] (**Table 2**). The use of multiple tests only partially overcomes the limitations of the individual tests [92] and conducting several tests together to determine iron status is costly and inconvenient and therefore not an option in resource poor settings [49, 93].

The definitive method of assessing iron stores in pregnancy is still by examination of a stained bone marrow preparation [6] but the procedure is invasive [94] and traumatic [91].

Serum ferritin (SF) is one of the few biochemical indices of which low levels reflect depleted iron stores [49] and it has been found to be a significant and convenient predictor of iron deficiency anaemia [94, 95]. However, ferritin is raised by infection and inflammation and

thus has very high false negative rates in least developed countries as it is highly specific but has low sensitivity [96].

Zinc protoporphyrin (ZnPP) levels can be used as an indicator for lack of iron to the developing red blood cells [91]. In general, elevated levels correlate well with low serum ferritin and can serve to screen for moderate iron deficiency without anaemia [97]. However, levels can be affected by infection and inflammation [94].

The concentration of soluble transferrin receptors (sTfR) provides a semi-quantitative measure of the severity of iron deficiency even in the presence of inflammatory disorders [91] and it shows a sensitive response during the early development of iron deficiency including mild tissue iron deficiency [98]. However, sTfR may be elevated when there is increased red cell production, turnover or both, and there is a lack of uniform standards and agreed references for its measurement [49].

The use of the sTfR/LogSF ratio for estimation of iron stores has been demonstrated in healthy adults to be a good measure of iron status [93]. Although the method has been validated, there is a lack of standardisation in the assay ranges, unit of measurement and reference samples. The serum ferritin component is also influenced by infection and chronic disease [91].

Serum iron, total iron binding capacity (TIBC) and transferrin indices are also often used but none of these are very reliable indicators because they show marked individual variations, are affected by recent iron ingestion and are also sensitive to infection [6]. Just like serum iron, transferrin saturation (TSAT) is also used for estimation of chronic iron estimation but lacked specificity as any alteration in plasma iron concentration will alter its level [91].

Table 2: Limitation of current methods of assessing IDA

Test	Measure	Limitation
Stain bone marrow preparation	Iron stores	Expensive, invasive and traumatic
Haemoglobin (Hb)	Anaemia	Does not measure ID per-se
Serum ferritin (SF)	Iron stores	Raised by infection and inflammation
Zinc protoporpherin (ZnPP)	Iron in new cells	Affected by infection and inflammation
Soluble transferrin receptor (sTfR)	Severe ID even with inflammation	Affected by increased red cell production. Lack of standardised reference for measure'
sTfR/logSF ratio	Iron stores	Lack standardised assay range. Ferritin affected by infection or inflammation
Transferrin saturation (TSAT)	Iron levels	Affected by increased plasma concentration
Serum iron	Iron in sera	Affected by recent iron ingestion and infection
Total iron binding capacity (TIBC)	Iron in serum	Affected by infection

The recently discovered hormone, hepcidin (Section 2.5) is the master regulator of iron metabolism and there is now a considerable enthusiasm in the potential role of plasma and urinary hepcidin concentration in the screening of iron deficiency [94, 99].

2.5 Potential for hepcidin to be an improved index of 'safe and ready to receive' iron:

Hepcidin is a peptide hormone that has been shown recently to be the master regulator of iron absorption and distribution in humans [100-103]. Hepcidin controls iron homeostasis by inhibiting dietary iron absorption, release of iron in the macrophages and reducing iron flow to the erythron [102, 104-107] (**Figure 4**). Hepcidin binds to the iron exporter ferroportin inducing its internalisation and degradation [108] (**Figure 5**). Ferroportin is the sole known

iron exporter from cells [105] and is also the only mammalian iron exporter identified to date for materno-fetal iron transfer [109]. Levels are known to be reduced by hypoxia and iron deficiency [103] and increased by iron sufficiency or overload and infections [110-112]. Schulze et al [113] provided strong evidence that iron status influences hepcidin concentrations among pregnant Bangladeshi women indicating an insight in to the role of hepcidin in iron deficiency associated with pregnancy. The potential for hepcidin as a superior marker for iron deficiency has been highlighted in many recent studies [114-118]. Pasricha et al [117] in evaluating hepcidin concentration as a test for iron deficiency in a large group of blood donors in a high risk anaemia area found that hepcidin shows a considerable promise as a diagnostic test for iron deficiency and appears to perform at least as well as available iron indices such as sTfR and reticulocyte haemoglobin. The team indicated that for a diagnosis of iron deficiency defined by a sTfR/log ferritin index, hepcidin less than 18 ng/ml has a sensitivity of 79.2% and a specificity of 85.6%. *“On-going analysis within our group is yielding an even better performance among rural Gambian children, suggesting that hepcidin will be a superior index in scenarios where infections are common”* [119]

A few studies have noted that over the course of a malaria season, hepcidin integrates signals arising from parasitaemia, inflammation and anaemia [96, 120]. The fact that hepcidin plays a crucial role in the above signals and acts both as a reporter of iron status and an effector of iron absorption, distribution and metabolism suggests it may be the ideal index for iron deficiency and form the basis of a point-of-care-diagnostic for iron deficiency for at-risk population groups in developing countries [120].

The human iron cycle

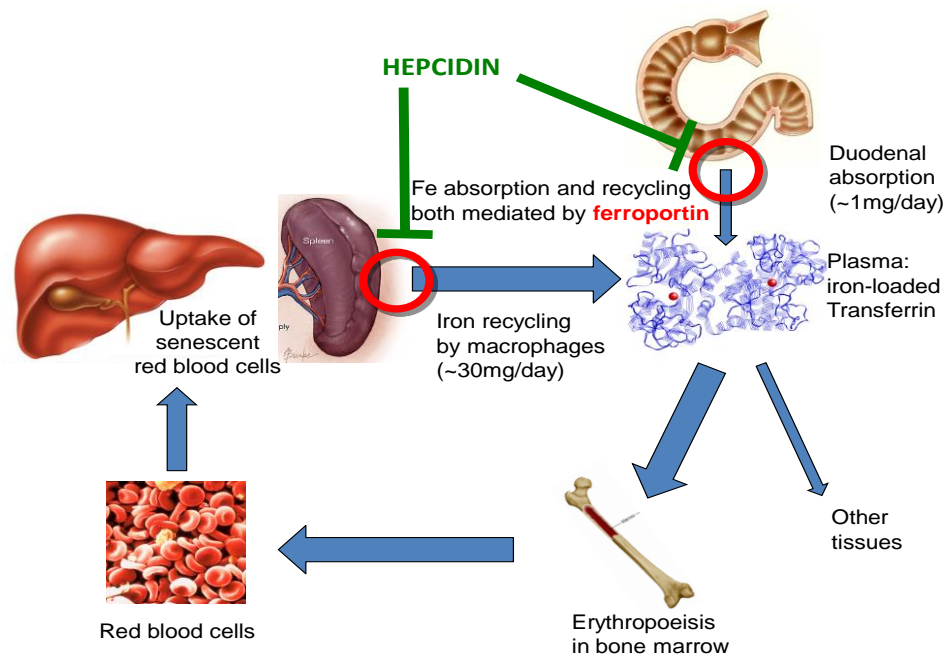


Figure 4: Hepcidin regulates intestinal iron absorption, iron recycling by macrophages, and iron release from hepatic stores.

Source: Adapted from Drakesmith Prentice [121]

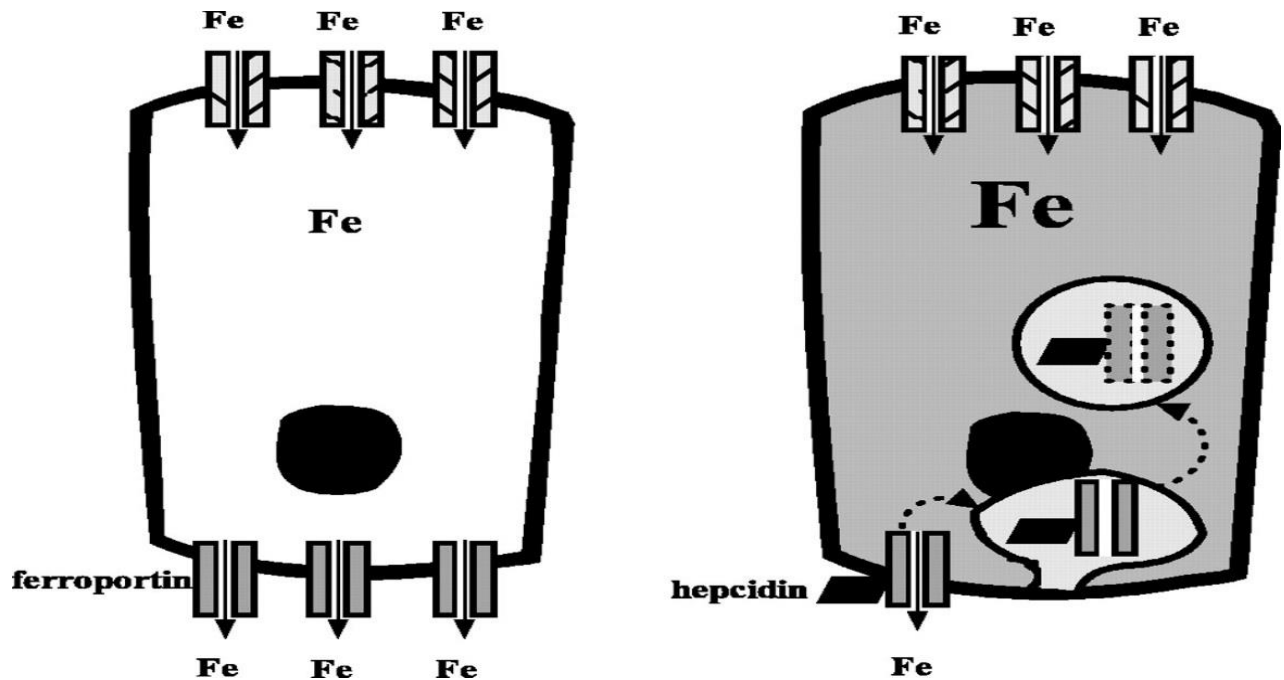


Figure 5: Hepcidin regulates ferroportin expression on the basolateral membrane of enterocytes. *Left:* iron deficiency, with hepcidin secretion suppressed and ferroportin strongly expressed on the basolateral membrane, iron absorption is maximal. *Right:* iron excess. The liver secretes hepcidin, which interacts with ferroportin molecules on the basolateral membrane, causing ferroportin to be endocytosed and degraded. Iron export from enterocytes is decreased, and the cells fill with iron. Eventually, iron-filled enterocytes will be shed into the lumen of the intestine. Adapted from: Ganz Nemeth [105].

2.6 Hepcidin and Pregnancy

A review of the literature on hepcidin in pregnancy revealed very little published data. However, Rehu et al [122] concluded that hepcidin concentration has been observed to be low at term during pregnancy allowing for the increased availability of iron to the fetus and that maternal and cord blood hepcidin are independently associated with maternal and cord blood iron status, respectively. Maternal serum hepcidin and maternal/neonatal iron status are thought to play a role in placental uptake of both haem and non-haem iron with a greater percentage of haem iron present in the neonates [123].

On the other hand, urinary hepcidin is not found to be significantly related to haemoglobin, erythropoietin or C-reactive protein [113, 124], although, Schulze et al [113] found urinary hepcidin among pregnant women in rural Bangladesh to be related to iron status and alpha-1 acid glycoprotein (AGP).

An apparent ineffectiveness of hepcidin has been highlighted in two studies; in one of the studies, neither placental infection nor maternal anaemia were related to maternal or cord blood hepcidin concentrations and iron status [48]. In the other study, plasma iron concentrations were found to be increased despite high hepcidin concentration in preeclampsia and this might indicate a resistance to iron decreasing action of hepcidin [125].

2.7 Need for a better point-of-care (PoC) diagnostic

Iron status can be considered as a continuum from iron deficiency; iron deficiency with no anaemia; iron deficiency with anaemia; to normal status with varying amounts of stored iron and finally to iron overload which can cause organ damage [87] or in the case of oral supplementation may lead to serious adverse consequences in infectious environments [126]. Since iron deficiency anaemia is a common cause of maternal anaemia, iron supplementation is a common practice to reduce the incidence of maternal anaemia [127, 128].

Placebo controlled studies of iron supplementation during gestation showed that women taking placebo have lower iron status (lower serum ferritin and haemoglobin) compared to women taking iron supplements and that differences in iron status persist for many months after delivery [129]. The studies also showed that a significant fraction of the women on placebo, developed iron deficiency and iron deficiency anaemia [129, 130]. In The Gambia, a double blind placebo controlled community based oral iron supplementation trial (200 mg

ferrous sulphate) showed that iron supplementation reduced the prevalence of anaemia and iron deficiency [131].

Hence the World Health Organisation recommends universal iron supplementation with all pregnant women to be given 60 mg iron and 400 ug folic acid daily [49]. which was later updated in 2012 [132] recommending that pregnant women should be supplemented with 30 - 60 mg elemental iron throughout pregnancy, starting as early in pregnancy as possible, with a preferred daily dose of 60 mg of elemental iron in settings where anaemia in pregnant women is a severe public health problem (prevalence of 40% or higher).

However, a recent review has shown that pregnant women who received daily iron and folic acid supplementation are at a greater risk of haemoconcentration (haemoglobin greater than 130 g/L) in the second and third trimester of pregnancy than those who received no treatment or placebo [130]. Although the effect of the haemoconcentration in the above review was uncertain, Ziaei et al [133] in a randomised placebo-controlled trial of over 700 participants (pregnant women) who took 50 mg elemental iron as ferrous sulphate daily throughout pregnancy found that small-for-gestational-age birth rate and the number of women with hypertension disorders increased significantly. They concluded that routine iron supplementation in non-anaemic women is not rational and may be harmful. Recently, two hazardous complications of pregnancy; gestational diabetes mellitus and preeclampsia have been recognized to be associated with elevated body iron levels [134]. Iron supplementation has also been found to be associated with glucose impairment and hypertension [135].

Recent studies and reviews have shown that pregnant women who are anaemic and iron deficient may be protected from malaria [136, 137]. With the use of flow cytometry our group demonstrated that *P. falciparum* erythrocytic stage growth *in vitro* is reduced in anaemic pregnant Gambian women at baseline, but increases during supplementation (**Figure 6**) [138]. It has been noted that although there has been significant reduction of malaria in

malaria endemic areas, anaemia still causes a high burden among pregnant women [139, 140].

Nonetheless, iron supplementation programmes for pregnant women are currently being implemented in 90 of the 112 countries that reported to WHO in 1992. However, most of these programmes are neither systematically implemented nor well monitored or evaluated [49].

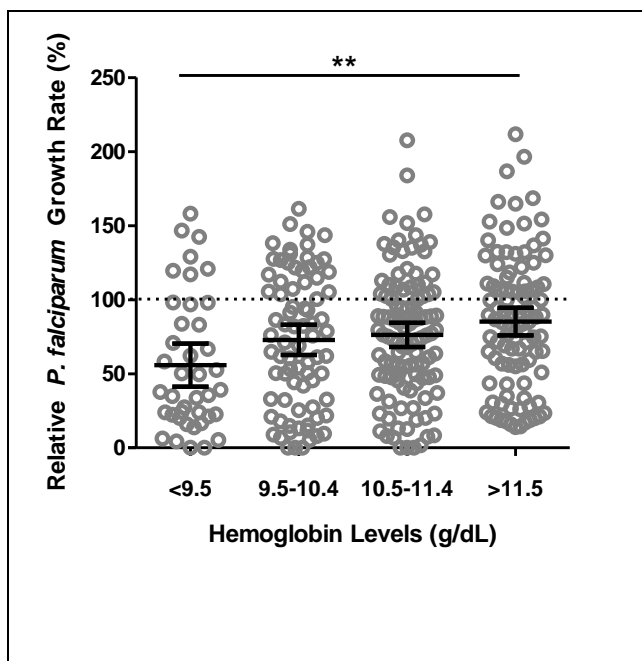


Figure 6: Malaria susceptibility increases transiently during iron supplementation and pregnant women receiving iron supplements have increased numbers of young RBCs (Goheen et al 2017) [138].

It is believed by many that one of the reasons national iron supplementation programmes have failed is because of women's non-compliance with taking iron supplements daily because of gastrointestinal upsets [141, 142] and other side effects [130, 141-143] that sometimes occur when taking iron. Despite the importance of side effects, recent studies

have shown that side effects have a limited influence on compliance [129, 144]. Other issues found to be more important than side effects affecting compliance include: inadequate supplies, inadequate counseling [142], poor access to prenatal services, beliefs against consuming the tablets, fears that consuming too much may cause too much blood or a big baby and make delivery difficult [3].

Much has been made of iron dosage and how much iron is adequate during pregnancy as a supplement. Milman [129] in a review of iron prophylaxis in pregnancy noted that *“there used to be a tradition of recommending high doses of iron supplements of 100 - 200 mg of ferrous iron daily in pregnancy where a daily dose of 100 mg will induce a maximum rise in haemoglobin concentration and a dose of 200 mg of ferrous iron increases serum ferritin and haemoglobin at term to the same or even higher level as in non-pregnant women. It is also noted that 66 mg ferrous iron daily from 18 weeks gestation can prevent iron deficiency anaemia in all pregnant women”*.

However, other studies found that 40 mg ferrous iron daily was adequate to prevent iron deficiency (France); a daily dose of 27 mg had a favourable influence in pregnant Norwegian women; in Australia a daily dose of 20 mg ferrous iron from 20 weeks gestation reduced the frequency of iron deficiency and iron deficiency anaemia at delivery; and in Denmark, a daily dose of 40 mg ferrous iron appeared to be adequate for preventing iron deficiency anaemia for 95% of the women [129]. In settings where prevalence of anaemia is lower than 20%, WHO has recommended the intermittent use of iron and folic acid supplements (one supplement of 120 mg iron and 2800 ug folic acid once a week) for non-anaemic pregnant women to prevent anaemia and improve gestational outcomes [145]. However, Moretti et al (2015) [146] showed that Iron supplementation at doses of 60 mg Fe as FeSO₄ or higher increase hepcidin for up to 24 hours and are associated with lower iron absorption on the following day. Providing lower dosages (40-80 mgFe) and avoiding twice-daily dosing

maximise fractional absorption. The duration of the hepcidin response supports alternate day supplementation.

There is now some evidence that smaller doses of 30 mg iron daily could achieve similar results as the daily 60 mg iron [49]. In a Cochrane review of the treatments of iron deficiency anaemia in pregnancy, Reveiz et al [147] as part of their conclusion indicate that daily low dose iron supplementation might be effective at treating anaemia in pregnancy with less gastrointestinal side effects compared with higher doses. WHO therefore recommended in 2012 that for countries exceeding 40% anaemia prevalence, daily supplementation with 30 - 60 mg elemental iron (as ferrous salt) be given throughout pregnancy, starting as early in pregnancy as possible, with a preferred daily dose of 60 mg of elemental iron in settings where anaemia in pregnant women is a severe public health problem [132].

2.8 Use of multiple micronutrient supplements

UNIMMAP formulation has been used in other pregnancy trials in developing countries with good patient compliance, acceptability and favourable outcomes. Micronutrient supplements with three or more micronutrients is associated with a 39% reduction in maternal anaemia compared with placebo or with two micronutrients or fewer (relative risk 0.61, 95% CI 0.52-0.71). Multiple micronutrient supplementation is also known to result in a decrease in the risk of low-birth weight babies (0.83, 0.76-0.91) and small-for-gestational-age babies (0.92, 0.86-0.99) [148]. In a 2017 Cochrane review, Haider and Bhutta [149] concluded that, in comparison with iron, with and without folic acid, daily multiple micronutrient supplementation during pregnancy reduced the risk for low birthweight and small-for-gestational-age births in LMICs.

Smith et al [150] identified several subgroups of mothers that might experience greater benefits from antenatal multiple micronutrient supplementation than from iron-folic acid supplementation alone. In anaemic women, multiple micronutrient supplementation resulted in greater reductions in the risk of low birthweight by 19%, small-for-gestational-age births by 8%, and infant mortality at 6 months of age by 29% than in non-anaemic women. In underweight women (BMI <18.5 kg/m²), multiple micronutrient supplementation reduced the risk of preterm birth by 16%. Furthermore, initiation of multiple micronutrient supplementation before 20 weeks' gestation decreased the risk of preterm birth by 11% and high adherence to regimen (≥95%) decreased the risk of infant mortality by 15%.

In summary, although iron supplementation has been recommended in the management of iron deficiency and iron deficiency anaemia, iron supplementation in developed countries still remains controversial and hence supplementation should include screening with ferritin in early pregnancy in order to identify women who can manage without prophylactic iron [151]. In developing countries, Brabin et al [152] revealed there is in vitro evidence that iron availability influences severity and chronicity of infections that cause such outcomes as stillbirth, preterm birth and congenital infection and that although reducing iron deficiency anaemia among women is beneficial and should improve the iron stores of babies, caution with maternal iron supplementation is desired in iron-replete women who have high infection exposure to avoid iron intervention strategies that result in detrimental birth outcomes for some groups of women.

However, iron supplementation combined with other measures depending on the aetiology of the anaemia remains a viable option in combating anaemia and hence the assessment of iron status at the point of care will help in determining who should receive iron and when it is safe to receive on the day and point where the pregnant woman is receiving antenatal care. Therefore, in an effort to contribute towards the reduction of maternal morbidity and mortality due to anaemia and iron deficiency, we conceived the idea of testing the hypothesis that a

screen-and-treat approach to iron supplementation below the pre-determined hepcidin cut-off value ($<2.5 \mu\text{g/L}$), is non-inferior to the reference arm (WHO-recommended universal iron supplementation of 60 mg iron) in preventing anaemia and iron deficiency at a lower dose and hence improve safety and tolerability after 12 weeks intervention with haemoglobin concentration as the primary endpoint. **Figure 7** shows the study location in rural Gambia.

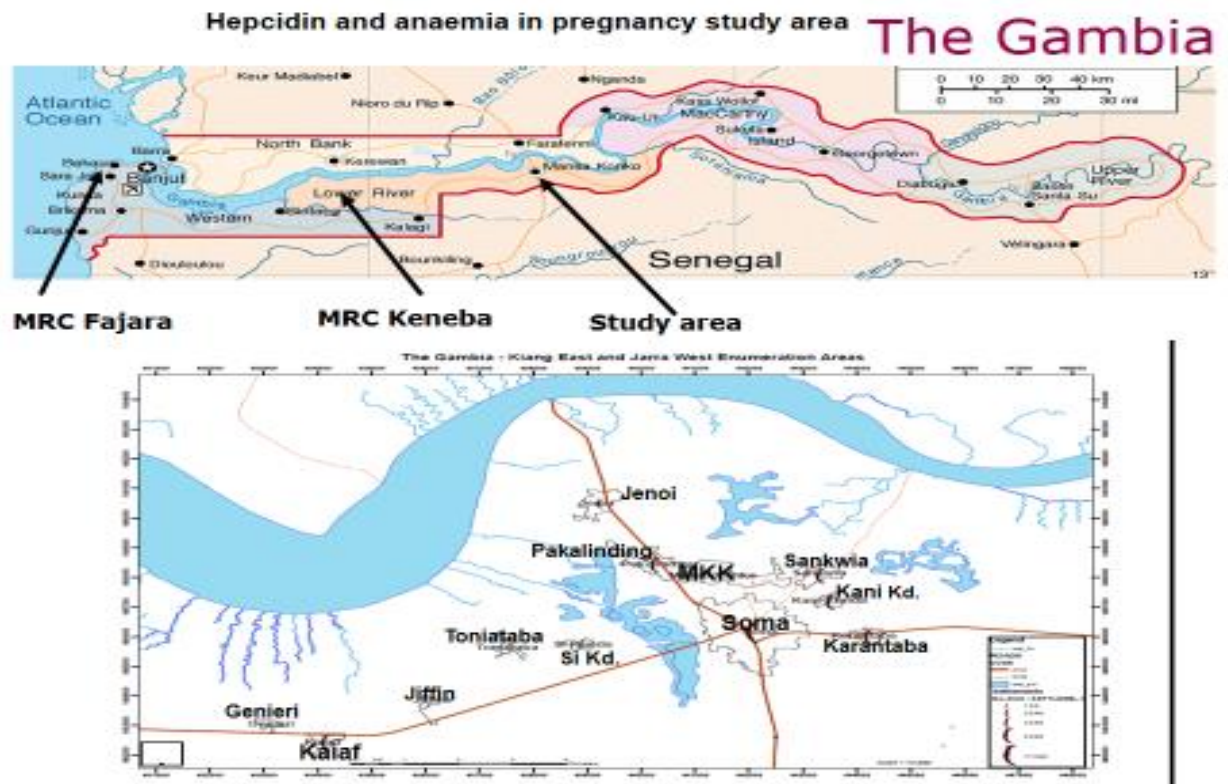


Figure 7: Study location

Chapter 3: Protocol paper for HAPn



London School of Hygiene & Tropical Medicine
Keppel Street, London WC1E 7HT

T: +44 (0)20 7299 4646

F: +44 (0)20 7299 4656

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RESEARCH PAPER COVER SHEET

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SECTION A – Student Details

Student ID Number	025190	Title	Mr
First Name(s)	AMAT		
Surname/Family Name	BAH		
Thesis Title	A DOUBLE BLIND RANDOMISED CONTROLLED TRIAL COMPARING STANDARD DOSE OF IRON SUPPLEMENTATION FOR PREGNANT WOMEN WITH TWO SCREEN-AND-TREAT APPROACHES USING HEPcidIN AS A BIOMARKER FOR READY AND SAFE TO RECEIVE IRON		
Primary Supervisor	PROF ANDREW M. PRENTICE		

If the Research Paper has previously been published please complete Section B, if not please move to Section C.

SECTION B – Paper already published

Where was the work published?	BMC Pregnancy and Childbirth		
When was the work published?	(2016) DOI:10.1186/s12884-016-0934-89		
If the work was published prior to registration for your research degree, give a brief rationale for its inclusion	Work was published after registration as part of the research degree		
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SECTION C – Prepared for publication, but not yet published


Where is the work intended to be published?	N/A
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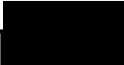
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For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper. (Attach a further sheet if necessary)	I am the main and corresponding author who contributed to the design of the protocol and drafted the manuscript.
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SECTION E

Student Signature	
Date	26/03/2019

Supervisor Signature	
Date	26/03/2019

STUDY PROTOCOL

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A double blind randomised controlled trial comparing standard dose of iron supplementation for pregnant women with two screen-and-treat approaches using hepcidin as a biomarker for ready and safe to receive iron

Amat Bah^{1*}, Rita Wegmuller¹, Carla Cerami¹, Lindsay Kendall², Sant-Rayn Pasricha³, Sophie E. Moore⁴ and Andrew M. Prentice¹

Abstract

Background: Until recently, WHO recommended daily iron supplementation for all pregnant women (60 mg/d iron combined with 400ug/d folic acid) where anaemia rates exceeded 40 %. Recent studies indicate that this may pose a risk to pregnant women. Therefore, there is a need to explore screen-and-treat options to minimise iron exposure during pregnancy using an overall lower dosage of iron that would achieve equivalent results as being currently recommended by the WHO. However, there is a lack of agreement on how to best assess iron deficiency when infections are prevalent. Here, we test the use of hepcidin a peptide hormone and key regulator of iron metabolism, as a potential index for 'safe and ready to receive' iron.

Design/Methods: This is a 3-arm randomised-controlled proof-of-concept trial. We will test the hypothesis that a screen-and-treat approach to iron supplementation using a pre-determined hepcidin cut-off value of <2.5 ng/ml will achieve similar efficacy in preventing iron deficiency and anaemia at a lower iron dose and hence will improve safety. A sample of 462 pregnant women in rural Gambia will be randomly assigned to receive: a) UNU/UNICEF/WHO international multiple micronutrient preparation (UNIMMAP) containing 60 mg/d iron (reference arm); b) UNIMMAP containing 60 mg/d iron but based on a weekly hepcidin screening indicating if iron can be given for the next 7 days or not; c) or UNIMMAP containing 30 mg/d iron as in (b) for 12 weeks in rural Gambia. The study will test if the screen-and-treat approach is non-inferior to the reference arm using the primary endpoint of haemoglobin levels at a non-inferiority margin of 0.5 g/dl. Secondary outcomes of adverse effects, compliance and the impact of iron supplementation on susceptibility to infections will also be assessed.

(Continued on next page)

* Correspondence: Amat.Bah@lshtm.ac.uk

¹MRC Unit The Gambia & MRC International Nutrition Group, PO Box 273, Banjul, The Gambia

Full list of author information is available at the end of the article



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Discussion: This trial is expected to contribute towards minimising the exposure of pregnant women to iron that may not be needed and therefore potentially harmful. If the evidence in this study shows that the overall lower dosage of iron is non-inferior to 60 mg/day iron, this may help decrease side-effects, improve compliance and increase safety. The potential for the use of hepcidin for a simple point-of-care (PoC) diagnostic for when it is most safe and effective to give iron may improve maternal health outcomes.

Trial registration: ISRCTN21955180

Keywords: Pregnancy, Hepcidin, Anaemia, UNIMMAP, Lower dose iron, Iron deficiency

Background

Anaemia is a global public health problem affecting all population groups, but especially pregnant women and young children [1]. For pregnant women, the consequences of anaemia include mortality, poor pregnancy and birth outcomes including premature delivery, low birth weight and increased perinatal mortality [1–3]. The most significant contributor to the onset of anaemia is iron deficiency [1]. The World Health Organisation (WHO) estimates that iron deficiency anaemia (IDA) affects almost half of the world's pregnant women and pre-school children with a prevalence of over 65 % in Africa and Asia, and that it causes (directly or indirectly) one fourth of all maternal deaths [3]. In The Gambia, iron deficiency anaemia among women and children has been found to be high and of public health significance with 73 % of pregnant women and 56 % of lactating women being anaemic [4].

Although iron deficiency with or without anaemia has important consequences for human health and child development, there has been an absence of international agreement on how best to assess the iron status of populations. Serum ferritin (SF) is one of the few biochemical indices of which low levels reflect depleted iron stores [5, 6] but it is known to be raised by infection and inflammation as it is an acute phase protein and thus has very high false negative rates in least developed countries [7]. Similar problems also arise with the other

commonly used iron status indicators as summarised in Table 1 below.

Hepcidin is a peptide hormone that has been shown recently to be the master regulator of iron absorption and distribution in humans [8–11]. The potential for hepcidin as a superior marker for iron deficiency has been highlighted in many recent studies [12–16]. Hepcidin controls iron homeostasis by inhibiting dietary iron absorption, release of iron in the macrophages and reducing iron flow to the erythron [8, 11, 17–19]. This it does by binding to the iron exporter ferroportin inducing its internalisation and degradation [18].

Recent studies have noted that over the course of a malaria season, hepcidin integrates signals arising from parasitaemia, inflammation and anaemia [7, 20]. The fact that hepcidin plays a crucial role in the above signals and acts both as a reporter of iron status and a regulator of iron absorption, distribution and metabolism suggests it may be the ideal index for iron deficiency and could form the basis of a PoC diagnostic for iron deficiency in at-risk population groups in developing countries [20].

WHO, originally recommended universal iron supplementation for all pregnant women with a dose of 60 mg iron and 400ug folic acid daily [21]. However, a recent study has shown that pregnant women who are anaemic and iron deficient may be protected from malaria [22]. A recent review also indicate that pregnant women who received daily iron and folic acid supplementation are at

Table 1 Limitation of current methods of assessing IDA

Test	Measure	Limitation
Stain bone marrow preparation	Iron stores	Expensive, invasive and traumatic
Haemoglobin (Hb)	Anaemia	Does not measure ID per-se
Serum ferritin (SF)	Iron stores	Raised by infection and inflammation
Zinc protoporpherin (ZnPP)	Iron in new cells	Affected by infection and inflammation
Soluble transferrin receptor (sTfR)	Severe ID even with inflammation	Affected by (>) red cell prod. Lack standardised reference for measure'
sTfR/logSF ratio	Iron stores	Lack standardised assay range. Ferritin affected by infection or inflammation
Transferrin saturation (TSAT)	Iron levels	Affected by (>) plasma concentration
Serum iron	Iron in sera	Affected by recent iron ingestion and infection
Total iron binding capacity (TIBC)	Iron in serum	Affected by infection

a greater risk of haemoconcentration (haemoglobin greater than 130 g/L) in the second and third trimester of pregnancy [23]. Although the effect of the haemoconcentration in the above review was uncertain, Ziaei et al. [24] in a randomised controlled trial (RCT) of over 700 pregnant women who took 50 mg iron as ferrous sulphate daily found that small-for-gestational-age birth rate and the number of women with hypertensive disorders increased significantly. They concluded that routine iron supplementation in non-anaemic women is not rational and may be harmful. Recently, two hazardous complications of pregnancy; gestational diabetes mellitus and preeclampsia have been recognized to be associated with elevated body iron levels [25].

There has been little specific evidence on the relationship between risk of malaria and other infections with iron status and iron supplementation in pregnant women. Yet, the benefits of iron supplementation must be carefully weighed against the risks in developing countries [26, 27].

There is now some evidence that smaller doses of 30 mg iron daily could achieve similar results as the daily 60 mg iron [21]. A Cochrane review on the treatments of iron deficiency anaemia in pregnancy [28] indicated that daily low dose iron supplementation may be effective at treating anaemia in pregnancy with fewer gastrointestinal side effects compared with higher doses. WHO has now recommended the use of doses between 30 and 60 mg for daily supplementation for pregnant women [29]. Further evidence suggest that the use of multiple micronutrient supplements with three or more micronutrients is associated with a 39 % reduction in maternal anaemia compared with placebo or with two micronutrients or fewer (relative risk 0.61, 95 % CI 0.52–0.71). Multiple micronutrient supplementation is also known to result in a decrease in the risk of low-birth weight babies (0.83, 0.76–0.91) and small-for-gestational-age babies (0.92, 0.86–0.99) [30].

In this proof of concept study we aim to test the hypothesis that a screen-and-treat approach to iron supplementation will achieve similar efficacy in combating ID and IDA at a lower overall dosage of iron. The assumption that lower doses will improve safety and tolerability will also be tested. The design will establish whether using screen-and-treat with UNIMMAP containing either 60 mg or 30 mg iron per day is non-inferior to UNIMMAP containing 60 mg/day as a universal daily supplement.

Design/Methods

Study design

This study is designed as a proof-of-concept, 3-arm, double blind, RCT over a period of 12 weeks with a sample of 462 pregnant women randomly assigned to receive: a) UNIMMAP containing 60 mg/day iron; b)

UNIMMAP containing 60 mg/day iron but based on a weekly hepcidin screening indicating if iron can be given for the next 7 days or not; c) UNIMMAP containing 30 mg/day iron based on screening as in (b).

Determining the hepcidin cut-off value

The hepcidin cut-off of <2.5 ng/mL as a threshold (to receive iron or not) is based on the analysis of sera from 270 pregnant women participating in the ENID study [31] with samples available for 3 time points (12–14 weeks, 20 weeks and 30 weeks gestation). A receiver operating characteristics (ROC)-curve was generated to measure the area under the curve (AUC^{ROC}). Method described elsewhere [12].

Study location and participants

The Heparidin and Anaemia in Pregnancy (HAPn) study will be carried out in 12 communities of Jarra West and Kiang East (rural Gambia) about 150 km from the capital city of Banjul. The Regional Health Team, the health facilities within the study area and the individual communities have been sensitised and their approval gained for conducting the study.

The study will involve 462 healthy pregnant women between the ages of 18 and 45 years (established by asking, use of birth certificates, identity cards or calendar events) who are pregnant (estimated at between 14 and 22 weeks gestation, by fundal height assessment and date of last menstrual period (LMP)) and are likely to be resident in the study area for the duration of the study period.

Pregnant women who are identified as potential participants will be excluded from the study if found to be (i) severely anaemic (<7 g/dL), (ii) seriously ill (infectious disease of clinical significance) at recruitment (iii) suffer from a chronic disease (iv) have pregnancy complications (e.g., pre-eclampsia) at enrolment or (v) already participating in another study.

Detailed study procedure

Screening and enrollment (Baseline)

Pregnant women living within the two health facility catchment areas will be identified as they visit the Reproductive and Child Health (RCH) clinics to register and book their pregnancies. As part of the routine services provided, a nurse midwife determines their stage of pregnancy. If a woman is within the window of the study (14–22 weeks gestation), she will be invited by the study team to take part in the study and informed consent will be sought. Once a signed informed consent is obtained and all of the inclusion and none of the exclusion criteria are met, she will be enrolled in the study, and asked to provide 5 mL venous blood (Day 0 below). Participants will thereafter be assigned to one of

the 3 study groups (see randomisation, below). All women enrolled in to the study will be provided with long lasting insecticide-treated bed nets (LLINs).

Follow-up

Following recruitment, women will be followed up weekly in their communities. Each week, trained MRC field assistants (FA) will invite the study participants to a central location within their communities for collection of a finger prick blood sample for analysis of haemoglobin (Hb) using a HemoCue Hb 301 analyser (HemoCueAB, Angelholm, Sweden), malaria parasitaemia using a SD Bioline One step malaria antigen *Pf* Test (SD Standard Diagnostics, Inc. Kyonggi-do, Korea) and hepcidin levels using the BACHEM Hepcidin-25 ELISA. Hb and malaria assessments will be performed immediately; samples for hepcidin measurements will be transferred on ice to a laboratory at MRC Keneba where analysis will commence within the hour of arrival. The following day hepcidin results will be available and a 7 day supply of supplements packed according to the hepcidin results (computer generated). The day after, participants will be provided with their supplements. While the supplements are being distributed, the FA will also assess beneficial effects, adverse events and compliance. All activities will be documented on a case report form (CRF) using electronic data capture in the form of a hand held device (SAMSUNG Galaxy Tab3 Model SM-T211). Data will be sent through a secure internet connection to the MRC database.

Ethics and safety monitoring

The trial has been approved by the Medical Research Council (MRC) Scientific Coordinating Committee (SCC) and the Joint Gambia Government MRC Ethics Committee. It will be overseen by a Data Safety Monitoring Board (DSMB) and a Trial Steering Committee assisted by a Trial Monitor (TM). Together they will be responsible for reviewing all interim data, treatment safety and efficacy including the protection of the rights and wellbeing of the participants. The trial will be conducted according to Good Clinical Practice (GCP) principles taking in to consideration the provisions of the World Medical Association (WMA) Declaration of Helsinki (October 2013).

Participants will be monitored on each scheduled follow up day for all adverse events (AEs) defined as any untoward or unfavourable medical occurrence in a human subject, including signs and symptoms which are temporally associated with the research procedure or trial intervention, whether or not considered related to the subject's participation in the research. All serious adverse events (SAEs) defined as any AE that is life-threatening or results in death or require hospitalisation or prolongation of hospitalisation, is a persistent or significant disability/

incapacity or is a congenital anomaly/birth defect or a reported maternal death, miscarriage or stillbirth will be recorded as SAEs and investigated by a physician. Monitoring of the participants will then continue until they deliver and the outcome of the pregnancy for both mother and child is known (postnatal check-up within 72 h after delivery).

Collection and analyses of biological samples during enrollment and follow-up visits

As described, finger prick blood samples will be collected weekly. Additional 5 mL venous blood samples will also be collected at 4 different time-points (Days 0, 14, 49 and 84) within the 12 week period of the study. As intermittent preventative treatment (IPT) is routine in this region, participants will receive their IPT dose immediately after blood draws are done at days 14 and 49 in order not to influence our *ex vivo* *P. falciparum* assays. All venous blood draws will be carried out by the study nurse and finger prick blood samples by the field assistants (FAs).

Full haematology including haemoglobin and reticulocytes will be assessed on samples collected on Days 0, 14, 49 and 84 in a 1.2 mL EDTA Sarstedt tube using the Medonic M Series analyser.

Biochemistry analysis of plasma ferritin, iron, transferrin saturation (TSAT), soluble transferrin receptor (sTfR), C-reactive protein (CRP), and alpha-1-acid glycoprotein (AGP) will be measured by Cobas Integra 400+ using 500 µl from -20 frozen samples. The Cobas measuring principle for ferritin, transferrin and CRP will be via turbidimetric principle at 552 nm, 340 nm and 552 nm respectively and for iron, FerroZine method without deproteinization. sTfR will be measured photometrically at 583 nm and alpha-1-acid glycoprotein will be through turbidimetric. Hb genotyping will be performed using Hb electrophoresis with Shandon Vokam 400 on all samples collected on Day 0.

This study is not powered to use clinical endpoints to assess safety. Instead the trial will use *in vitro* assays to assess safety on each of the four venous samples per subject. *Ex vivo* growth of *P. falciparum* will be assessed in washed red blood cells (RBCs) using a field-ready 96-well plate method with fluorescence-activated cell sorting (FACS) readout [32]. A small subset of RBCs will be lysed for measurement of riboflavin status by the erythrocyte glutathione reductase activation coefficient (EGRAC) test because this may affect RBC stability.

The *ex vivo* growth of sentinel organisms (*Escherichia coli*, *Yersinia enterocolitica*, *S. enterica* serovar (Typhimurium), *Staphylococcus epidermidis*, *Staphylococcus aureus* and *Candida albicans*) analyses will be performed in frozen (-20 °C) plasma (400 µl) as previously described in the investigation of the effects of

iron supplementation on pathogen virulence in human serum [33].

DNA will be extracted from baseline whole blood samples to study the genes implicated in iron metabolism. Known genetic risk factors for malaria assays will include alpha-thalassemia, G6PD and sickle traits. Furthermore, putative functional and key tagging variants in iron regulatory and inflammatory pathways will be screened.

Randomisation and blinding

Randomisation

Recruited women will be randomly assigned (computer generated) to one of the 3 treatment arms (equal number in each arm) balanced by the Hb concentration of the baseline blood sample and gestational age. To achieve this, at each day of recruitment, subjects will be categorised into two Hb classes (above and below the median Hb of the respective day) and according to 2 gestational age periods (14–18 weeks, 19–22 weeks) making 4 classes. In each of the 4 classes, the women will be randomly assigned to the 3 treatment arms using a predetermined block randomisation.

The randomisation database of treatment arms (A, B, C) will be password protected with the database developer and his assistant knowing the password. If a subject needs to be unblinded at the request of the DSMB, their treatment can be easily identified without unblinding the whole study.

Blinding

Participants, field workers, study nurse and PI will be blinded as to which treatment group participants belong to and which supplement participants receive each week. The supplements will be pre-packed on a weekly basis by the field coordinator in Keneba using lists automatically (computer) generated by the data office taking into account the hepcidin results of the participants. The list will indicate the letter of the supplement the participant receives for the following 7 days but the field coordinator will not know which code is allocated to which supplement. The capsules are coded (2 codes for treatment arm A (60 mg and 60 mg iron), 2 codes for arm B (60 mg and 0 mg iron) and 2 codes for arm C (30 mg and 0 mg iron), see Fig. 1. The pre-packed weekly supplies labelled with each participant's ID will be handed over to the PI who will be responsible for handing them

over to the field workers who will distribute to the individual participants. The laboratory staff and data entry clerks will also be blinded.

The allocation of the colour code will be done by 2 people independent of the study and the key will be kept in a locked cabinet in Keneba. The blinding for the study may be broken if in any of the 3 treatment arms, safety issues arise and the trial team is advised by the DSMB to do so.

Investigational product

The investigational product to be used is the UNICEF/WHO/UNU international multiple micronutrient preparation (UNIMMAP). Three products will be administered (UNIMMAP with 60 mg iron, UNIMMAP with 30 mg iron, UNIMMAP with 0 mg iron). All formulations also contain 400 ug folic acid and 14 other micronutrients (Table 2). The UNIMMAP supplement has already been used safely in other pregnancy trials [34]. The formulations are produced by DSM South Africa under Good Manufacturing Practice (GMP) conditions where it will also be dosed into gelatin capsules and packed in tubs. The labelling will include a statement that 'trial medications are only for use of trial participant'.

The products will be stored under controlled conditions (in an air-conditioned storage at around 20 °C) at MRC Keneba. The product is stable for 18 months if kept under these conditions.

Each participant will receive 1 capsule per day. Each week field workers will be visiting study participants to distribute the respective weekly supply (7 capsules) to each study participant. The participants will be instructed to take 1 capsule a day with water or another drink. Each time the field workers distribute the new weekly supply of capsules they will account for the number of capsules consumed/not consumed from the previous week in order to check for compliance. Any left-over capsules will be collected by the field workers.

Sample size and statistical analysis plan

Sample size determination

Using the haemoglobin data obtained from pregnant women enrolled in the ENID study in West Kiang [31], a SD of 1.28 was derived. This was used to obtain a sample size of 154 participants for each of the 3 arms

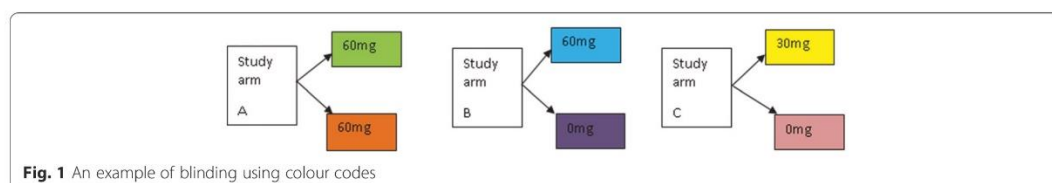


Fig. 1 An example of blinding using colour codes

Table 2 Intervention product - Formulation based on UNU/ UNICEF/WHO supplement called UNIMMAP

Micronutrients	Dose/day
Vitamin A (ug RE)	800
Vitamin D (IU)	200
Vitamin E (mg)	10
Thiamine (mg)	1.4
Riboflavin (mg)	1.4
Niacin (mg)	18
Folic acid (ug)	400
Vitamin B6 (mg)	1.9
Vitamin B12 (ug)	2.6
Vitamin C (mg)	70
Zinc (mg)	15
Iron (mg)	60 or 30 or 0 (placebo)
Iodine (ug)	150
Selenium (ug)	65
Copper (mg)	2

(Table 3) calculated using a 1-sided α (alpha) of 2.5 with a conservative-Bonferroni type correction ($\div 3$) so as not to inflate the type 1 error rates while performing multiple tests. A total sample size of 462 pregnant women followed up for 12 weeks with a less than 10 % loss to follow-up will provide 80 % power to establish that:

- 1) arm B is non-inferior to arm A on the primary endpoint defined below.
- 2) arm C is non-inferior to arm A at the same level as described in the statistical analysis plan.
- 3) arm C is non-inferior to arm B.

Note: it is being stated *a priori* that arm C will be compared with B for non-inferiority to explore if the results can influence policy on the further lowering of the dose of iron for those not iron deficient. The study (sample size) is powered for this analysis.

Statistical analysis plan

The approach to the analysis for this trial will be a test of non-inferiority. As recommended for the acceptance of

Table 3 Study arms

Group	Dose (mg/day Fe)	Universal (N)	Screen with Hepcidin (Yes or No)
A	60	154	No
B ^a	60	154	Yes
C ^a	30	154	Yes

^aGroups B and C will be tested weekly and only given their next seven day supply of iron if plasma hepcidin falls below cut-off for 'safe and ready'

non-inferiority analysis, a per-protocol (PP) analysis will be performed. Additional analysis including all Hb measurements (not only day 84) will be explored. These will be described in a more detail statistical analysis plan.

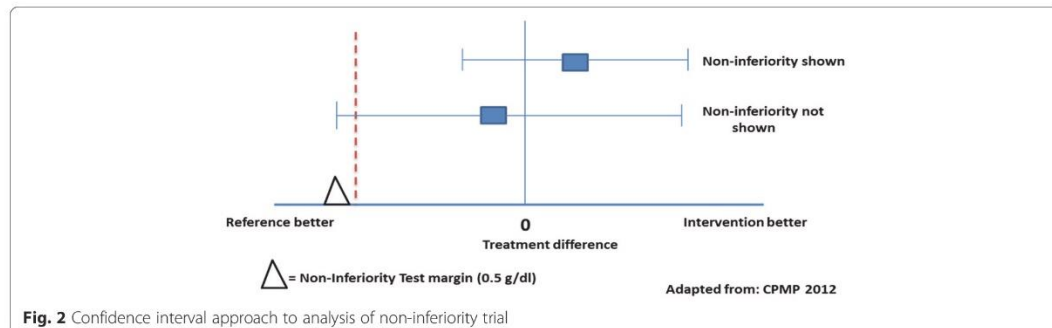
Primary endpoint

The primary non-inferiority endpoint is pregnancy-adjusted haemoglobin at Day 84. To adjust for multiple testing (3 arms), non-inferiority will be tested with a 96.7 % CI of the lower 0.83 % (2.5 %/3) limit for the difference. The lower confidence limit for the difference in haemoglobin concentration between the universal and screened treatments on Day 84 will be above -0.5 g/dL (-5 g/L), the smallest value considered to be of minimum public health relevance. See illustration in Fig. 2.

Secondary endpoints

- i. Proportion of Hb < 11 g/dL (%) at Day 84.
- ii. Hepcidin at Day 84 (as a continuous variable but also using a cut-off point of >2.5 ng/mL to calculate the proportion).
- iii. sTfR/log-ferritin ratio (ferritin index <2.0) at Day 84 (continuous variable but a proportion will also be calculated).
- iv. Iron deficiency anaemia (IDA) defined as Hb < 11 g/dL and ferritin < 15 ug/L when CRP is < 5 mg/L OR Hb < 11 g/dL and ferritin < 30 ug/L when CRP is > 5 mg/L and ferritin index < 2.0.
- v. Iron dosage (number of weeks supplemented).
- vi. Adverse events that may include nausea/vomiting, dizziness, constipation, black stool, stomach discomfort assessed weekly will be evaluated at Day 28 and Day 84. In addition, an aggregate score will also be assessed.
- vii. Compliance.
- viii. *P. falciparum* growth in serum (difference between in vitro growth rates at Days 0, 14, 49 and 84).
- ix. *Ex vivo* growth of sentinel bacteria (difference between in vitro growth rates at Days 0, 14, 49 and 84).

Analysis of the continuous variables (ii) and (iii) will be based on comparing means using a t-distribution where 95 % CI is calculated for the difference in arm A compared to B and C respectively and also between B and C. A logarithmic transformation will be applied to non-normally distributed variables and unpaired t-tests done on the transformed data. For variables (i), (iv), (v), (vi), (vii), (viii) and (ix), a frequency distribution using χ^2 comparing proportions between the arms will be performed with a statistical test for significance for the difference between the arms set at 5 % ($P < 0.05$). Additionally, adverse events will be analysed using multiple



regression analysis controlling for possible confounders to see which events or aggregate score are associated to which arm. Compliance will be assessed comparing proportions consumed in each arm with number consumed as numerator and the total number of capsules prescribed between enrolment and end of study as the denominator. STATA 12.1 or any of its latest versions will be used for all the analyses.

Further exploratory analysis will be conducted and the endpoints include:

Primary endpoints adjusted for CRP, AGP and malaria (we decided *a priori* that the primary endpoints will not be adjusted for the above, however, we wish as part of an exploratory analysis to adjust for them), sTfR, Ferritin, and TSAT.

Informed consent and confidentiality

All field workers taking part in the recruitment of participants will be trained on translating and issuing of the informed consent documentation. The information sheet will be translated to all the illiterate participants in a language that they understand in the presence of an independent witness. The literate participants will be allowed to read the information sheet in their own time. Participants will also be encouraged to ask questions and seek clarification from the field workers and the PI. If the participant agrees to take part in the study, informed consent is recorded by a signature or thumbprint.

Participants will be allocated an individual identification number. Participant identification numbers will be used on all samples and data forms generated during the course of the study. Following sample collection and data entry, linkage of the ID back to the study participant will not be possible without a lookup table, which will be held by the data manager only and his designated data staff during the course of the study. The field worker will have a printout version of the list. Once data collection is complete, analysis will be performed on an anonymised copy of the data. All forms and case report forms (CRF) will be kept in locked files. At all stages,

staff/collaborators responsible for sample analysis will be blinded as to the subject's identification. Together, these processes will ensure complete confidentiality of the data gathered and impartiality of data analyses.

Discussion

WHO has identified the need for a lower dose in iron supplementation as recommendations being used by countries can pose risks to some pregnant women. This trial will test the efficacy of employing a screen-and-treat approach to minimise iron exposure whilst achieving a similar therapeutic effect.

As 50 % of anaemia in pregnancy is assumed to be due to ID, the assessment of iron status (not only anaemia) in supplementation programmes is critical and hepcidin has shown the potential of being an improved biomarker for iron status and therefore a signal for the safe administration of iron in pregnancy. In this study we will explore this potential of hepcidin with a pre-determined cut-off value of <2.5 ng/ml to screen for the readiness to receive iron. When iron is needed, we will supplement using oral, tablet form UNIMMAP formulation containing 60 mg iron daily (universal) on one hand or provide 60 or 30 mg iron daily only when hepcidin levels are below the threshold cut-off value mentioned above (screen-and-treat). We hypothesise that a screen-and-treat approach to iron supplementation will achieve similar efficacy in combating ID and IDA at a lower overall dosage of iron as therapy will be targeted to periods when the enterocyte iron absorption channels are open. We assume that lower doses will improve safety and tolerability and these will be tested as secondary outcomes.

In summary, this trial will contribute towards minimising exposure to excessive iron that may not be needed and may indeed be harmful and allow the pregnant woman to maximise the absorption and utilisation of iron when it is most needed. In addition, the overall lower dosage may help decrease side-effects and increase compliance. The exploration of hepcidin as a potential for a simple PoC diagnostic to screen for the readiness

to receive iron will assist health workers to make the right decisions for iron supplementation which will help in improving health care delivery and reduce maternal morbidity and mortality as part of efforts to meet the Millennium Development Goal 5 and the forthcoming Sustainable Health Agenda.

Abbreviations

AGP, alpha-1 acid glycoprotein; CRF, case report form; CRP, C-reactive protein; DSM, Dutch State Mines; ELISA, enzyme-linked immunosorbent assay; FA, field assistant; FACS, fluorescence-activated cell sorting; FW, field worker; GMP, good manufacturing practice; Hb, haemoglobin; ID, iron deficiency; IDA, iron deficiency anaemia; LLINs, long lasting insecticide-treated nets; MRC-ING, Medical Research Council International Nutrition Group; MRC, Medical Research Council; represents Medical Research Council Unit, The Gambia; PoC, point of care; RCH, reproductive and child health; RCT, randomised control trial; RDT, rapid diagnostic test; SAT, transferrin saturation; SCC, Scientific Coordinating Committee; SF, serum ferritin; sTR, soluble transferrin receptors; TM, trial monitor; UNIMMAP, UNICEF/WHO/UNU International Multiple Micronutrient Preparation; WHO, World Health Organisation; WIMM, Weatherall Institute of Molecular Medicine

Acknowledgement

The trial will not be possible without the funding provided by the Bill and Melinda Gates Foundation to the MRC-ING. The trial is also supported by the UK MRC and the UK Department for International Development (DFID) under the MRC/DFID Concordant agreement. We appreciate the valuable contribution of Momodou W. Jallow and Ebrima Ceasay for analysing the blood samples for the determination of the hepcidin cut-off point used in this trial.

Authors' contributions

AB is the main and corresponding author as this is part of his PhD. He put together the proposal. AMP is a substantial contributor to the concept and design of the protocol. He is also the supervisor of the PhD student. RCW is a contributor to the design of the protocol as well as reviewing the contents. SEM contributed to the reviewing of the protocol and she is also the associate supervisor of the PhD student. CC is a collaborator who is contributing to the ex-vivo analyses. LK is the statistician of the project and contributed to the statistical write-up of the protocol. SP is the collaborator who did the analysis for the hepcidin cut-off point being used and contributed to the design of the protocol. All authors read and approved the final manuscript.

Authors' information

AB is the Deputy Executive Director of the National Nutrition Agency [NaNA] in The Gambia who is seconded to MRC-ING to pursue a PhD. He has a BSc in Applied Human Nutrition from Queen Margaret University, Edinburgh and an MSc in Public Health Nutrition from the LSHTM.

AMP is a Professor of nutrition at the LSHTM and Theme leader for the MRC-ING. RW is the Head of Station for Keneba Field Station MRC Unit The Gambia. SEM is Group Leader, Maternal and Child Health Group, MRC Human Nutrition Research.

CC is an Assistant Professor at the Gillings School of Global Public Health, University of North Carolina and Senior Investigator Scientist, MRC Unit The Gambia.

LK is a statistician at the MRC Gambia Unit The Gambia.

SP is a Fellow at Weatherall Institute of Molecular Medicine (WIMM), University of Oxford.

Competing interests

The authors declare that they have no competing interests.

Author details

¹MRC Unit The Gambia & MRC International Nutrition Group, PO Box 273, Banjul, The Gambia. ²MRC Unit The Gambia, PO Box 273, Banjul, The Gambia.

³The Weatherall Institute of Molecular Medicine, University of Oxford, John Radcliffe Hospital, Oxford OX3 9DS, UK. ⁴MRC Human Nutrition Research, Elsie Widdowson Laboratory, 120 Fulbourn Road, Cambridge CB1 9NL, UK.

Received: 28 July 2015 Accepted: 14 June 2016

Published online: 13 July 2016

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Chapter 4: Hepcidin cut-off paper



London School of Hygiene & Tropical Medicine
Keppel Street, London WC1E 7HT

T: +44 (0)20 7299 4646

F: +44 (0)20 7299 4656

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First Name(s)	AMAT		
Surname/Family Name	BAH		
Thesis Title	A DOUBLE BLIND RANDOMISED CONTROLLED TRIAL COMPARING STANDARD DOSE OF IRON SUPPLEMENTATION FOR PREGNANT WOMEN WITH TWO SCREEN-AND-TREAT APPROACHES USING HEPCIDIN AS A BIOMARKER FOR READY AND SAFE TO RECEIVE IRON		
Primary Supervisor	PROF ANDREW M. PRENTICE		

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SECTION B – Paper already published

Where was the work published?	Journal of Nutrition. https://doi.org/10.3945/jn.116.245373		
When was the work published?	19 APRIL 2017		
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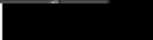
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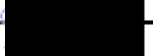
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SECTION E

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Serum Hepcidin Concentrations Decline during Pregnancy and May Identify Iron Deficiency: Analysis of a Longitudinal Pregnancy Cohort in The Gambia¹⁻³

Amat Bah,^{4,8*} Sant-Rayn Pasricha,^{5,8} Momodou W Jallow,⁴ Ebrima A Sise,⁴ Rita Wegmuller,⁴ Andrew E Armitage,⁵ Hal Drakesmith,⁵ Sophie E Moore,^{6,8} and Andrew M Prentice^{7,8}

⁴Medical Research Council (MRC) Unit The Gambia–MRC International Nutrition Group, Banjul, Gambia; ⁵MRC Human Immunology Unit, MRC Weatherall Institute of Molecular Medicine, University of Oxford, Oxford, United Kingdom; ⁶Division of Women's Health, King's College London, London, United Kingdom; and ⁷MRC Unit The Gambia–MRC International Nutrition Group and London School of Hygiene and Tropical Medicine, London, United Kingdom

Abstract

Background: Antenatal anemia is a risk factor for adverse maternal and fetal outcomes and is prevalent in sub-Saharan Africa. Less than half of antenatal anemia is considered responsive to iron; identifying women in need of iron may help target interventions. Iron absorption is governed by the iron-regulatory hormone hepcidin.

Objective: We sought to characterize changes in hepcidin and its associations with indexes of iron stores, erythropoiesis, and inflammation at weeks 14, 20, and 30 of gestation and to assess hepcidin's diagnostic potential as an index of iron deficiency.

Methods: We measured hemoglobin and serum hepcidin, ferritin, soluble transferrin receptor (sTfR), and C-reactive protein (CRP) at 14, 20, and 30 wk of gestation in a cohort of 395 Gambian women recruited to a randomized controlled trial. Associations with hepcidin were measured by using linear regression, and hepcidin's diagnostic test accuracy [area under the receiver operating characteristic curve (AUC^{ROC}), sensitivity, specificity, cutoffs] for iron deficiency was analyzed.

Results: The prevalence of anemia increased from 34.6% at 14 wk of gestation to 50.0% at 20 wk. Hepcidin concentrations declined between study enrollment and 20 wk, whereas ferritin declined between 20 and 30 wk of gestation. The variations in hepcidin explained by ferritin, sTfR, and CRP declined over pregnancy. The AUC^{ROC} values for hepcidin to detect iron deficiency (defined as ferritin <15 µg/L) were 0.86, 0.83, and 0.84 at 14, 20, and 30 wk, respectively. Hepcidin was superior to hemoglobin and sTfR as an indicator of iron deficiency.

Conclusions: In Gambian pregnant women, hepcidin appears to be a useful diagnostic test for iron deficiency and may enable the identification of cases for whom iron would be beneficial. Hepcidin suppression in the second trimester suggests a window for optimal timing for antenatal iron interventions. Hemoglobin does not effectively identify iron deficiency in pregnancy. This trial was registered at www.isrctn.com as ISRCTN49285450. *J Nutr* doi: 10.3945/jn.116.245373.

Keywords: hepcidin, anemia, iron deficiency, pregnancy, diagnostic

Introduction

More than 38% of pregnant women worldwide are anemic, with the prevalence greatest in sub-Saharan Africa and parts of Asia (1). In Africa and Asia, anemia directly or indirectly contributes to one-quarter of maternal deaths (2). Consequences of antenatal anemia include premature delivery and low birth

weight (3). Universal iron supplementation is recommended by the WHO for all pregnant women in a setting where anemia prevalence in this population exceeds 40% (4). However, only half of anemia cases in pregnancy worldwide (including only 44% of cases in Africa and 47% of cases in Asia) are attributed to iron deficiency and amenable to iron supplementation (1). The use of iron to treat other causes of anemia (including

¹ The ENID (Early Nutrition and Immune Development) trial was supported by the Medical Research Council (MRC; United Kingdom), through core funding to the MRC International Nutrition Group (MC-A760-5QX00) and the UK Department for International Development (DFID) under the MRC/DFID Concordat agreement.

² Author disclosures: A Bah, S-R Pasricha, MW Jallow, EA Sise, R Wegmuller, AE Armitage, H Drakesmith, SE Moore, and AM Prentice, no conflicts of interest.

³ Supplemental Table 1 is available from the "Online Supporting Material" link in the online posting of the article and from the same link in the online table of contents at <http://jn.nutrition.org>.

⁸ These authors contributed equally to this study.

*To whom correspondence should be addressed. E-mail: amat.bah@lshtm.ac.uk.

malaria, inflammation, and hemoglobinopathy) may not be beneficial, may represent a missed opportunity to identify and treat an alternative condition, and in some cases, may be actively harmful because it could exacerbate the risk of infection (5) or iron overload (6). However, such cases would likely be misdiagnosed and iron supplementation provided if a decision for iron intervention was based on a diagnosis of anemia, or if universal iron interventions are provided. Therefore, an appropriate case definition of iron deficiency in pregnancy could be of value in correctly identifying women who would most benefit from iron interventions.

Although there are several well-established tests for iron status, accurate assessment with the use of a single test is difficult (7, 8). Definitions of iron deficiency with the use of conventional markers, such as ferritin, remain under review (9) and may require adjustment for levels of inflammation. However, deploying multiple tests in the field is complex and costly, requires sophisticated interpretation, and is of limited value in resource-poor settings (7, 10). A biomarker that enables accurate diagnosis of iron status is needed.

Hepcidin, a peptide hormone produced by the liver, is the master regulator of systemic iron homeostasis (11). Hepcidin binds to the iron exporter ferroportin, inducing its internalization and subsequent degradation (12). Hepcidin concentrations are suppressed in iron deficiency, facilitating increased iron absorption and utilization, and elevated in iron loading and inflammation, preventing access of iron to the plasma. There is therefore considerable interest in pursuing hepcidin as a diagnostic test for iron status (13). We have previously found that hepcidin is a promising tool to identify individuals who might gain the most benefit from iron supplementation and defined putative thresholds that could help define iron deficiency in young children (14) and in women (15).

Small longitudinal studies showed that hepcidin is suppressed in pregnancy (16), likely facilitating the recognized increase in iron absorption seen during this period (17). Whether hepcidin suppression is mediated chiefly by iron deficiency (18) or maternal or fetal erythropoiesis, or another fetal, placental, or maternal factor, is currently unclear (16). Experimental data indicate that hepcidin may be directly transcriptionally regulated by estrogen and that steroid hormones may directly upregulate hepcidin expression (19, 20). The value of hepcidin as an index for iron deficiency in pregnancy has not been previously established, and putative cutoffs have not been defined. In a longitudinal cohort of pregnant Gambian women, we sought to evaluate associations between variables of maternal iron status and erythropoiesis with hepcidin at 3 distinct time periods and then to determine the diagnostic test accuracy and estimate potential cutoffs of hepcidin as an index of iron deficiency.

Methods

Participants and study design. Samples were derived from the Early Nutrition and Immune Development (ENID)⁹ trial in rural Gambia, which is a randomized trial assessing whether nutritional supplementation to pregnant women and their infants can enhance infant immune development (trial registration: ISRCTN49285450) (21). For

the main ENID trial, all of the women in the 36 villages who were registered within the West Kiang Demographic Surveillance System and aged between 18 and 45 y were invited from February 2010 to January 2013 to participate in the study. Women with confirmed pregnancy between 10 and 20 wk by ultrasound were randomly assigned to 1 of 4 intervention groups: 1) iron-folic acid (standard care), 2) multiple micronutrients (including iron-folic acid), 3) protein energy plus iron-folic acid, and 4) protein energy plus multiple micronutrients (including iron-folic acid). Women who were 1) currently beyond 20 wk of gestation at the first clinic attendance (by ultrasound), 2) enrolled in another study, 3) severely anemic at recruitment (hemoglobin <7 g/dL), or 4) menopausal were excluded. Samples from the first 400 women recruited to the ENID trial were included in this analysis. Thus, all of the participants in this substudy analysis received 60 mg Fe and 400 µg folic acid/d as part of the ENID intervention, from enrollment to delivery, as per current WHO guidelines (4, 21). The national prevalence of anemia in pregnancy was estimated at 67.9% (22). It would therefore not have been ethical to deny iron to women in pregnancy given the established benefits from iron supplementation.

Analytical methods. Maternal blood samples collected at enrollment (booking) of mean gestational ages of 14, 20, and 30 wk of gestation were used for sample analysis. All of the samples were collected after an overnight fast, before 0900. Hemoglobin was analyzed from whole-blood samples by using a Medonic M-Series automated hematology analyzer (Boule Medical) shortly after sample collection. Serum ferritin, soluble transferrin receptor (sTfR), iron, and C-reactive protein (CRP) were analyzed from serum samples by using an automated biochemistry analyzer (COBAS Integra 400 plus; Roche Diagnostics). Serum hepcidin was quantified on the same samples by using a competitive ELISA (Bachem Hepcidin-25; now marketed by Peninsula Laboratories International), with a detection range of 0.049–25 ng/mL. Concentrations were interpolated from a 4-parameter curve fitted from a 2-fold, 10-point serial dilution made from a manufacturer-provided standard peptide. Samples outside the standard curve were re-analyzed at a higher dilution, and the final concentration was calculated on the basis of the dilution factor (23). The concentrations were obtained from the standard curve by using Dynex Revelation software (Dynex Technologies). Hepcidin measurements were performed in duplicate. All of the analyses were performed in the MRC (Medical Research Council) Keneba Laboratory. Results of conventional iron indexes were not available to staff measuring hepcidin, and vice versa.

Statistical analysis. Variables were summarized at each time point, and means or proportions between time points compared by using *t* tests or 2-sample tests of proportions (2-sided, $\alpha = 0.05$; significance defined as $P < 0.05$). Next, we modeled determinants of hepcidin concentrations at each of the 3 time points with the use of multiple linear regression, with variables log-transformed if the distribution was skewed. We estimated β -coefficients (which normalize the mean and SD), enabling comparison of associations between variables. We then generated nonparametric receiver operating characteristic curves (ROCs) and calculated the AUC^{ROC} together with Bamber and Hanley CIs for hepcidin concentration as a test of iron deficiency as defined by 2 recognized reference standards: 1) serum ferritin <15 µg/L (24) and 2) estimated body iron stores <0 mg/kg (based on the ratio of sTfR and ferritin) (10). The sensitivity and specificity of hepcidin as an index of iron deficiency were determined for each possible cutoff for hepcidin. We calculated the Youden index [(sensitivity/100 + specificity/100) - 1] at each value of hepcidin to assist in the selection of an optimal cutoff. Missing data were analyzed by list-wise deletion. The sample size of the study was predicated on the size of the cohort. Analyses were undertaken by using Stata 13 (StataCorp).

Ethics. Informed consent (including permission to undertake future related analysis of the samples) was obtained from all participants through either a signature or a thumbprint. The ENID trial was approved by the Gambia Government/MRC Unit The Gambia joint ethics committee (SCC1126v2).

⁹ Abbreviations used: CRP, C-reactive Protein; ENID, Early Nutrition and Immune Development; IDA, iron deficiency anemia; ROC, receiver operating characteristic curve; sTfR, soluble transferrin receptor.

Results

Samples from a total of 395 pregnant women were analyzed at recruitment (~14 wk of gestation) and at 20 and 30 wk of gestation (Table 1). The mean age of the women was 29.6 y (95% CI: 29.1, 30.3), mean weight was 54.9 kg (95% CI: 53.9, 55.9 kg), mean height was 161.4 cm (95% CI: 160.9, 162.1 cm), and mean BMI (in kg/m²) was 21.0 (95% CI: 20.6, 21.3). Hematologic and iron indexes at each time point are shown in Table 1. Hepcidin concentrations were lower at 20 wk than at 14 wk and even lower at 30 wk of gestation. Hemoglobin declined from 14 wk through 30 wk of gestation, whereas serum ferritin decreased, sTfR increased, and hence total body iron declined, between 20 and 30 wk of gestation. CRP increased significantly between 14 and 20 wk of gestation.

The prevalence of anemia, iron deficiency, and inflammation across pregnancy is presented in Table 2. The prevalence of anemia was 34.6% at 14 wk and increased to 50.0% at 20 wk and remained stable thereafter. Iron deficiency defined as ferritin <15 µg/L was 37.6% at 14 wk and 34.3% at 20 wk, increasing to 50.6% at 30 wk of gestation. Iron deficiency defined as body iron <0 mg/kg was 27.35%, 24.78%, and 35.05% at 14, 20, and 30 wk of gestation, respectively. The prevalence of elevated sTfR likewise increased between 20 and 30 wk. The prevalences of iron deficiency anemia (anemia and low ferritin) were 18.8%, 20.16%, and 32.83% at 14, 20, and 30 wk of gestation, respectively. Thus, these data indicate that the prevalence of anemia increases during the second trimester of pregnancy, whereas the prevalence of iron deficiency increases between the second and early third trimester.

Changes in concentrations of hepcidin and iron indexes at different gestation durations led us to evaluate the relation between hepcidin and these variables at each time point. Thus, we undertook multiple linear regression to compare associations between hepcidin at each of the 3 time points with 3 factors likely to influence it: ferritin (reflecting iron stores), sTfR (reflecting tissue iron demand including erythropoiesis), and CRP (reflecting inflammation). We used standardized coefficients to enable comparison in slope and the strength of association between each variable at each time point. As shown in Table 3, the slope of association between hepcidin and ferritin diminished over the course of pregnancy, whereas the association between hepcidin and sTfR was strengthened. Finally, the overall amount of variation in hepcidin explained by these variables diminished over the course of pregnancy.

We then graphed the ROC curves and estimated AUC^{ROC} for hepcidin to detect iron deficiency. With the use of a standard definition of iron deficiency of ferritin <15 µg/L, the AUC^{ROC} values for hepcidin to detect iron deficiency were 0.86, 0.83, and 0.84 at 14, 20, and 30 wk, respectively. With the use of body iron <0 mg/kg, the AUC^{ROC} values for hepcidin to detect iron deficiency were 0.85, 0.86, and 0.80 at 14, 20, and 30 wk, respectively. During pregnancy, iron supplementation should be routinely administered when body iron stores and dietary iron cannot meet maternal, fetal, and placental demands (4, 25). However, hemoglobin remains the most commonly deployed initial test to determine the need for treatment doses of iron in pregnancy (26), even though it is a test for anemia rather than iron deficiency per se, because the conditions have often been considered synonymous (27). sTfR is another increasingly widely available index of iron status. We therefore compared the capacity of hepcidin with hemoglobin concentration and sTfR to detect iron deficiency (defined by low body iron stores) and found that hepcidin was superior to hemoglobin at 14 and 20 wk, and similar at 30 wk, and was superior to sTfR at each time point when using ferritin alone as a gold standard (Figure 1).

Sensitivity, specificity, and the Youden index for a range of potential hepcidin cutoffs are shown in Table 4. Hepcidin appears to have an optimal cutoff (based on the tradeoff between sensitivity and specificity calculated by the Youden index) that fluctuates over the duration of pregnancy (from 1.5–2.0 ng/mL at booking, to 0.5 ng/mL at 20 wk, to 1.0–1.5 ng/mL by 30 wk). However, these lower thresholds are associated with reductions in sensitivity. The prevalence of iron deficiency at each hepcidin threshold is presented in Supplemental Table 1.

Discussion

In this longitudinal study in pregnant women, we measured hepcidin concentrations together with traditional iron biomarkers, investigated the changes that occurred between 14 and 30 wk of gestation, and assessed the diagnostic performance of hepcidin for iron deficiency. We observed that hepcidin concentrations decreased by 20 wk of gestation, whereas iron stores (measured by ferritin and body iron stores) declined most substantially at 30 wk of gestation. These changes occurred despite the distribution of routine iron supplementation to all of

TABLE 1 Iron and hematologic indexes in pregnant Gambian women¹

Index	14 wk	20 wk	<i>P</i>	30 wk	<i>P</i>
<i>n</i>	395	375		367	
Gestational age, wk	14.1 (8.0, 21.3)	20.4 (15.0, 26.9)		30.5 (25.4, 34.4)	
Hemoglobin, g/dL	11.55 (7.2, 17.9)	11.00 (7.4, 14.5)	<0.001	10.77 (6.2, 14.4)	<0.0001
MCV, fL	81.9 (60.9, 98.6)	83.8 (62.9, 104.0)	<0.001	83.6 (61.8, 99)	NS
Serum ferritin, µg/L	20.69 (0.1, 237.2)	19.20 (0.1, 273.8)	NS	14.29 (0.1, 315.5)	<0.001
Serum sTfR, mg/L	4.41 (0.58, 17.97)	4.25 (1.12, 15.42)	NS	4.80 (1.49, 17.81)	<0.001
Serum sTfR-F index	3.29 (–14.80, 111.63)	3.26 (–5.63, 56.43)	NS	3.86 (–87.19, 76.26)	NS
Serum CRP, mg/L	1.70 (0.02, 43.48)	2.41 (0.00, 59.32)	0.001	2.29 (0.01, 126.68)	NS
Total body iron, mg/kg	2.70 (–18.38, 14.40)	2.50 (–17.57, 14.06)	NS	1.18 (–17.84, 11.02)	<0.001
Serum hepcidin, ng/L	1.59 (0.03, 49.79)	1.23 (0.02, 45.70)	0.006	1.09 (0.04, 135.69)	NS

¹ Values are arithmetic (hemoglobin, MCV, sTfR, CRP, and total body iron) or geometric (ferritin, sTfR-F index, and hepcidin) means (ranges). *P* values are for 2-sided paired *t* tests comparing analytes between 14 and 20 wk and between 20 and 30 wk of gestation. CRP, C-reactive protein; MCV, mean cell volume; sTfR, soluble transferrin receptor; sTfR-F index, sTfR/log₁₀ (ferritin).

TABLE 2 Prevalence of anemia, iron deficiency, and inflammation at 14, 20, and 30 wk of gestation in pregnant Gambian women¹

Condition	14 wk	20 wk	<i>P</i>	30 wk	<i>P</i>
Anemia, <i>n</i> /total <i>n</i>	135/390	189/378	<0.001	201/368	0.207
Hemoglobin <11 g/dL, % (95% CI)	34.62 (30.04, 39.50)	50.00 (44.95, 55.04)		54.62 (49.47, 59.66)	
Iron deficiency, <i>n</i> /total <i>n</i>	145/385	117/341	0.348	159/314	<0.0001
Ferritin <15 µg/L, % (95% CI)	37.66 (32.93, 42.63)	34.31 (29.43, 39.53)		50.64 (45.09, 56.16)	
IDA, <i>n</i> /total <i>n</i>	70/387	73/362	0.470	110/335	<0.001
Anemia + low ferritin, % (95% CI)	18.09 (14.54, 22.26)	20.16 (16.33, 24.64)		32.83 (27.99, 38.07)	
Elevated sTfR, <i>n</i> /total <i>n</i>	152/382	118/347	0.106	133/310	0.019
sTfR >4.4 mg/L, % (95% CI)	39.79 (34.97, 44.80)	34.01 (29.18, 39.17)		42.90 (37.47, 48.50)	
Absent body iron, <i>n</i> /total <i>n</i>	102/373	84/339	0.436	102/291	0.005
<0 mg/kg, % (95% CI)	27.35 (23.04, 32.11)	24.78 (20.45, 29.67)		35.05 (29.75, 40.74)	
Inflamed, <i>n</i> /total <i>n</i>	83/375	100/345	0.035	69/302	0.076
CRP >5 mg/L, % (95% CI)	22.13 (18.20, 26.63)	28.99 (24.41, 34.02)		22.85 (18.43, 27.95)	

¹ *P* values are for 2-sided tests of proportion comparing proportions between 14 and 20 wk and between 20 and 30 wk of gestation. CRP, C-reactive protein; IDA, iron deficiency anemia; sTfR, soluble transferrin receptor; sTfR-F index, sTfR/log₁₀ (ferritin).

the women in the cohort. We observed that hepcidin performs well as a diagnostic test for iron deficiency, and indeed outperforms hemoglobin at all time points. On the basis of the AUC^{ROC}, the diagnostic performance of hepcidin to detect iron deficiency was good (AUC^{ROC} >0.80) and was similar from 14 to 30 wk of gestation.

Few studies, to our knowledge, have previously reported on hepcidin concentrations during human pregnancy, and those that did generally included relatively small sample sizes. Van Santen et al. (28) measured hepcidin in 31 women across the 3 trimesters of pregnancy and observed that hepcidin concentrations decreased from the second trimester of pregnancy and became essentially undetectable by the third trimester; hepcidin concentrations correlated with iron status. A study in 37 Danish women receiving iron supplementation in pregnancy likewise found that hepcidin concentrations were suppressed during pregnancy, occurring between the first measurement at 13–20 wk and the second measurement at 21–28 wk; hepcidin concentrations were observed to remain suppressed during pregnancy and to increase at delivery and thereafter (29). In contrast, Simavli et al. (30) measured hepcidin concentrations across pregnancy in healthy Turkish women and observed no reduction in hepcidin across pregnancy, nor an association between hepcidin and iron status. Interestingly, in a similar study, the authors found evidence that elevated hepcidin in the second trimester may be associated with adverse pregnancy outcomes, such as pre-eclampsia and intrauterine growth retardation (31). The importance of hepcidin in facilitating increased iron absorption was confirmed in a stable isotope iron study that showed a correlation between maternal hepcidin concentrations and maternal iron absorption and transfer of iron to the neonate (32).

By studying hepcidin concentrations across gestation in a large cohort of women with uncomplicated singleton pregnancies, all of whom were randomly assigned to receive iron supplementation, in a population at high risk of anemia, we confirm that hepcidin concentrations decline by 20 wk of pregnancy, before the onset of biochemical evidence of iron deficiency or clear evidence of changes in iron stores (assessed by ferritin, sTfR, and total body iron). Conversely, the reduction in iron stores observed between 20 and 30 wk of pregnancy was not accompanied by a further reduction in hepcidin concentrations. At all times in pregnancy, hepcidin is associated with

ferritin, sTfR, and CRP, but the proportion of variation in hepcidin attributable to these factors decreases during gestation. These findings suggest that iron deficiency itself is not solely responsible for the reduction in hepcidin during pregnancy and raise the possibility of an additional, as yet unidentified, regulator of hepcidin concentrations during pregnancy. The expansion of maternal erythropoiesis with the expression of the erythroid-derived hepcidin-suppression hormone erythroferrone represents one hypothesis (33), which is supported by the increasing regression coefficient between hepcidin and sTfR (an indicator of RBC production) as pregnancy progresses. Alternatively, an as-yet-undiscovered placenta- or fetus-derived factor may act to suppress hepcidin expression in the maternal liver. Estrogen and progesterone appear to upregulate hepcidin transcription in cellular and animal models (20), but their role in regulating iron homeostasis in human pregnancy requires further investigation. The expansion of plasma volume during pregnancy causes reductions in concentrations of many analytes, including hemoglobin (hemodilution), and has been considered a potential mechanism for reductions in concentrations of ferritin (34). However, such a mechanism would not explain concordant increases in concentrations of sTfR, suggesting that the changes in measured iron status are related to changes in iron stores and metabolism, rather than exclusively to hemodilution.

The suppression of serum hepcidin is an essential part of the physiologic response to iron need, and pregnant women with

TABLE 3 Factors associated with (log) hepcidin at 14, 20, and 30 wk of gestation in pregnant Gambian women by multiple regression¹

	14 wk	<i>P</i>	20 wk	<i>P</i>	30 wk	<i>P</i>
Ferritin	0.60	<0.001	0.51	<0.001	0.42	<0.001
sTfR	-0.16	<0.001	-0.23	<0.001	-0.33	<0.001
CRP	0.04	0.327	0.02	0.696	0.07	0.139
Overall <i>r</i> ²	0.48		0.40		0.39	

¹ Values are β-coefficients (regression coefficients adjusted for a mean and SD of 1) and *P* values for association. CRP, C-reactive protein; sTfR, soluble transferrin receptor.

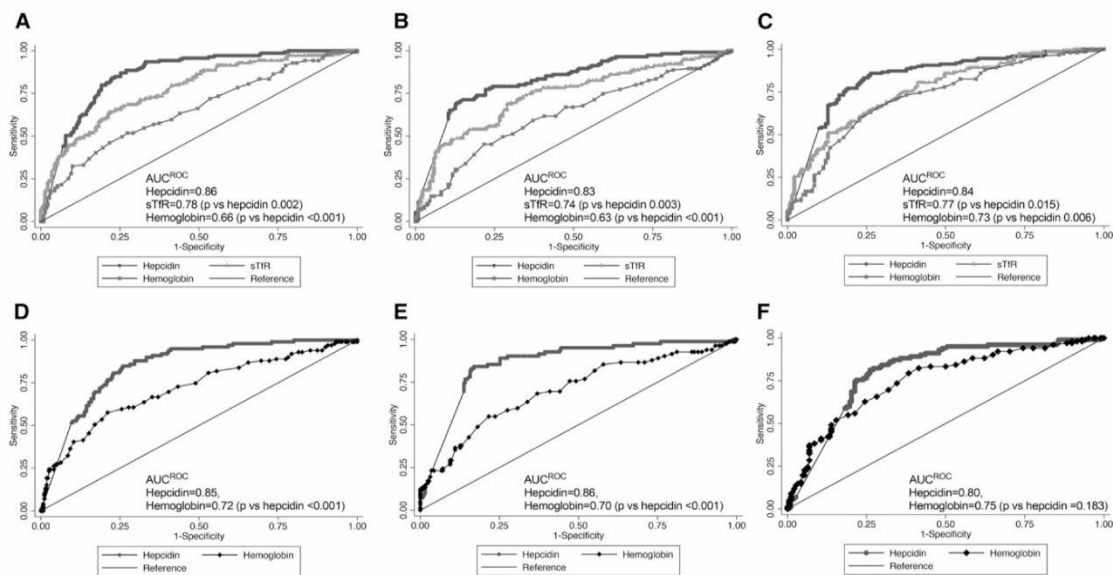


FIGURE 1 ROCs and the corresponding AUC^{ROC} values for hepcidin as a test for iron deficiency defined by 2 reference standards in pregnant Gambian women. (A–C) Capacity of hepcidin to detect iron deficiency as defined by ferritin $<15 \mu\text{g/L}$ at 14 (A), 20 (B), and 30 wk (C) of gestation, respectively; the performance of hepcidin is compared with sTfR and hemoglobin. (D–F) Capacity of hepcidin to detect iron deficiency defined by body iron stores $<0 \text{ mg/kg}$ at 14 (D), 20 (E), and 30 wk (F) of gestation, respectively; the performance of hepcidin is compared with hemoglobin. ROC, receiver operating characteristic curve; sTfR, soluble transferrin receptor.

undetected concentrations of serum hepcidin transfer dietary iron to their fetus more effectively (32). As the direct regulator of systemic iron homeostasis through its role in governing cellular iron export, and hence absorption across the enterocyte (12), hepcidin is an intriguing candidate as a potential guide to determine which individuals should be considered for iron

interventions. Our data indicate a hepcidin threshold with the use of this assay that fluctuates over pregnancy, from between 1.5 and 2.0 ng/mL at 14 wk to lower thresholds at 30 wk of gestation; reductions in these cutoffs are associated with improved specificity but come at the necessary expense of sensitivity. Importantly, reductions in hepcidin concentrations in pregnancy

TABLE 4 Sensitivity, specificity, and the Youden index at putative hepcidin thresholds at each time point in pregnancy in pregnant Gambian women¹

Hepcidin cutoff, ng/mL	14 wk			20 wk			30 wk		
	Sensitivity	Specificity	Youden	Sensitivity	Specificity	Youden	Sensitivity	Specificity	Youden
Ferritin $<15 \mu\text{g/L}$ as standard									
0.16	42.1	93.3	0.354	60	89.7	0.497	52.2	90.9	0.431
0.5	56.6	87.9	0.445	72.2	81.7	0.539	69.2	85.1	0.543
1.0	76.6	81.3	0.579	79.1	71.4	0.505	76.1	80.5	0.566
1.5	84.1	77.1	0.612	80	65.2	0.452	83.6	76	0.596
2.0	88.3	70.8	0.591	83.5	59.4	0.429	85.5	70.1	0.556
2.5	93.1	66.3	0.594	86.1	52.2	0.383	86.2	64.9	0.511
3.0	93.8	62.1	0.559	89.6	48.2	0.378	88.7	59.7	0.484
Total body iron $<0 \text{ mg/kg}$ as standard									
0.16	50	90.4	0.404	69.5	86.3	0.558	58.8	82	0.408
0.5	63.7	83.4	0.471	85.4	78.8	0.642	78.4	74.6	0.530
1.0	82.4	74.2	0.566	90.2	68.6	0.588	84.3	69.3	0.536
1.5	87.3	68.6	0.559	90.2	62.7	0.529	88.2	61.9	0.501
2.0	91.2	83.9	0.751	92.7	56.9	0.496	90.2	56.6	0.468
2.5	95.1	57.6	0.527	95.1	50.2	0.453	91.2	52.4	0.436
3.0	95.1	53.5	0.486	95.1	45.1	0.402	95.1	48.1	0.432

¹ Sensitivity = true positive detection rate; specificity = true negative detection rate; Youden index = (sensitivity/100 + specificity/100) - 1 for each time point by using the 2 gold standards of iron deficiency considered.

reflect a physiologic state of high iron requirement. Thus, although low hepcidin concentrations likely appropriately identify individuals in whom iron supplementation may be beneficial, it is unclear whether depriving women of iron supplements on the basis of hepcidin concentrations that are low but above the threshold we identified is beneficial. Given the established benefits from iron supplements on maternal and child health and the paucity of data indicating that iron is harmful in this group (35), it may be reasonable to use a higher hepcidin threshold to identify women in whom iron should be withheld, which is more “sensitive.” This would need to be confirmed in prospective studies. Our data indicate that hepcidin generally outperforms hemoglobin measurement as an index of iron deficiency, reinforcing the concept that hemoglobin testing alone is not an adequate approach to determining iron stores and the need for iron interventions. As we showed previously in children (14), testing for anemia is an inaccurate approach for the detection of iron deficiency. However, in both high-income (36) and low-income (37) settings, individual decisions to treat iron deficiency are routinely based on hemoglobin, rather than on iron deficiency testing. In population health, the dose of iron for the universal distribution of iron interventions is based on the prevalence of anemia, not iron deficiency (4). Our data suggest that measurement of an iron variable (including hepcidin) may better guide individual or public health approaches. Although rural Gambia is a malaria-endemic region, recent data from both the health center and community surveys showed that malaria endemicity in The Gambia is now low, heterogeneous, and seasonal (38), and malaria did not affect this cohort. Approximately one-fifth of the cohort had inflammation as defined by CRP >5 mg/L. CRP did not correlate with hepcidin in this population, indicating that inflammation was not an important regulator of hepcidin in these women. However, CRP-based definitions of inflammation are indistinct in pregnancy because CRP is elevated over the course of gestation (39) and may hence be an imperfect biomarker for inflammation in this context.

To our knowledge, the diagnostic performance of hepcidin to detect iron deficiency has not been previously evaluated in pregnancy. However, studies in nonpregnant women found hepcidin to be a promising indicator of iron deficiency. When hepcidin was compared with the sTfR/log₁₀ (ferritin) index as a standard, hepcidin showed an AUC^{ROC} of 0.89, and when compared with ferritin, it showed an AUC^{ROC} of 0.87 (15). With the use of the same hepcidin ELISA kit as the current study (Bachem), we assessed the diagnostic performance of hepcidin as an index of iron deficiency and the need for iron supplementation in West and East African preschool children and found that the AUC^{ROC} for hepcidin to identify iron deficiency was 0.85, with a threshold that used the Bachem ELISA of 5.5 ng/mL to distinguish iron deficiency across the overall population and among anemic children (14). Given the suppression of hepcidin concentrations in pregnancy, it is unsurprising that a lower optimal threshold would be identified in this population. There are several assays available for hepcidin measurement, which use ELISA or MS methodology. Hepcidin concentrations measured by these different assays are correlated but differ in absolute values (40). If hepcidin assays could be harmonized, the thresholds identified by our study may serve as a platform for a value that could be used to detect iron deficiency in pregnancy. A key advantage of hepcidin measurement is that it directly interrogates systemic iron handling, and hence predicts absorption and utilization of ingested iron (41). Given that hepcidin transcription is directly regulated by iron stores, erythropoiesis, and inflammation (42), hepcidin measurements

represent the net integration of these signals, which currently have to be measured by individual biomarkers [e.g., ferritin, hemoglobin (or sTfR), and CRP or α 1-glycoprotein, respectively]. Thus, a single biomarker may be able to replace multiple indexes, which may obviate the costs associated with the more current, relatively expensive assays.

Our study compares hepcidin with established reference standards frequently recommended for the diagnosis of iron deficiency in pregnancy. However, the optimal ferritin thresholds used to define iron deficiency remain uncertain and continue to be reviewed (9). Validation of iron deficiency in a large field study with gold-standard assessments, such as measurement of bone marrow iron stores or stable isotope iron incorporation, would not have been achievable, and hence our analysis represents the most pragmatic approach. Factors beyond changes in body iron stores, such as plasma dilution, could potentially explain reductions in ferritin over the course of pregnancy; however, these effects are unlikely to explain the changes in both hepcidin and ferritin because reductions in the biomarkers were seen at different time intervals (whereas hemoglobin declined constantly from 14 to 30 wk). Changes in transferrin saturation may acutely modulate hepcidin expression, but we were unable to include these data in this study (43).

An improved understanding of the complex and distinctive mechanisms of regulation of iron absorption, utilization, and transfer during pregnancy will enable improved targeting of clinical and public health interventions. Our data show the suppression of hepcidin concentrations among pregnant rural women by 20 wk of gestation, in advance of the onset of low iron stores, and suggest a window at the commencement of the second trimester before iron stores have declined when iron utilization may be greatest. In this population, the commonly used approach of identifying participants in need of iron treatment by screening for anemia (by measuring hemoglobin) is only modestly accurate. Associations between hepcidin and iron stores are maintained, although modified, across pregnancy and are reflected by the capacity of hepcidin to distinguish individuals in the population with iron deficiency. An approach for hepcidin-directed iron supplementation in both pregnant women and children is currently being tested in the field (44, 45).

Acknowledgments

We thank the ENID study team members [Medical Research Council (MRC) Unit The Gambia], especially the fieldworkers, clinical staff, laboratory technicians, and data office staff who tirelessly collected the data and samples. The authors' responsibilities were as follows—SEM and AMP: designed the ENID trial; SEM: led the implementation of the trial in the field; MWJ, EAS, AB, and AEA: performed the laboratory analysis of the samples; S-RP: performed the statistical analysis; AB, S-RP, RW, HD, SEM, and AMP: wrote the manuscript; AB, S-RP, and AMP: had primary responsibility for the final content; and all authors: read and approved the final manuscript.

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Chapter 5: Hepcidin and anaemia in pregnancy (HAPn) Paper



London School of Hygiene & Tropical Medicine
Keppel Street, London WC1E 7HT

T: +44 (0)20 7299 4646
F: +44 (0)20 7299 1656
www.lshtm.ac.uk

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Stage of publication	Undergoing revision

SECTION D – Multi-authored work

For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper. (Attach a further sheet if necessary)	Contributed to the design of the concept, led the HAPn trial as part of the PhD, oversaw all aspects of its implementation and drafted the manuscript.
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SECTION E

Student Signature	
Date	26/03/2019

Supervisor Signature	
Date	26/03/2019

Title

Hepcidin-guided screen-and-treat interventions against iron deficiency anaemia in pregnancy: a randomised controlled trial in Gambian women

Authors

Amat Bah, MSc^{1,2}, Abdul Khalie Muhammad, MSc¹, Rita Wegmuller, PhD^{1,3}, Hans Verhoef, PhD^{4,2}, Morgan M. Goheen, MD/PhD^{1,5}, Saikou Sanyang¹, Ebrima Danso, BSc¹, Ebrima A. Sise, BSc¹, Sant-Rayn Pasricha, MD/PhD⁶, Andrew E. Armitage, PhD⁷, Hal Drakesmith, PhD⁷, James H. Cross, BSc^{1,2}, Sophie E. Moore, PhD^{1,8}, Carla Cerami, MD/PhD^{1,2}, Andrew M. Prentice, PhD, FMedSci^{1,2}

Affiliations

- ¹ MRC Unit The Gambia at London School of Hygiene and Tropical Medicine, Atlantic Boulevard, Fajara, Banjul, The Gambia.
- ² London School of Hygiene and Tropical Medicine, Keppel Street, London, WC1E 7HT, UK.
- ³ GroundWork LLC, Hintergass 1, Flaesch 7306, Switzerland.
- ⁴ Division of Human Nutrition/Cell Biology and Immunology Group, P.O. Box 17, Wageningen University, 6700 AA Wageningen, The Netherlands.
- ⁵ University of North Carolina at Chapel Hill School of Medicine, Department of Microbiology and Immunology, Chapel Hill, North Carolina, USA.
- ⁶ Population Health and Immunity Division, The Walter and Eliza Hall Institute of Medical Research; and Department of Medical Biology, University of Melbourne, Parkville VIC 3052, Australia.
- ⁷ MRC Human Immunology Unit, MRC Weatherall Institute of Molecular Medicine, University of Oxford [John Radcliffe Hospital], Oxford, OX3 9DS, UK.
- ⁸ Department of Women & Children's Health, King's College London, 10th floor North Wing, St Thomas' Hospital, Westminster Bridge Road, London, SE1 7EH, UK.

Corresponding author:

Prof Andrew M. Prentice, PhD, FMedSci, MRC Unit The Gambia at LSHTM and London School of Hygiene and Tropical Medicine, Atlantic Boulevard, Fajara, The Gambia.
E-mail: aprentice@mrc.gm Tel: +220-7236903

Running title:

Hepcidin and anaemia in pregnancy (HAPn Trial).

Research in Context

Evidence before this study

Based upon regularly updated meta-analyses, the World Health Organisation (WHO) recommends that pregnant women should take supplements containing 30 to 60mg elemental iron and 400µg folic acid daily to prevent maternal anaemia, puerperal sepsis, low birthweight and pre-term birth. If daily supplementation is not acceptable due to side effects, weekly supplementation with 120mg iron and 2800µg folic acid is an acceptable alternative in areas where the prevalence of anaemia is less than 20%.

Side effects (including constipation, black stool, dizziness, nausea, vomiting and epigastric discomfort) are frequently reported and contribute to poor adherence. These might be caused by unabsorbed iron passing to the large intestine and colon and causing oxidative stress and/or shifts in the gut microbiome (dysbiosis). There are additional concerns that iron might predispose to gestational diabetes, pre-eclampsia and infections, especially malaria where it is known that anaemia and iron deficiency protect against *P falciparum* infections in pregnancy.

We reasoned that a screen-and-treat approach to combatting anaemia in pregnancy would be advantageous if it could achieve equivalent or better efficacy at a lower overall dose of iron and with fewer side effects. We determined a threshold for the iron-regulatory hormone hepcidin that would indicate 'ready-and-' to receive iron and used this, in a double-blind randomised trial, to test two hepcidin-guided screen-and-treat approaches against the standard 60mg per day regime. There have been no prior studies of hepcidin-guided antenatal iron supplementation and no other similar trials have been registered.

Added value of this study

With the primary outcome of maternal haemoglobin, we demonstrated that a weekly screen-and-treat approach administering 60mg/d iron to women whose hepcidin indicated ready-and-safe to receive iron was non-inferior to the 60mg/d standard of care using a pre-defined non-inferiority margin of -5.0g/L haemoglobin. A screen-and-treat approach using 30mg/d was not conclusively non-inferior. Secondary outcomes of prevalence of anaemia and iron deficiency showed clear evidence of inferiority in both screen-and-treat groups at both dose levels. There was a decreased frequency of iron administration in the screen-and-treat groups relative to the control group. Side effects were fewer in the 30mg/d screen-and-treat arm, but there was no substantive evidence that the screen-and-treat regimens were likely to be safer according to adverse events or ex vivo tests of *P falciparum* growth in red blood cells or sentinel bacterial growth in plasma. Compared to baseline plasma, the growth of all pathogens was markedly higher in plasma taken after commencement of iron supplementation in all trial arms.

Implications of all the available evidence

We were unable to demonstrate any clear advantages of a hepcidin-guided screen-and-treat approach to maternal iron supplementation over the current WHO-recommended standard of care. These data, together with the available evidence suggests that that efforts should be directed towards developing low-cost iron supplements with better side effect profiles to help overcome the poor adherence that currently undermines antenatal iron supplementation programmes.

Abstract:

BACKGROUND: WHO recommends daily iron supplementation for pregnant women but adherence is limited by side-effects, effectiveness is low, and there are concerns around possible harm. The iron-regulatory hormone, hepcidin, signals 'ready-and-safe' to receive iron. We tested whether a hepcidin-guided screen-and-treat (S&T) approach to combat iron deficiency anaemia (IDA) could achieve equivalent efficacy to universal administration but with lower exposure to iron.

METHODS: We conducted a 3-arm randomised-controlled double-blind trial in rural Gambia to assess non-inferiority of two S&T interventions versus WHO standard of care (enrolment June 2014 to March 2016). Participants received daily, either: a) UNU/UNICEF/WHO international multiple-micronutrient preparation (UNIMMAP) containing 60mg iron (reference group, REF); b) UNIMMAP containing 60mg iron for 7d if weekly hepcidin was $<2.5\mu\text{g/L}$ or UNIMMAP without iron if hepcidin was $\geq 2.5\mu\text{g/L}$ (S&T60); or c): as b), but with 30mg iron (S&T30). We randomised 498 pregnant women (18-45y) recruited between 14-22wks gestation using a block design stratified by haemoglobin and stage of gestation (REF n=167; S&T60 n=166; S&T30 n=165). Participants and investigators were blinded. Primary endpoint was haemoglobin at D84 with a non-inferiority margin of -5.0g/L . Secondary outcomes were anaemia, iron deficiency (ID), IDA, adherence and side-effects, and *ex vivo* assays of malaria and sentinel bacterial growth. Trial registration was ISRCTN21955180.

FINDINGS: In per protocol analysis of the primary outcome the screen-and-treat approaches did not exceed the preset non-inferiority margin of -5.0g/L (endpoint haemoglobin: S&T60 - 2.2g/L , 95%CI: $-4.6, 0.1\text{g/L}$ (n=133); S&T30 -2.7g/L , 95%CI: $-5.0, -0.5\text{g/L}$ (n=147) versus REF (n=140)). Intention-to-treat analysis yielded similar results (endpoint haemoglobin: S&T60 - 1.3g/L , 95%CI: $-3.5, 1.0\text{g/L}$ (n=164); S&T30 -2.9g/L , 95%CI: $-5.1, -0.7\text{g/L}$ (n=165) versus REF (n=165). For secondary outcomes at D84, anaemia (haemoglobin $<110\text{g/L}$) was more

common in the S&T groups (S&T60=57.3%, S&T30=59.3% vs REF=45.3%) and ID and IDA were also more common in the S&T groups assessed using ferritin, transferrin saturation, soluble transferrin receptor or hepcidin. S&T60 received 54% and S&T30 74% less iron than REF. Adherence, reported side effects and adverse events were similar between groups. *Ex vivo* tests of malarial growth in erythrocytes and bacterial growth in serum were elevated after iron but did not differ by treatment.

INTERPRETATION: The hepcidin-guided screen-and-treat approaches were less efficacious in combatting ID and IDA than universal daily iron supplementation and had no advantages in terms of adherence, side effects or safety outcomes. Our results suggest that the current WHO policy for iron administration to pregnant women should remain unchanged.

FUNDING: Bill & Melinda Gates Foundation and UK Medical Research Council.

Keywords: Pregnancy, anaemia, iron deficiency, iron supplementation, hepcidin, screen-and-treat, UNIMMAP, adverse effects.

INTRODUCTION

Iron deficiency and its associated anaemia (IDA) is the most prevalent micronutrient deficiency worldwide affecting an estimated 1.24 billion people.¹ It is the leading cause of years lived with disability in most of sub-Saharan Africa and many parts of Asia.¹ WHO recommends universal daily iron and folic acid supplementation (IFAS) in pregnancy² based on Cochrane evidence that it provides maternal and neonatal health benefits.³ Recommended dosage ranges between 30-60mg elemental iron daily with a preferred dose of 60mg in countries where anaemia prevalence exceed 40%.² In low and middle-income countries, IFAS has greater benefits for iron-deficient women⁴ and is increasingly being combined in multiple micronutrient formulations.⁵ However, even when supplements are made available, adherence is low⁴⁻⁷ due in large part to gastrointestinal side effects (constipation, nausea, vomiting, black stools and epigastric discomfort).^{8,9} This led WHO to also recommend intermittent supplementation 'if daily iron is not acceptable due to side effects'.^{10,11} There are also concerns that iron supplementation can predispose to haemoconcentration³ and gestational diabetes.¹² In low income settings, there is the additional possibility that iron supplementation might increase gastro-intestinal and other infections,⁴ especially malaria. Anaemia and low iron status are associated with protection against falciparum malaria in pregnant women^{13,14} and there are clear pathways by which iron administration abrogates this protection.¹⁵ Thus, lowering the dose of supplemental iron could be beneficial, if it could be achieved without compromising efficacy.

We reasoned that hepcidin, the hepatic iron-regulatory peptide that acts as a master regulator of iron metabolism, could signal when women are 'ready-and-safe' to receive iron, and hence could form the basis of a screen-and-treat IFAS regime. Hepcidin is the homeostatic regulator of body iron absorption, distribution and metabolism.¹⁶ Circulating

hepcidin is suppressed during iron deficiency, anaemia, and increased erythropoiesis, and increased by high serum and hepatic iron, and during infection and inflammation.¹⁷ By integrating these competing signals a low hepcidin level indicates when the body is iron deficient^{18,19} and will efficiently absorb iron.²⁰ Conversely, raised hepcidin would block duodenal iron absorption thereby rendering supplementation ineffective and exposing the gut microbiota to unnecessary iron that may cause dysbiosis and side effects.²¹

We hypothesised that a hepcidin-guided screen-and-treat approach to iron supplementation would be non-inferior to the WHO-recommended universal daily supplementation and by lowering the total exposure to iron would have a better adherence, side effect and safety profile. We assessed this in a 12-week randomised-controlled, double-blind, non-inferiority trial in Gambian pregnant women with Day 84 haemoglobin as the primary outcome.

PARTICIPANTS AND METHODS

Full details are in Supplementary Methods and the published trial protocol paper.²²

Study design

The Hepcidin and Anaemia in Pregnancy (HAPn) study was a randomised, double-blind, proof-of-concept, non-inferiority trial with pregnant women randomly allocated to: a) daily supplementation with UNU/UNICEF/WHO international multiple micronutrient capsules (UNIMMAP) containing 60mg iron as ferrous fumarate (Reference, REF); b) weekly screening of plasma hepcidin for 12 weeks, each time succeeded by daily supplementation for 7 days with UNIMMAP containing 60mg iron (as ferrous fumarate) if plasma hepcidin concentration was $<2.5\mu\text{g/L}$, or no iron if hepcidin was $\geq 2.5\mu\text{g/L}$ (S&T60); c) screen-and-treat supplementation as in b), but with UNIMMAP containing 30mg/day iron (S&T30).

Calculation of the hepcidin threshold of $<2.5\mu\text{g/L}$ to define 'ready-and-safe' to receive iron has been described previously¹⁹. The intervention started at Day 0 (the day of screening, enrolment and randomisation) and continued for 84 days or until delivery, whichever came first. Data collection started 16th June 2014 and ended 3rd March 2016.

Primary and secondary outcomes

The primary outcome was haemoglobin at Day 84. Secondary outcome measures were: proportion of anaemia (Hb $< 11\text{g/dl}$) at Day 84; prevalence of iron deficiency at Day 84; prevalence of iron deficiency anaemia (IDA) at Day 84; total iron dosage; adverse events; and compliance²².

Ethics, governance, safety monitoring and informed consent

The trial was approved by the Medical Research Council (MRC) Unit The Gambia Scientific Coordinating Committee (SCC), Joint Gambia Government/MRC Ethics Committee (SCC 1357, amendments L2014.56v2) and London School of Hygiene and Tropical Medicine Ethics Committee (7168), overseen by a Data Safety Monitoring Board (DSMB), Trial Steering Committee and Trial Monitor, and conducted according to Good Clinical Practice (GCP) standards supervised by the MRCG@LSHTM Clinical Trials Office. All participants gave written, informed consent.

Study setting

We conducted the study in 19 rural communities in the Jarra West and Kiang East Districts where anaemia is common. Malaria endemicity is low, heterogeneous and seasonal.

Recruitment, screening and enrolment

Nurse midwives and fieldworkers identified and screened pregnant women at first antenatal care visits at two health facilities, obtained informed consent and collected demographic information. Qualified personnel recorded the medical history, performed a medical examination and collected 5-7mL venous blood for field measures of haemoglobin (HemoCue Hb301 analyser, Sweden) and a malaria rapid test (SD Bioline MalariaAgPf, Standard Diagnostics, Korea) followed by microscopy of positive samples. Blood samples were transferred on ice to the laboratory at MRCG@LSHTM Keneba fieldstation for full blood count (Medonic M Series) and assessment of plasma hepcidin (see below). At Days 0,14,49 and 84 freshly washed red blood cells (RBCs) were used for malaria growth assays. Remaining plasma was stored at -20°C for iron and bacterial growth assays. Day 14 was selected for the *ex vivo* malaria assays as a time when there would likely be a high level of reticulocytosis. Day 49 was then selected as the midpoint between Days 14 and endpoint at Day 84.

Women aged 18–45y were eligible for randomisation if gestational age was 14–22wks assessed by reported first date of last menstrual period or, in absence of recall, by fundal height. Exclusion criteria were: unlikely to remain in the area; severe anaemia (haemoglobin concentration $<70\text{g/L}$); serious illness; chronic disease; and self-reported history of previous pregnancy complications (repeated miscarriage, or abortions, pre-eclampsia/eclampsia). At enrolment, women were provided with long-lasting insecticide-treated bed nets. Any woman found to have haemoglobin $<70\text{g/L}$ during the trial was treated as per the national protocol.

Randomisation

On the day of screening, eligible women were randomly allocated using computer-generated numbers to one of 3 intervention arms based on a stratified, permuted block design (n=9) with a 1:1:1 allocation ratio, balanced by haemoglobin (above and below median haemoglobin of the respective day) and gestational age (14–18wks or 19–22wks; to account for natural differences in haematological and iron status).

Investigational product and blinding to intervention

The UNICEF/WHO/UNU international multiple micronutrient preparation (UNIMMAP) was produced in 3 variants (containing 60mg, 30mg or no iron) by DSM South Africa as identical gelatine capsules, packed in tubs under Good Manufacturing Practice (GMP) conditions. All formulations contained 400µg folic acid and 13 other micronutrients (**see Supplementary Table 1**). Participants and the research team, with exception of the data manager, were blinded to the group allocation and supplementation type throughout the fieldwork. The supplements were pre-packed weekly by the field coordinator using computer-generated lists accounting for each participant's preceding hepcidin value. Participants were instructed to take 1 capsule a day with water or another drink.

Follow-up

On Day 2 and weekly thereafter, each woman was seen by a fieldworker who counted remaining supplements, measured axillary temperature, recorded self-reported side effects, and gave the next week's supply of tablets. At Days 14, 49 and 84, 5-7mL venous blood was collected for assessments and processing as described for baseline. At Day 7 and weekly thereafter (except when venous blood was collected), field staff collected finger-prick capillary blood samples. At each time point, haemoglobin was measured by HemoCue, *P*

falciparum infection by rapid test, and hepcidin concentrations were measured to determine their subsequent allocation of iron or no iron in groups S&T60 and S&T30. To maintain blinding, participants in the REF group also had weekly finger-prick blood samples collected and hepcidin concentrations analysed, even though it did not influence their subsequent supplement allocation.

Gambian national guidelines stipulate that pregnant women should receive intermittent preventative treatment against malaria with sulfadoxine-pyrimethamine beginning at 16wks gestation as first dose and at least 2 other doses with one month interval between them. So as not to interfere with the malaria susceptibility assays, we arranged that participants received their first dose immediately after blood draws on Day 49.

Participants were monitored until delivery and the outcomes of the pregnancy were registered for both mother and child (postnatal check-up within 72h after delivery). Where possible, reasons for being lost to follow up were recorded. Adverse events were defined as any untoward or unfavourable medical occurrence, including signs and symptoms which are temporally associated with the research procedure or trial intervention, whether or not considered related to the subject's participation in the research. Serious adverse events were investigated by a physician and defined as any adverse event that was life-threatening or resulted in death or required hospitalisation or prolongation of hospitalisation; was a persistent or significant disability/incapacity; was a congenital anomaly/birth defect; or a reported maternal death, miscarriage or stillbirth.

Laboratory analyses

Plasma hepcidin was assayed by ELISA (hepcidin-25 (human) EIA Kit, Bachem; now sold by Peninsula Laboratories International, USA) with a detection range 0.049-25.0µg/L. The assay was validated as part of a worldwide harmonization exercise²³. Heparin was quantified as single measurements to allow results within 24h after blood collection and due to cost.

Serum ferritin, iron, unbound iron binding capacity, transferrin saturation (TSAT), soluble transferrin receptor (sTfR), C-reactive protein (CRP), and α_1 -acid glycoprotein (AGP) were measured by an automated analyser (CobasIntegra400plus, Roche Diagnostic, Switzerland).

Ex vivo growth rates of *P falciparum* parasites in fresh red blood cells and 4 sentinel bacterial species in heat-inactivated serum were conducted as proxy safety indices using methods described previously.^{15,24} The bacteria were selected as frequent causes of sepsis in low-income settings and as representing a range of iron acquisition mechanisms. Assays for one of the bacteria (*Staphylococcus epidermidis*) proved unreliable with frequent absence of any growth so have been excluded from the results. The technical reasons for this were discovered in hindsight and there was insufficient sample to rerun them.

Sample size determination

Haemoglobin concentration data obtained from a prior study in neighbouring villages²⁵ yielded a standard deviation of 12.8g/L. This value was used to calculate a sample size of 154 participants for each of the 3 arms using a 1-sided α of 2.5 percent with a conservative-Bonferroni type correction. Initially, a total sample size of 462 pregnant women was

calculated assuming <10% loss to follow-up. This was to provide 80% power to establish that, for the primary endpoint haemoglobin, at a non-inferiority margin of 5.0g/L: 1) S&T60 is non-inferior to REF; 2) S&T30 is non-inferior to REF; and 3) S&T30 is non-inferior to S&T60. After the first 2 cohorts, permission was obtained from the Ethics Committee to increase the sample size to 498 as loss to follow up exceeded 10%.

Statistical analysis

The following definitions were used: anaemia = haemoglobin concentration <110g/L; iron deficiency (ID) = plasma ferritin concentration <15µg/L if CRP<5mg/L or ferritin<30µg/L if CRP>5mg/L; iron deficiency anaemia (IDA) = Hb<110g/L and ferritin<15µg/L when CRP is <5mg/L OR Hb<110g/L and ferritin<30µg/L when CRP is >5mg/L and ferritin index >2.0. Adherence was calculated as described in the Supplementary Methods.

Per-protocol analysis was used to assess non-inferiority of the primary end point (haemoglobin at D84). All missing values and outliers present after the locking of the data were maintained. In the intention-to-treat analysis, missing values were replaced by multiple imputation (see Supplementary Methods). Intervention effects on continuous variables were measured as the difference in means, with logarithmic transformation as appropriate. A modified intent-to-treat analysis was also performed (excluding the 3 participants withdrawn before the first dose of supplement) and groups were compared using linear regression analysis, with intervention entered as a dummy-coded categorical variable.

The number of adverse events was too low to allow meaningful analysis by type of adverse events. For each woman, we added the counts for various types of adverse events. We used negative binomial regression to assess group differences in observed counts. Negative binomial regression was used instead of Poisson regression to account for over-dispersion (i.e. where the variance exceeds the mean). Effect sizes thus obtained are reported as the relative change in observed counts. Adherence was assessed as the extent to which the participant's history of supplementation coincided with the prescribed supplementation (see supplementary material).

RESULTS

Participant flow

Between June 2014 and March 2016, we identified 527 pregnant women with gestational age 14–22wks who consented to take part in the trial. To ensure that the study was conducted across different seasons and to ensure that detailed monitoring could be achieved, the study was conducted in 6 cohorts starting June 2014 (n= 52), September 2014 (n=87), January 2015 (n= 99), April 2015 (n=75), August 2015 (n=96), December 2015 (n=91). Of those, 29 were excluded for reasons stated in the CONSORT diagram (**Figure 1**). Of the 498 participants who were enrolled and randomly allocated to intervention arms, 78 (15.7%) were withdrawn or lost to follow up before the scheduled completion of the intervention, with no evidence of a marked imbalance in non-completion between groups (Figure 1). Three participants were excluded before the first supplement was received, resulting in 495 women being included in the modified intention-to-treat analysis.

Group characteristics at baseline

Baseline characteristics were similar between intervention groups (**Supplementary Table 2**), and indicated a population with high prevalence of anaemia (>50%) in all three groups. One third of all women were iron deficient using ferritin thresholds adjusted for inflammation. Poor population iron status was confirmed by the high prevalence of other iron markers with abnormal values (hepcidin (54%), mean corpuscular volume (82%), transferrin saturation (35%), sTfR (36%)) (Supplementary Table 2). Inflammation measured by CRP and AGP was high (32%). Sickle cell disorder was absent. There was only one positive test for *Plasmodium* infection.

Effect of Intervention

Figure 2 shows the per protocol non-inferiority analysis for the primary endpoint of haemoglobin concentration at Day 84 (with confidence intervals listed in **Table 1**). The screen-and-treat approaches did not exceed the preset non-inferiority margin of -5g/L (endpoint Hb: S&T60 -2.2g/L, 95%CI: -4.6, 0.1g/L (n=133); S&T30 -2.7g/L, 95%CI: -5.0 - 0.5g/L (n=147) versus REF (n=140)). On the other hand, the upper limit of the confidence interval for S&T60 was borderline lower than REF and for S&T30 was clearly lower. The effect of S&T30 was not substantively different to S&T60 (-0.5g/L, 95%CI -2.8, 1.8g/l) Intention-to-treat analysis was similar (endpoint Hb: S&T60 -1.3g/L, 95%CI: -3.5, 1.0g/L (n=164); S&T30 -2.9g/L, 95%CI: -5.1, -0.7g/L (n=165) versus REF (n=165).

Table 1 also shows the intervention effects for secondary outcomes assessed as continuous variables. Hpcidin, ferritin and the sTfR/log-ferritin 'ferritin index' (measures of iron deficiency) were all significantly lower than REF in both S&T groups. The other iron markers

(serum iron, transferrin, sTfR, and unbound iron binding capacity) confirmed these results (see Supplementary Results and Supplementary Table 3).

The prevalence of anaemia and iron deficiency showed a similar picture though the contrasts appear more striking (**Table 2**). In the REF group the anaemia prevalence dropped from 58.2% to 45.3%; and rose in the other two groups (S&T60, 52.2% to 57.3%; S&T30, 52.8% to 59.3%) such that the S&T arms were clearly inferior to REF. Similarly the prevalence of 'ready-and-safe' to receive iron (defined as hepcidin <2.5µg/L) declined substantially (from 56.0% to 21.4%) in the REF arm and at endpoint was lower than both S&T groups, indicating better iron status (S&T60: 41.7%; S&T30: 52.4%; REF: 21.4%)(see **Supplementary Figure 3**). The prevalence of iron deficiency (defined as ferritin <15µg/L when CRP<5 or <30µg/L when CRP>5) declined more in the REF group (38.6 to 17.1%) than in the S&T60 (39.6 to 29.0%) and S&T30 group where it increased slightly (37.0 to 39.7%). Iron deficiency prevalence defined using the sTfR threshold of >4.4mg/L showed a very similar pattern where the prevalence of iron deficiency anaemia were also higher in the S&T groups at the end of intervention.

Adherence and supplement use

Adherence exceeded 86% in all groups and was similar between groups (**Table 2**). Participants in S&T60 and S&T30 groups received 46% and 52% of the number of supplemental iron doses received by their peers in REF, respectively.

Adverse events

The risk of self-reported illnesses and side effects (black stool, constipation, dizziness, fatigue, nausea and stomach ache) was similar in the S&T60 group (13.7%) to that in the

REF group (11.1%; difference 2.5, 95%CI 0.3%, 4.8%); in the S&T30 group it was lower than in REF (7.8%; difference -3.5%, 95%CI -5.4%, -1.6%) (Table 2).

The frequency of adverse events or serious adverse events was similar between the groups (Table 2).

Safety assays

Figure 3A shows that the growth of malaria parasites in fresh RBCs was suppressed at baseline (compared to the non-anaemic controls used in the assay), was greatly stimulated at Day 14 and gradually declined to Day 84, with no differences between the intervention groups at any timepoint.

Figure 3B illustrates the *ex vivo* growth of *Escherichia coli*, *Salmonella enterica* and *Staphylococcus aureus* in heat inactivated sera from the participants. These sentinel bacteria were selected on account of their differing iron acquisition mechanisms. For each organism replication rates were significantly faster in serum drawn 14, 49 and 84 days after the commencement of iron supplementation. On Days 14 and 49 there were no differences between treatment arms. On Day 84 sera from women in the REF arm supported faster bacterial growth than in S&T30 for *E coli* and *S aureus*, and for *E coli* growth in REF was also faster than in S&T60. This effect was due to the acute effect of iron administered to those women in S&T60 and S&T30 whose hepcidin measured 7 days previously was below the 2.5µg/L threshold. This is verified in **Supplementary Figure 4** which shows that there is no difference in bacterial growth across intervention groups among women who received iron 3h prior to the blood draw, and significantly lower growth in those in S&T60 and S&T30 who did not have iron.

Discussion

The World Health Organisation recommends universal daily oral iron and folic acid supplementation for pregnant women to prevent maternal anaemia, puerperal sepsis, low birth weight, and preterm birth.² Implementation of this policy in low-income countries is highly variable and adherence is notoriously poor; the latter in large part due to the common side-effects of gastric discomfort, black stools, nausea and dizziness.² In recognition of these problems WHO also endorses intermittent supplementation¹⁰, which might help overcome the fact that iron administration raises hepcidin and reduces the absorption of iron on the following day^{26,27}. However, efficacy of weekly supplementation in reducing anaemia is generally low.¹¹ There is clear evidence that iron supplementation has the greatest benefit in iron deficient women,⁴ and moderately strong evidence that administration of iron might be harmful in women who are iron replete;⁴ all of which suggests that a screen-and-treat approach would be beneficial.

In this study, we reasoned that hepcidin would be the ideal marker for defining 'ready-and-safe' to receive iron because it is a highly specific measure of iron status^{18,19} and additionally records if women, due to on-going infection or inflammation, might be at risk from iron^{15,16} and would anyway not absorb it.²⁰ We simulated a point-of-care test using weekly finger-prick blood sampling and overnight ELISA analysis.

The hepcidin-guided approach approximately halved the iron exposure in the two screen-and-treat groups; S&T60 and S&T30 received iron in 46.2% and 52.6% of weeks in the intervention period, respectively. Since the amount of iron was halved in S&T30 the overall

iron intake was only 26.3% as much as in REF. The primary outcome (haemoglobin at Day 84) was lower in both S&T groups. In S&T60 the lower limits of the 95% CI were within our pre-set non-inferiority margin of -5g/L (-2.2g/L, 95%CI: -4.6, 0.1g/L, n=133). In the S&T30 group the lower limit of the 95% CI touched on the non-inferiority margin (-2.7g/L, 95%CI: -5.0, -0.5g/L, n=147). All of the secondary outcomes for iron status showed evidence of inferiority. Anaemia prevalence declined in the universal supplementation REF arm and increased in both S&T arms. Likewise, the prevalence of ID determined by ferritin, sTfR, ferritin index or hepcidin thresholds was higher in both S&T groups than the REF group and similarly for IDA.

As in many trials,^{3,4} even the REF arm had low apparent efficacy, with only a 3.3g/L improvement in haemoglobin and only a 13% reduction in anaemia, despite being implemented under the ideal conditions of an efficacy trial. However, true efficacy in ameliorating the haemodilution of pregnancy cannot be judged in the absence of a placebo arm.

Iron is a problematic nutrient with both beneficial and potentially harmful effects. Some of these effects are potentially serious especially in low-income settings where infections are common.²⁸ Detection of differences in event rates for serious infections would require a very large trial and, in the case of malaria, would be unethical since intermittent preventive therapy for pregnant women is advised, and in The Gambia mandated. We also issued insecticide-treated bed nets to all participants at enrolment. In light of these constraints we used proxy assays of likely infection potential for malaria and for three sentinel bacteria that use a range of iron acquisition mechanisms. By conducting these *ex vivo* assays at baseline, Days 14 and 49 we were able to capture the short- and medium-term effects of chronic iron

administration. On Day 84 blood was drawn 3h after the last oral iron (or placebo) dose and hence results at Day 84 capture both the chronic and acute post-absorptive effects of iron. The malaria parasite assays have previously provided a robust mechanism to explain how IDA protects against *P falciparum* infection (parasite invasion and growth rates are poor in older microcytic RBCs) and why supplementation abrogates this effect (parasite invasion and growth rates are high in reticulocytes and large young RBCs).^{15,29,30} These effects are clearly replicated in Figure 3A and concur with the associated changes in CD71 (a reticulocyte marker). There was no difference between treatment arms at any timepoint. This can be explained by the fact that the most iron deficient subjects in all groups received iron early in the trial and this elicited a broadly similar reticulocyte surge despite the poorer overall performance of the two S&T arms. Reticulocytosis is also a natural response to the expansion of blood volume in mid-pregnancy and may have contributed to the increased risk.³¹ Note that the absence of an acute effect of iron administration at Day 84 is entirely consistent with the fact that the assay uses washed RBCs and their susceptibility is governed by cell morphology rather than iron content.²⁴

Growth rates of all three bacteria rose markedly in all treatment groups after commencement of iron supplementation. In the absence of a placebo group we cannot conclude that this is an effect of the iron (or other micronutrients), but it seems highly likely. Pregnancy-related changes in humoral immunity are an unlikely explanation since the plasma was heat-inactivated prior to inoculation. Furthermore, the growth-stimulatory effect of iron is clearly illustrated by the response to the acute iron and micronutrient administration 3h before the blood draw on Day 84 shown in Supplementary Figure 4. This corroborates our previous results in adult men where growth rates were promoted by prior iron (without additional micronutrients) and were highly correlated with serum iron and transferrin saturation.²⁴

These *ex vivo* assays may not equate to the situation *in vivo* but are highly suggestive that blood-stream bacteria would grow faster at higher levels of iron and transferrin saturation, and would therefore have a greater chance of overcoming immune defences.

The real or perceived side effects of taking oral iron supplements are less serious than the threat of a major infection but are important insofar as they lead to poor adherence to iron supplementation. The poorer performance of the S&T arms in resolving ID and IDA might have been acceptable if there was evidence that they were safer or had fewer side effects, as we initially hypothesised. In fact there was a higher prevalence of self-reported illnesses and side effects in the S&T60 group; possibly because women in the REF arm adapted to the iron better than when administration was intermittent (with on and off weeks). As might be expected the prevalence of illnesses and side effects was lower in the S&T30 group. Note that the unusually high adherence in this study may reflect the influence of sensitization and fieldworker encouragement, and the fact that subjects were aware that adherence was being monitored.

There are several possibilities why the screen-and-treat approach failed. First, it is possible that weekly screening fails to capture the dynamics of inter-current infections and inflammation, and that more frequent screening is needed. Even if this were the case and a point-of-care test were available, it would be entirely impractical to screen more frequently. A second possibility is that our hepcidin threshold, determined to diagnose iron deficiency,¹⁹ did not adequately differentiate iron absorbers from iron blockers (because the derivation did not include information on iron absorption). A higher threshold might have yielded more frequent dosing and a higher efficacy, but would have been less effective at total number of women given iron, and should not have been necessary because we already prioritised

sensitivity over specificity in selecting the threshold (see Supplementary Methods). A lower threshold would have reduced efficacy yet further. Our surmise is that the large bolus doses of highly-absorbable ferrous fumarate override the physiological mechanism of hepcidin-induced iron blockade evolved to regulate duodenal iron absorption from foods; and hence iron continued to be absorbed in the REF group even in the face of raised hepcidin.

Our study had numerous strengths and some weaknesses. Hepcidin is theoretically the ideal index of 'ready-and-safe' to receive iron and it very effectively reduced the amount of iron administered in an area with high anaemia prevalence. The study had adequate statistical precision for the main outcomes, conducted to GCP standards, had high adherence and relatively few drop outs. A limitation is that sample size was insufficient to capture potentially rare adverse events and the trial was conducted in an area with low malaria transmission, and high use of insecticide-treated bed nets and intermittent preventive treatment for pregnant women, and hence could not assess to what extent the S&T approach reduced the risk of malaria. Our proxy safety outcomes for malarial and bacterial infections provide intuitively solid outcomes, but may not reflect *in vivo* susceptibility. Provision of the iron with multiple other micronutrients can be viewed as both a strength and a weakness; a strength because other nutrient deficiencies that might limit the acquisition and/or utilisation of iron should be eradicated or a weakness because of possible nutrient-nutrient interactions (eg that the zinc in UNIMMAP might compete with iron for absorption). Note also that the UNIMMAP capsules are not enteric coated; this will not affect aggregate iron availability but may cause loss of other micronutrients. Because all subjects received the same UNIMMAP excepting for differences in iron content the latter concern would not affect comparison between the intervention arms.

Prior evidence demonstrates that intermittent iron supplementation in pregnancy is somewhat less efficacious than daily supplementation^{4,11} and we conclude from this study that a hepcidin-guided screen-and-treat strategy does not overcome this limitation. These results are likely to be generalizable at least to other populations in LMICs with high levels of anemia and iron deficiency and in a low malaria setting. Future alternatives to universal oral iron supplementation in pregnancy may include use of parenteral iron formulations such as ferric carboxymaltose which can deliver up to 1000mg elemental iron over a 15-minute infusion; this will require evidence of cost effectiveness and safety in low income settings together with development of infrastructure to overcome barriers to implementation. We therefore support continued application of the current WHO guidelines, but urge development of novel iron formulations with a much better side-effect profile in order to encourage better adherence. The findings from our RBC malaria susceptibility assays underscore the importance of the WHO guideline that iron administration in malarious areas should ideally be implemented in conjunction with adequate measures to prevent, diagnose and treat malaria.³²

Abbreviations:

AGP	Alpha-1 acid Glycoprotein
CONSORT	Consolidated Standards of Reporting Trials
CRP	C-reactive protein
DSM	Dutch State Mines
DSMB	Data Safety Monitoring Board
ELISA	Enzyme-linked Immunosorbent Assay
ENID	Early Nutrition and Immune Development

GMP	Good Manufacturing Practice
HAPn	Hepcidin and anaemia in pregnancy
ID	Iron deficiency
IDA	Iron deficiency anaemia
IFAS	Iron and folic acid supplementation
MRCG at LSHTM	Medical Research Council Unit, The Gambia at London School of Hygiene and Tropical Medicine
RBC	Red blood cell
REF	Reference group
S&T	Screen-and-treat
S&T30	Screen-and-treat with 30mg iron
S&T60	Screen-and-treat with 60mg iron
SCC	Scientific Coordinating Committee
SD	Standard deviation
sTfR	Soluble transferrin receptors
UNIMMAP	UNICEF/WHO/UNU International Multiple Micronutrient Preparation
WHO	World Health Organisation

Competing interest:

The authors declare that they have no competing interest.

Authors' contribution:

AB contributed to the design of the concept, led the HAPn Trial as part of his PhD, oversaw all aspects of its implementation and drafted the manuscript.

AMP was PI of the HIGH Consortium, designed the concept, raised the funding and was overall PI for the trial with inputs to all aspects of design, analysis and write-up.

RW contributed to the design of the protocol, provided management support and supervision.

SEM contributed to the reviewing of the protocol and associate supervision of the PhD student.

CC designed, oversaw and analysed the ex-vivo malaria and bacterial analyses.

MMG contributed to the design, execution and analysis of the malarial growth assays.

AKM was the trial statistician.

HV supervised and contributed to the statistical analyses and the manuscript.

AEA advised on hepcidin measurement and contributed to the design of the protocol.

HD was co-PI of the HIGH Consortium that funded the trial and contributed to design and interpretation.

SRP conducted the analysis for the hepcidin cut-off point used and contributed to the design of the protocol.

SAS conducted the hepcidin and haemogram analysis

ED Oversaw and conducted the hepcidin and haemogram analysis

EAS conducted the iron analysis

JAC contributed to the design, execution and supervision of the bacterial growth assays

Authors' information:

AB is the Deputy Executive Director of the National Nutrition Agency [NaNA] in The Gambia who is seconded to MRC-ING to pursue a PhD.

AMP is a Professor of International Nutrition at LSHTM and Nutrition Theme Leader at MRCG at LSHTM.

RW was the Head of Station for Keneba Field Station MRCG and is currently at GroundWork and ETH Zurich.

SEM is a Reader in Global Women and Children's Health, King's College London

CC is senior Investigator Scientist at MRCG at LSHTM and Group Leader for Iron, Infection and Anaemia.

MMG is an MD/PhD student at the University of North Carolina at Chapel Hill School of Medicine, Department of Microbiology and Immunology, Chapel Hill, North Carolina, USA.

AKM is a statistician at MRCG at LSHTM.

AEA is a senior postdoctoral scientist at the MRC Human Immunology Unit, MRC WIMM, University of Oxford.

HV is Visiting Associate Professor at the Division of Human Nutrition, Wageningen University, The Netherlands

HD is a Programme Leader at the MRC Institute of Immunology in Oxford.

SP is a Laboratory Head at the Walter and Eliza Hall Institute in Melbourne.

SS is a senior laboratory technician at MRCG at LSHTM.

ED is a scientific officer at MRCG at LSHTM.

EAS is a scientific officer at MRCG at LSHTM.

JAC is a PhD student with MRCG at LSHTM.

Acknowledgements:

The HAPn Trial was supported by the HIGH (Hepcidin and Iron in Global Health) Consortium funded by the Bill & Melinda Gates Foundation (OPP 1055865) awarded to the MRC Unit the Gambia at LSHTM which is core-funded by MCA760-5QX00 from the UK Medical Research Council (MRC) and the UK Department for International Development (DFID) under the MRC/DFID Concordat agreement. All authors were funded through this arrangement apart from HD, SRP and AA who were funded by the UK Medical Research Council (Grant MC_UU_12010/1) and the NIHR Biomedical Research Centre, Oxford for HD. We appreciate the valuable contributions of Momodou W. Jallow, Alasana Saidykhan, Momodou K. Darboe, Muhammed Ngom, Kabiru Ceesay (field co-ordinator), Musa Jarjou and Lindsay Kendall of MRCG at LSHTM in the study. We thank the HAPn field team of MRCG at LSHTM and the communities of Kiang and Jarra.

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Table 1: Primary and secondary trial outcomes; continuous variables (per-protocol).

Outcome	Intervention Group	n (%) of randomised	Estimate	SE[†]	Effect [95% CI]
Primary outcome					
Haemoglobin, g/L	REF	139 (83.7)	110.1	0.8	
	S&T60	131 (79.9)	107.9	0.8	-2.2 [-4.6, 0.1]
	S&T30	145 (87.9)	107.4	0.8	-2.7 [-5.0, -0.5]
Secondary outcomes					
Hepcidin, µg/L *†	REF	140 (84.3)	6.3	3.4	
	S&T60	132 (80.5)	3.3	4.2	0.52 [0.37, 0.75]
	S&T30	147 (89.1)	2.3	4.7	0.37 [0.26, 0.52]
Ferritin, µg/L †	REF	139 (83.7)	34.6	1.9	
	S&T60	130 (79.3)	23.1	1.8	0.67 [0.58, 0.77]
	S&T30	145 (87.9)	21.4	1.7	0.62 [0.54, 0.71]
Ferritin (inflammation adjusted)	REF	139 (83.7)	31.6	0.1	
	S&T60	130 (79.3)	21.2	0.1	0.67 [0.58, 0.77]
	S&T30	145 (87.9)	19.3	0.1	0.61 [0.53, 0.70]
Ferritin index†	REF	139 (83.73)	2.2	1.5	

S&T60	129 (78.66)	2.9	1.5	1.35 [1.23, 1.49]
S&T30	145 (87.88)	3.1	1.5	1.43 [1.31, 1.58]

* Estimates obtained using Tobit regression on the natural-log transformed hepcidin concentration was left-censored at 0.049 µg/L (limit of detection) and right-censored at 25 µg/L.

Values indicate mean (SE) or † geometric mean (GSD as geometric standard deviation). Exponentiation of log-transformed variables † yielded effect estimates that are expressed as ratios of geometric means versus REF.

† SE = standard error obtained by the Delta method.

Table 2: Secondary trial outcomes; categorical variables (per-protocol)

Outcome	Intervention Group	Prevalence, %	n/N	Effect [95% CI]
Anaemia (haemoglobin <110g/L)	REF	45.3	63/139	
	S&T60	57.3	75/131	11.9 [0.1, 23.8]
	S&T30	59.3	86/145	14.0 [2.5, 25.5]
Ready-and-safe to receive iron (hepcidin <2.5µg/L)	REF	21.4	30/140	
	S&T60	41.7	55/132	20.24 [9.42, 31.05]
	S&T30	52.4	77/147	30.95 [20.40, 41.51]
Ferritin index (sTfR/logferritin ratio >2.0)	REF	58.6	82/140	
	S&T60	87.2	116/133	28.6 [18.7, 38.6]
	S&T30	89.1	131/147	30.5 [21.0, 40.1]
Iron deficiency anaemia (see legend ¹ , %)	REF	17.1	24/140	
	S&T60	29.0	38/131	11.9 [1.9, 21.8]
	S&T30	39.7	58/146	22.6 [12.5, 32.7]
Iron dosage (% of weeks in which iron was received)	REF	100.0	1974/1974	

	S&T60	46.2	1025/1905	-53.8 [-56.0, -51.6]
	S&T30	52.6	952/2009	-47.4 [-49.6, -45.2]
Adherence/compliance (%)	REF	86.1	275/1974	
	S&T60	86.3	260/1905	0.3 [0.3, 0.3]
	S&T30	87.8	246/2009	1.7 [1.7, 1.7]
Reported side effects (aggregate score ²) ³	REF	111	220/1974	
	S&T60	135	261/1906	1.2 [0.8, 1.8]
	S&T30	78	154/2009	0.7 [0.5, 1.0]
Adverse events ³	REF	89	167/1902	
	S&T60	82	149/1861	-7.4 [-26.0, 11.1]
	S&T30	89	175/1945	1.6 [-17.2, 20.3]
Serious adverse events (DSMB notified) ⁴	REF	29	9/1904	
	S&T60	47	14/1861	18.7 [-12.3, 49.8]
	S&T30	18	6/1945	-10.2 [-34.0, 13.6]

¹ IDA defined as Hb<110g/L and ferritin <15ug/L when CRP is <5mg/L OR Hb <110g/L and ferritin <30ug/L when CRP is >5mg/L and ferritin index >2.0.

² Individual complaints and events are listed in Supplementary Table 5.

³Prevalence = Observed number of events per 1000 person-weeks; n/N = Cases/Person-weeks

⁴ Prevalence = Observed number of events per 10,000 person-weeks; n/N = Cases/Person-weeks

The estimates from 3 & 4 above were based on a negative binomial model, accounting for differences in exposure. The effect and its accompanying 95% CI are the respective exponentiated relative changes in observed counts and their CIs.

Figure Legends

Figure 1: CONSORT diagram for participant flow

Figure 2: Non-inferiority tests for the primary outcome at endline

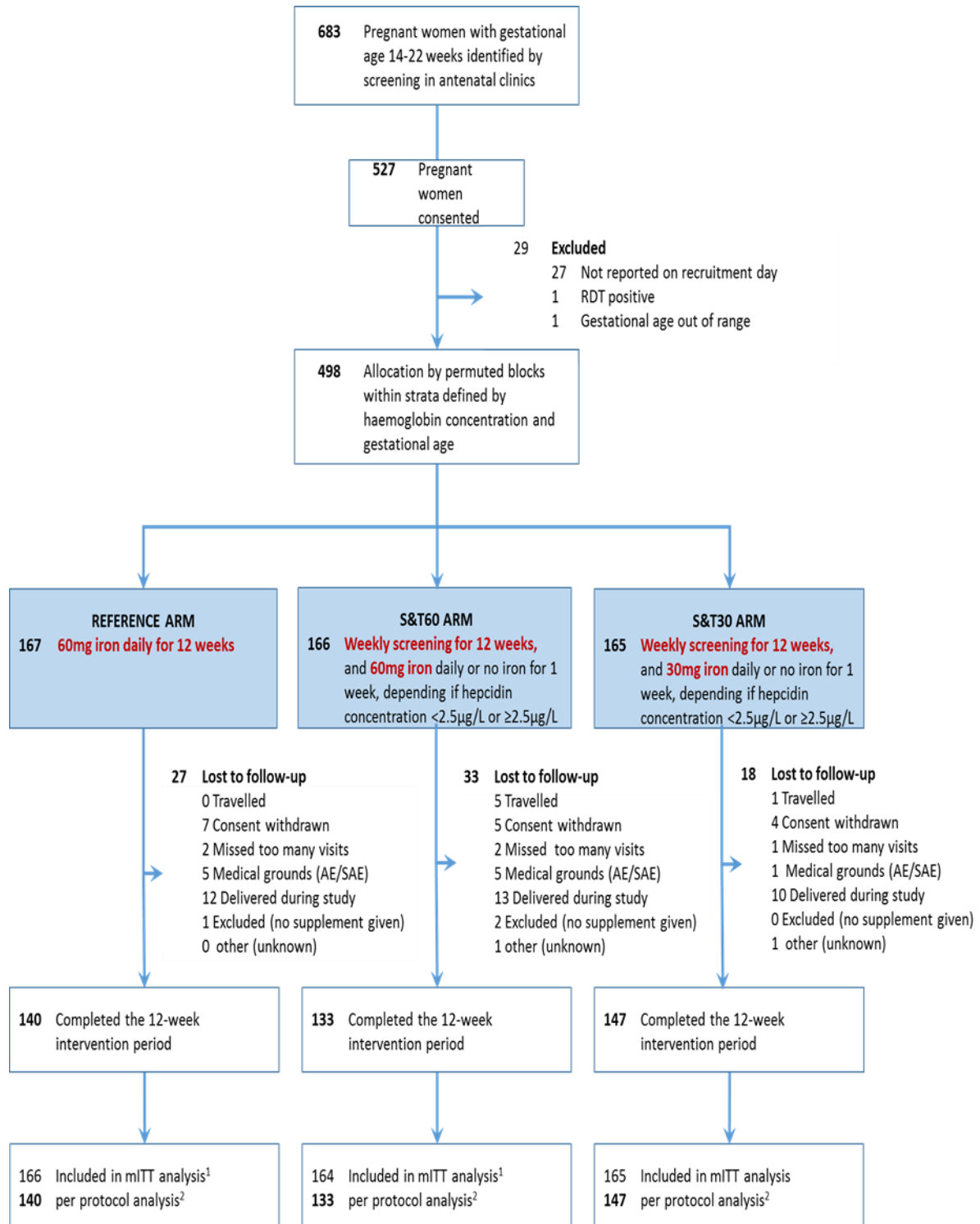
Per protocol analysis of change in haemoglobin from baseline to Day 84. Values are means \pm 95% CI. Dotted line shows the pre-set non-inferiority margin of -5g/L.

Figure 3: *Ex vivo* assays of malaria growth in erythrocytes and sentinel bacteria growth in serum

3A: Malarial growth assays in fresh red blood cells. Left panel shows growth rates of *P falciparum* strain FCR3-FMG in fresh red blood cells (RBCs) relative to the growth in RBCs from non-anaemic controls. Right panel shows reticulocyte counts assessed by FACS counting of CD71+ cells relative to non-anaemic controls. Parasite growth and reticulocyte counts were significantly higher at Days 14 and 49 compared to baseline ($P < 0.001$) with no differences between treatment groups. Blue = REF, red = S&T60, green = S&T30.

3B: Sentinel bacterial growth assays in serum. Upper section shows individual patient data with means \pm SE. Growth rates were assessed as change in optical density at 6h post inoculation for *E coli*, 7h for *S enterica* and 8h for *S aureus*. Blue = REF; red = S&T60; green = S&T30. *** $P < 0.0001$; ** $P < 0.001$. Differences between time points were assessed by repeat measures ANOVA and Scheffé's post-hoc tests. Days 14, 49 and 84 showed faster growth rates than at baseline for all species ($P < 0.0001$ for all times). The lower plots show the percentage of patient sera displaying *ex vivo* growth rates greater than the 95% centile calculated at baseline across all groups. All organisms showed significant increases following iron supplementation ($P < 0.01$). Differences between the intervention groups were not significant by Chi-squared tests. Blue = REF, red = S&T60, green = S&T30.

Fig 1



¹Randomised but excluding those who did not receive any supplement

²Lower numbers reported elsewhere are due to missing values

Fig 2

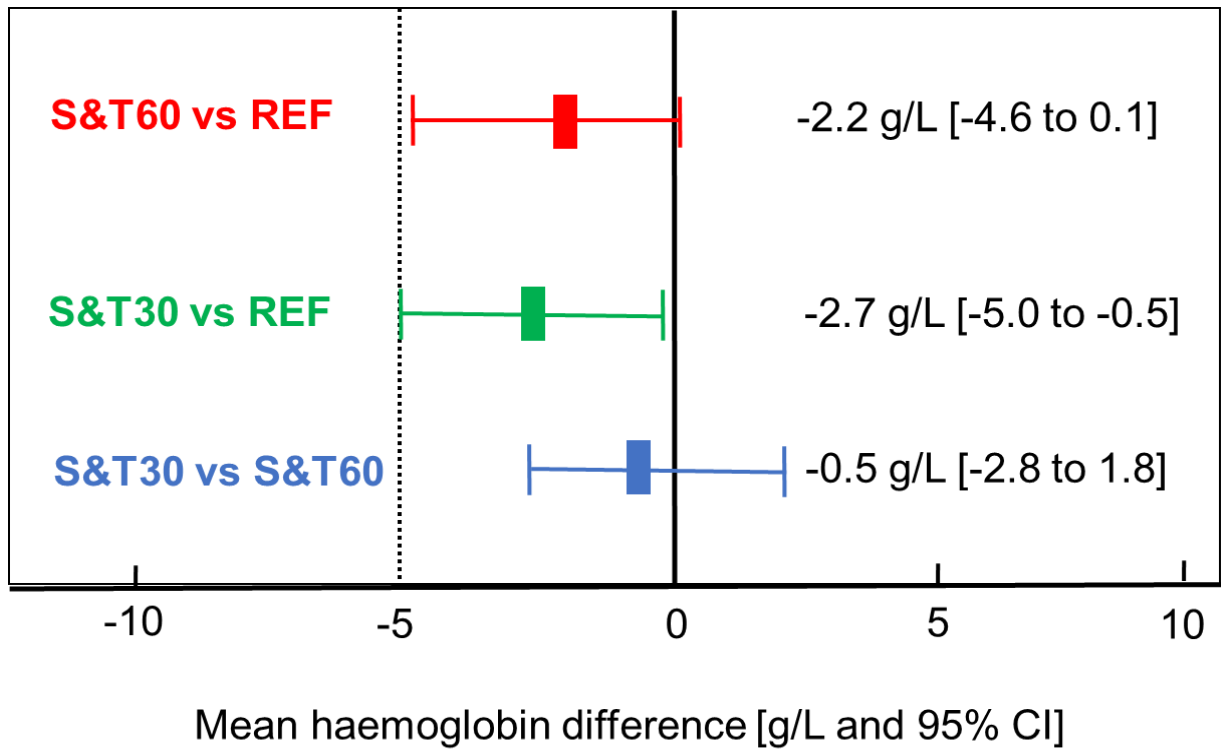


Fig 3A

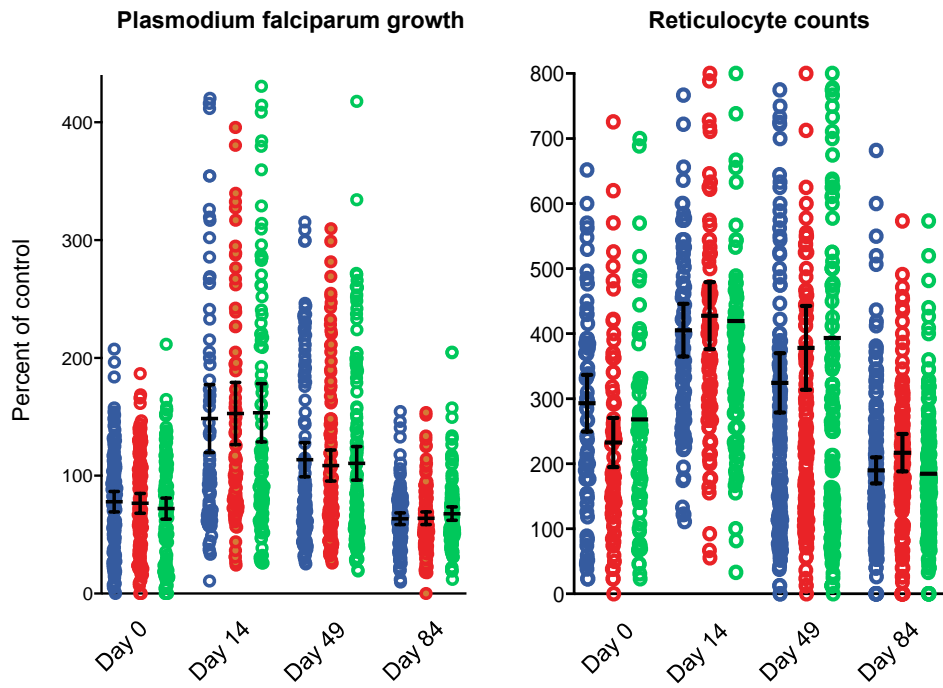
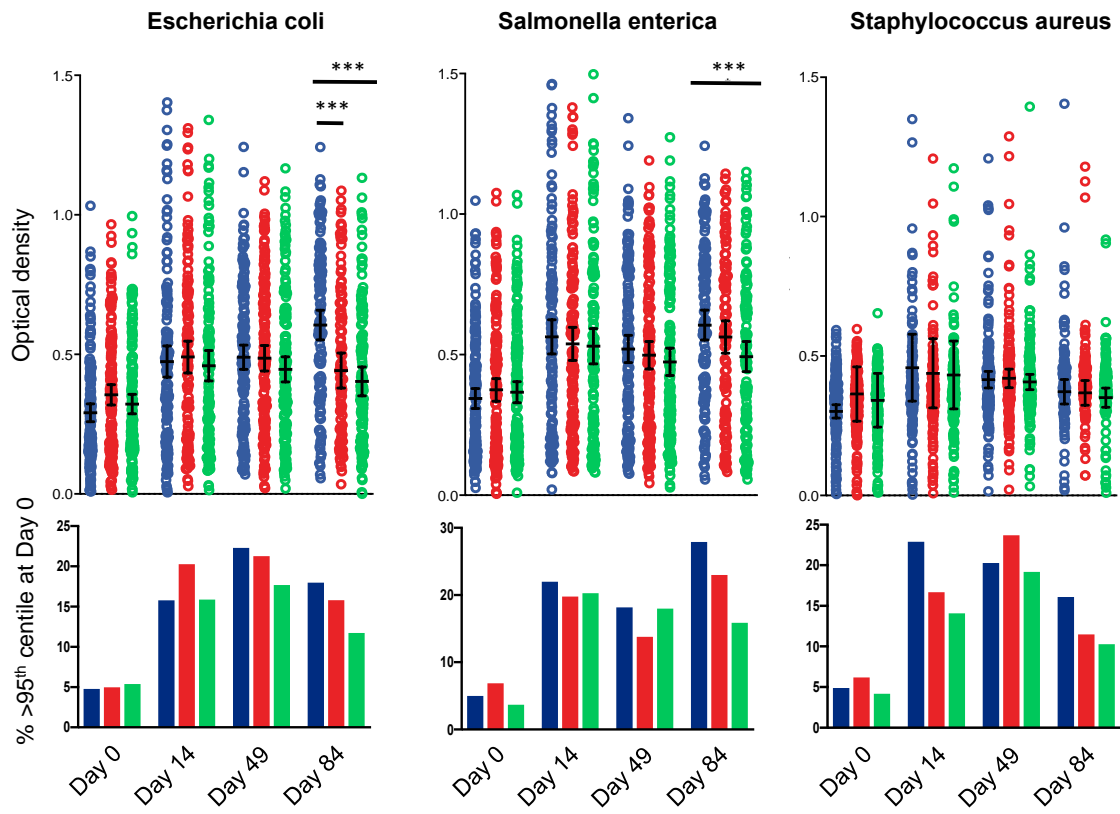


Fig 3B



Hepcidin-guided screen-and-treat interventions against iron deficiency anaemia in pregnancy: a randomised controlled trial in Gambian women

Authors

Amat Bah, Abdul Khalie Muhammad, Rita Wegmuller, Hans Verhoef, Morgan M. Goheen, Saikou Sanyang, Ebrima Danso, Ebrima A. Sise, Sant-Rayn Pasricha, Andrew E. Armitage, Hal Drakesmith, James H. Cross, Sophie E. Moore, Carla Cerami, Andrew M. Prentice

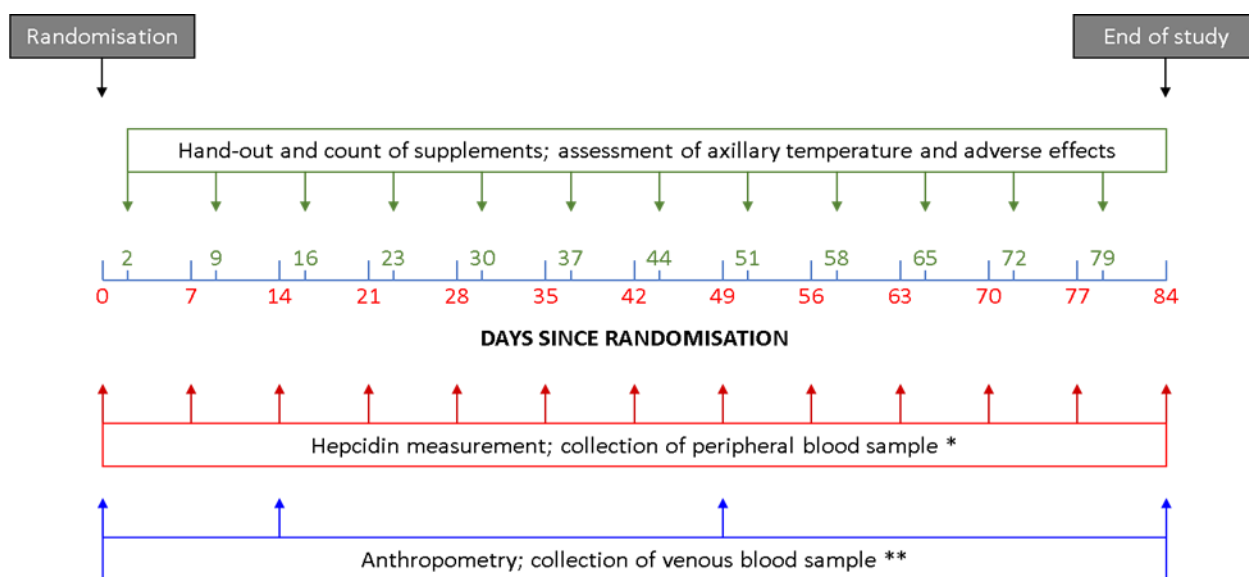
SUPPLEMENTARY MATERIAL

SUPPLEMENTARY METHODS

The full trial protocol has been previously published ¹.

Schematic representation of trial design

Supplementary Figure 1



* For determination of haemoglobin concentration (HemoCue) and *P. falciparum* antigenaemia by rapid dipstick test

** For haemogram by automated blood analyser and to determine concentrations of iron markers, inflammatory markers, malaria and bacterial growth in plasma or serum

Derivation of the hepcidin threshold to define 'ready-and-safe' to receive iron

The hepcidin cut-off value of $<2.5\mu\text{g/L}$ as a threshold to receive iron was based on the analysis of plasma from 395 pregnant women participating in the ENID study ² with samples available for 3 time points (14wks, 20wks and 30wks gestation). Based on a reference standard of ferritin concentration $<15\mu\text{g/L}$ and body iron content $<0\text{mg/kg}$, we constructed a receiver operating characteristic (ROC)-curve and calculated the area under the curve (AUC^{ROC}). The general suppression of hepcidin in pregnancy indicated that many women were ready to utilise iron. To make sure these women were not missed, we optimised for sensitivity over specificity, across the duration of pregnancy. At a hepcidin concentration of $<2.5\mu\text{g/L}$ with a high Youden

index, we had corresponding sensitivity and specificity values of 93.1% and 66.3% at 14wks, 86.1% and 52.2% at 20wks, and 86.2% and 84.9% at 30wks. The full method is described elsewhere ³.

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Additional information on informed consent procedures

The Regional Health Team, local health staff and individual communities were informed and approved the study. We trained all field workers who took part in the recruitment of participants on translating the informed consent documents. We also translated the information sheet to all the non-literate participants in a language they understand in the presence of an independent witness. The literate participants read the information sheet in their own time. Participants were encouraged to ask questions and seek clarification from the field workers and the PI. We recorded by a signature or thumbprint the informed consent of all the participants who agreed to take part in the study.

Investigational product

The composition of the three formulations of UNIMMAP (containing 0, 30 and 60mg iron) is listed below.

Supplementary Table 1

Composition of the experimental supplement based upon the UNIMMAP formulation

Micronutrients	Dose/day	Ingredients
Vitamin A ($\mu\text{g RE}$)	800	Dry vitamin A acetate 325
Thiamine (mg)	1.4	Thiamine mononitrate
Riboflavin (mg)	1.4	Riboflavin
Niacin (mg)	18	Niacinamide
Vitamin B6 (mg)	1.9	Pyridoxine hydrochloride
Folic acid (μg)	400	Folic acid food grade
Vitamin B12 (μg)	2.6	Vitamin B12 0.1%
Vitamin C (mg)	70	Ascorbic acid
Vitamin D (IU)	200	Dry vitamin D3
Vitamin E (mg)	10	Dry vitamin E 50%
Zinc (mg)	15	Zinc oxide
Iron (mg)	60 or 30 or 0 (placebo)	Ferrous fumarate
Iodine (μg)	150	Potassium iodide 10% on Potato Maltodextrin
Selenium (μg)	65	Sodium selenite anhydrous
Copper (mg)	2	Copper gluconate

Additional details on blinding to intervention

Participants and the research team, with exception of the data manager, were blinded to the group allocation and supplementation type throughout the fieldwork. The supplements were pre-packed on a weekly basis by the field coordinator in Keneba using lists automatically computer generated by the data office taking into account the hepcidin results of the participants. The list indicated a participant's identity number, a letter and number (code W1 to W6) of the supplement type to be received by the participant in the subsequent 7 days,

but the field coordinator did not know which code was allocated to which supplement or who belonged to which group.

Additional details on laboratory analyses

We measured hepcidin concentration in plasma from finger prick blood or from venous blood by competitive enzyme-linked immunosorbent assay (ELISA) (hepcidin-25 (human) EIA Kit, Bachem; now sold by Peninsula Laboratories International, San Carlos, USA) using a microplate photometer (Multiskan FC, Thermofisher Scientific, Waltham, MA, USA) with a detection range 0.049-25.0µg/L. Concentrations were interpolated from a 4-parameter curve fitted from a 2-fold, 10-point serial dilution made from a manufacturer-provided standard peptide. We quantified hepcidin as single measurements to allow results within 24 hours after blood collection and due to cost.

We prepared a haemogram from whole blood collected in EDTA tubes (Medonic M Series, Boule Diagnostics, Spånga, Sweden), and measured plasma concentrations of ferritin, iron, transferrin, soluble transferrin receptor (sTfR), C-reactive protein (CRP), and α_1 -acid glycoprotein (AGP) using an automated analyser (Cobas Integra 400 plus, Roche Diagnostic, Rotkreuz, Switzerland). TSAT and UIBC were calculated.

We determined sickle cell status by performing haemoglobin electrophoresis in a Hu15 Standard Horizontal Gel Unit (Scie-Plas Ltd, Cambridge, UK) with a Shandon Vokam 400 power pack (Astmoor Rancorn, Cheshire, UK) in blood samples collected at baseline.

Bacterial growth assays

Staphylococcus aureus (strain NCTC8325), *Staphylococcus epidermidis* (FDA strain PCI1200, ATCC12228), *Salmonella enterica serovar* Typhimurium (strain LT2, ATCC19585) and *Escherichia coli* (strain Crooks, ATCC8739) were grown overnight for 18 hours at 37 °C

in 5mL iron-free minimal growth media, Iscove's Modified Dulbecco's Medium (IMDM, Invitrogen). This was conducted in air with continuous shaking (250 rpm). All growth assays were run in triplicate in IMDM containing 50% heat-inactivated human serum. Bacterial growth was monitored by measuring the optical density at 620 nm (OD₆₂₀) hourly for 12 hours (*Staphylococcus aureus*, *Salmonella enterica serovar Typhimurium*, and *Escherichia coli*) and then at 20, 28, 36 hours (*Staphylococcus epidermidis*) using a Multiscan FC ELISA plate reader (Thermo Scientific).

Plasmodium growth assays

In vitro growth of the FCR3-FMG laboratory strain of *P falciparum* was assessed in fresh, washed RBCs as in⁴ for 96h (performed in triplicate for RBCs from each study participant). RBCs from healthy, non-pregnant, adult iron replete donors of normal haemoglobin genotype and G6PD status not undergoing iron supplementation served as controls. Growth rates represent final 96h parasitaemia divided by initial 0h parasitaemia, analysed by flow cytometry⁴.

1.5.1 Quantification of CD71-positive reticulocytes

CD71-positive reticulocytes in fresh RBCs were counted using PE-conjugated anti-human CD71 antibody (Clone M-A712, BD) and isotype control (Clone G155–178, BD), and analysed by flow cytometry for CD71-positive reticulocyte percentage relative to non-anaemic control as in⁵.

Additional details on statistical analysis

Because plasma ferritin concentrations can be increased by inflammation independent of iron status, we adjusted for inflammation (concentrations of C-reactive protein and α_1 -acid glycoprotein) measured in the same plasma samples, using approaches based on a Higher ferritin cutoff, and Excluding individuals. The higher ferritin cutoff means changing the cutoff

to 30µg/L among those with inflammation, whilst the excluding individuals involves stratifying women into groups with and without inflammation and use ferritin values only among those without inflammation.

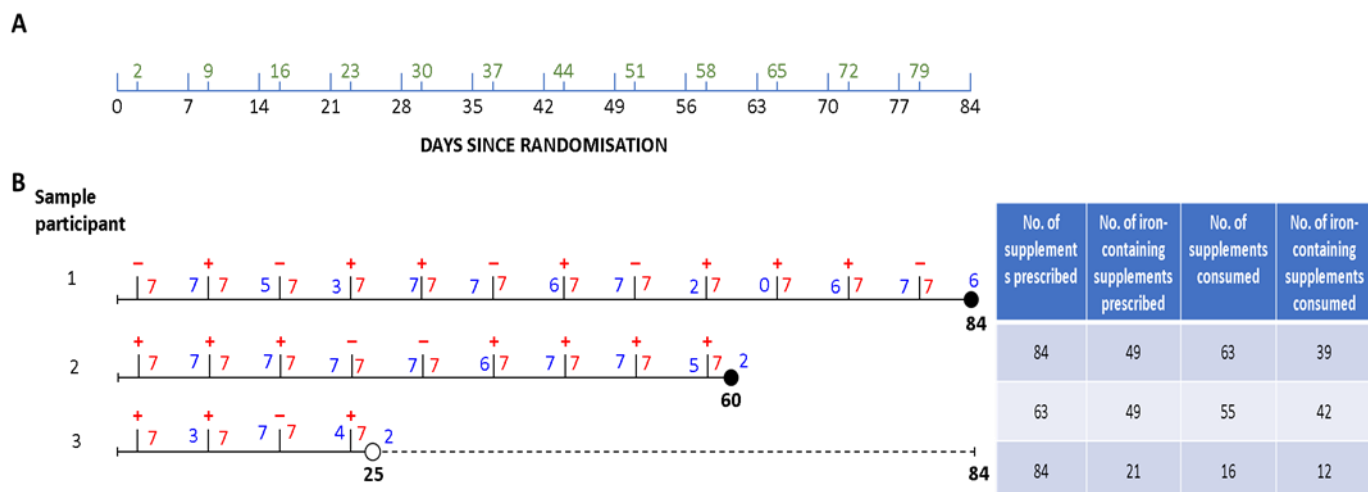
In the intention-to-treat analysis, missing values were replaced by multiple imputation using a Multiple Imputation Chained Equation with a burn-in of 100 and 100 imputations, including the following variables: gestational age, HemoCue haemoglobin concentration, hepcidin, red blood cells, mean corpuscular volume, red cell distribution width, haematocrit, mean platelet volume, white blood cells, Medonic haemoglobin, mean corpuscular haemoglobin concentration, lymphocytes, granulocytes, parity, gravida and age at start of study.

For individual women, we estimated adherence as the number of days that supplements were taken according to the capsule count (minus 2 days to account for the first two days after randomisation when supplementation was put on hold depending on the results of the first hepcidin concentration assessment) divided by the number of days between enrolment and leaving the study for reasons that were unrelated (or likely to be unrelated) to supplementation use (i.e., attaining the end of the 85-day intervention period, delivery, or emigration, whichever came first). Thus, as the denominator, we used the 85-day intervention period for women who refused, who were withdrawn for medical or unknown reasons, or who were withdrawn because of poor compliance. For groups, we calculated adherence by dividing the pooled number of days that supplements were taken as assessed by capsule count for all women by the pooled number of days until the end of the intervention period for all women. Details of calculation methods are shown in

Supplementary Figure 2.

Supplementary Figure 2

Methods used to describe supplement use in groups allocated to screen-and-treat supplementation with iron



C

$$\text{Group adherence} = \frac{\text{Sum of number of supplements consumed by all participants}}{\text{Sum of number of supplements prescribed for all participants}}$$

$$\text{No. of supplements prescribed} = 84 + 63 + 84 = 231$$

$$\text{Percentage of prescribed supplements containing iron} = \frac{(49) + (49) + (21)}{(84) + (63) + (25)} = 69.2\%$$

LEGEND: Panel A: schematic representation of the scheduled intervention period and visits made to hand out supplements and to assess adherence. Panel B: follow-up time and exposure to supplementation for three individual study participants in the screen-and-treat groups (hypothetical data). Each participant was provided with 7 supplements per week for the duration of the study, but whether these supplements contained iron or placebo depended on hepcidin concentrations in plasma or serum samples collected two days earlier. Weekly measurement of hepcidin concentrations started at Day 0; weekly provision of supplements started at Day 2. Results of the plasma/serum tests are shown by +/- signs, indicating hepcidin concentrations $<2.5\mu\text{g/L}$ or $\geq 2.5\mu\text{g/L}$, respectively. Values in red font indicate the number of iron-containing supplements provided each week; values of 0 indicate that

participants were provided with placebo. Values in blue font indicate the number of supplements consumed in a single week (calculated as the number prescribed and handed out minus the number returned unconsumed), regardless of whether they contained iron or placebo. Values in bold font indicate the number of days that each participant took part in the study. Circles indicating the end of follow-up show whether the participant left the study for reasons considered unrelated to intervention (i.e., completed the 84-day intervention period, delivered, or migrated; closed circles), or for reasons that could be related to the use of supplementation (i.e. refusal, withdrawn for medical reasons, because of poor adherence, or for unknown reasons; open circles). In the latter case, the period between leaving the study and the scheduled end of intervention (dashed line) contributed to the follow-up time that was used as the denominator of the formula to calculate adherence. For example, participant 1 completed the intervention period of 84 days. In the first week of supplementation, which started at Day 2, she was provided with supplements containing placebo (hence, 0 iron-containing supplements), which she all consumed. Participant 3 refused further cooperation at Day 25; to calculate adherence, however, a follow-up period of 84 days was maintained. Panel C: Calculation of results (data from panel B).

All missing values and outliers present after the locking of the data were maintained and analysis performed with them for per protocol analysis. Since the data is longitudinal and at baseline most of the observations were non-missing, hence it would be ideal to use these values for the predictions of subsequent observations. Firstly, we set the data to identify the missing values, the imputation number (which would be zero at this point) and the multiple imputation identity. We start by registering the variables that would be required to impute the missing values. These variables included gestational age, HemoCue haemoglobin concentration, hepcidin, red blood cells, mean corpuscular volume, red cell distribution width,

haematocrit, mean platelet volume, white blood cells, Medonic haemoglobin, mean corpuscular haemoglobin concentration, lymphocytes, granulocytes, parity, gravida and age at start of study. With these variables, we reshaped the data from long to wide, which simplified the data such that each individual now has only one row in the dataset. It also means that it is easy to use complete outcomes of some of the variables at some of the timepoints to predict the values of subsequent outcomes. We used a Multiple Imputation Chained Equation for the imputation with a burn-in of 100 and 100 imputations. This would also ensure that the Monte Carlo (MC) error is small enough to be unimportant⁶. The MC error was small enough to consider the number of imputations acceptable. The burn-in was adequate to show a convergence to a stationary state.

In the intention-to-treat analysis, missing values were replaced by multiple imputation. Intervention effects on continuous variables were measured as the difference in means, with logarithmic transformation as appropriate. We based the analysis of the primary end point (haemoglobin at Day 84) on the evaluation for non-inferiority with a per-protocol analysis. We also as per acceptable practice performed a modified intent-to-treat analysis (i.e., excluding participants who were lost to follow-up for being withdrawn before the first dose of supplement was received) on the randomised population and compared the groups using linear regression analysis, with intervention entered as a dummy-coded categorical variable and using the control arm (universal daily supplementation) as the reference group. To indicate non-inferiority, we used the lower limit of the 95% confidence interval for the difference in mean haemoglobin concentration between either of the screen-and-treat arms and the daily reference arm which should be above -5.0g/L (non-inferiority margin).

SUPPLEMENTARY RESULTS

Baseline characteristics of the subjects

See **Supplementary Table 2**. Groups were similar in baseline characteristics.

SUPPLEMENTARY TABLE 2: Baseline characteristics by intervention group

Characteristics	REFERENCE		S&T60		S&T30	
	n	Mean(SD)	n	Mean(SD)	n	Mean (SD)
Age, years	166	27.1 (6.0)	164	27.1 (5.7)	165	27.1 (5.8)
Number of pregnancies*	166	3.0 [2.0, 5.0]	164	3.0 [2.0, 5.0]	165	4.0 [2.0, 5.0]
Number of previous live births*	166	2.0 [1.0, 4.0]	164	2.0 [1.0, 4.0]	165	3.0 [1.0, 4.0]
Gestation age, weeks	166	18.4 (2.5)	164	18.6 (2.6)	165	18.5 (2.7)
Height, cm	166	163.1 (6.5)	164	161.7 (6.1)	165	162.4 (6.4)
Weight, kg	166	59.5 (11.4)	164	59.9 (11.2)	165	59.1 (11.3)
Body mass index, kg/m ²	166	22.3 (3.8)	164	22.8 (3.7)	165	22.4 (4.0)
Sickle cell genotype (AS) ‡	33/162	20.4	24/162	14.8	29/162	17.9
Haemoglobin concentration, g/L						
By Medonic analyser	166	106.8 (13.7)	164	108.5 (14.2)	165	107.6 (14.5)
By HemoCue field photometer	166	112.0 (12.4)	164	113.9 (12.9)	165	113.1 (12.8)
Anaemia (haemoglobin <110g/L) ‡						
By Medonic analyser	96/165	58.2	85/163	52.2	87/165	52.8

By HemoCue field photometer	66/166	39.8	57/164	34.8	61/165	37.0
Haematocrit, %	166	29.3 (3.9)	163	29.8 (4.1)	164	29.4 (3.9)
Mean corpuscular volume (MCV), fL	166	79.6 (7.2)	163	79.1 (6.4)	164	78.8 (7.2)
MCV <85 fL‡	129/166	77.71	136/163	83.44	140/164	85.37
Mean corpuscular haemoglobin (MCH), pg	166	29.1 (3.1)	163	29.0 (2.7)	164	28.9 (3.1)
MCH <27 pg‡	31/166	18.7	32/163	19.6	31/164	18.9
Mean corpuscular haemoglobin concentration, (MCHC) g/dL	166	365.1 (11.2)	163	364.9 (10.8)	164	366.2 (10.7)
Erythrocyte distribution width, %*	166	13.3 [12.7, 14.4]	163	13.2 [12.6, 14.5]	164	13.2 [12.6, 14.8]
Leukocyte count, ×10 ⁹ /L	166	7.5 (2.0)	163	7.5 (2.3)	164	7.2 (1.9)
Lymphocytes, ×10 ⁹ /L†	166	1.9 (0.3)	163	1.9 (0.3)	164	1.8 (0.3)
Lymphocytes, %	166	27.7 (7.0)	163	27.8 (7.5)	164	27.1 (6.2)
Granulocytes, ×10 ⁹ /L†	166	4.7 (0.4)	163	4.7 (0.4)	164	4.6 (0.3)
Granulocytes, %	166	65.6 (7.8)	163	65.4 (8.3)	164	66.2 (6.9)
Plasma marker concentrations						
Hepcidin, µg/L*	166	1.6 [0.4, 7.9]	164	2.5 [0.5, 8.4]	165	2.0 [0.5, 8.1]
Hepcidin <2.5 µg/L‡	93/166	56.0	83/164	50.6	92/165	55.8

Ferritin, µg/L*	161	21.2 [12.2, 40.3]	156	23.5 [11.5, 42.6]	152	22.4 [12.9, 42.6]
Iron deficiency (ferritin <15µg/L CRP <5 mg/L) OR (ferritin <30µg/L CRP >5 mg/L) ‡	64/166	38.6	65/164	39.6	61/165	37.0
Iron deficiency anaemia (Hb < 110 g/L ferritin < 15µg/L CRP <5mg/L) OR (Hb <110g/L ferritin < 30µg/L CRP >5mg/L & ferritin index > 2) ‡	42/165	25.5	46/162	28.4	36/163	22.1
Transferrin, g/L	162	3.3 (0.7)	160	3.3 (0.6)	160	3.3 (0.6)
Unsaturated iron binding capacity (UIBC), µmol/L	164	55.0 (18.3)	160	55.5 (15.7)	163	56.2 (16.5)
Iron, µmol/L	164	15.7 (8.6)	161	14.4 (7.0)	162	14.7 (7.0)
Iron <8.95 µmol/L‡	30/166	18.1	40/164	24.4	35/165	21.2
Transferrin saturation (TSAT) <16%‡	53/164	32.3	56/160	35.0	57/162	35.2
Soluble transferrin receptor, mg/L*	164	3.87 [2.85, 4.93]	160	3.99 [3.09, 5.10]	162	3.74 [2.93, 5.01]
Iron-deficient erythropoiesis (sTfR concentration > 4.4 mg/L) ‡	58/164	35.4	63/160	39.4	52/162	32.1
sTfR log10Ferritin ratio (ferritin index)	154	2.78 (0.60)	149	2.91 (0.65)	149	2.83 (0.57)
Ferritin index >2‡	121/166	72.9	113/164	68.9	120/165	72.7
C-reactive protein (CRP), mg/L	163	5.1 (6.2)	161	7.1 (17.7)	161	5.6 (12.0)

α_1 -acid glycoprotein (AGP), g/L	164	0.6 (0.2)	162	0.6 (0.3)	163	0.7 (0.3)
Inflammation‡						
CRP >5.0mg/L	52/163	31.9	45/161	28.0	45/161	28.0
AGP >1.0g/L	9/164	5.5	18/162	11.1	13/163	8.0
CRP >5.0mg/L OR AGP >1g/L	54/162	33.3	51/161	31.7	50/161	31.1
Current or recent <i>P falciparum</i> infection§	0/166	0.0	1/164	0.6	0/165	0.0

AGP: α_1 -acid glycoprotein; CRP: C-reactive protein; sTfR: soluble transferrin receptor. Values indicate mean (SD), * median [IQR], or † geometric mean (SD), ‡ proportion in percentage, § As indicated by the presence in whole blood of histidine-rich protein II (HRP-II) antigen of *P falciparum*,

Full listing of all primary and secondary continuous variables at Day 84

See **Supplementary Table 3**. The HemoCue results were consistently about 5.0g/L higher than the Medonic results, but the relative differences between treatment arms were similar, though slightly more pronounced by HemoCue. In the S&T60 group the lower confidence interval for the difference against REF was close to the non-inferiority margin at Day 84 (-2.7g/L [-5.0g/L, -0.5g/L]). In the S&T30 group the lower confidence limit for the difference against REF was below the non-inferiority margin (-3.5g/L [-5.7g/L, -1.4g/L]).

Red cell counts (erythrocytes) were similar across the groups and the lower haemoglobin in the S&T groups was accounted for by lower mean corpuscular volume and Mean corpuscular haemoglobin as is consistent with their greater iron deficiency. With the exception of plasma iron in the S&T60 group, all measures of iron status were worse in the S&T groups than REFERENCE. There were no differences in markers of inflammation.

Supplementary Table 3: Trial outcome measures at Day 84 of intervention (per-protocol analysis), continuous variables

Outcome	Intervention Group	n (%) of randomised	Estimate	SE[†]	Effect [95% CI]
Haemoglobin concentration, g/L					
By Medonic analyser	REFERENCE	139 (83.7)	110.1	0.8	
	S&T60	131 (79.9)	107.9	0.8	-2.2 [-4.6, 0.1]
	S&T30	145 (87.9)	107.4	0.8	-2.7 [-5.0, -0.5]
By HemoCue field photometer	REFERENCE	140 (84.3)	116.6	0.8	
	S&T60	132 (80.5)	113.8	0.8	-2.7 [-5.0, -0.5]
	S&T30	147 (89.1)	113.0	0.8	-3.5 [-5.7, -1.4]
Haematocrit, %	REFERENCE	139 (83.7)	30.3	0.2	
	S&T60	131 (79.9)	29.8	0.2	-0.5 [-1.19, 0.13]
	S&T30	145 (87.9)	29.6	0.2	-0.7 [-1.3, -0.1]
Erythrocyte distribution width, % *	REFERENCE	139 (83.7)	13.55	0.01	
	S&T60	131 (79.9)	13.70	0.01	1.01 [0.98, 1.04]
	S&T30	145 (87.9)	13.67	0.01	1.01 [0.98, 1.04]
Erythrocyte count, $\times 10^{12}/L$	REFERENCE	139 (83.7)	3.7	0.0	

	S&T60	131 (79.9)	3.7	0.0	-0.0 [-0.1, 0.1]
	S&T30	145 (87.9)	3.7	0.0	-0.0 [-0.1, 0.1]
Mean corpuscular volume, fL	REFERENCE	139 (83.7)	82.53	0.5	
	S&T60	131 (79.9)	81.45	0.5	-1.08 [-2.6, 0.4]
	S&T30	145 (87.9)	81.14	0.5	-1.39 [-2.8, 0.1]
Mean corpuscular haemoglobin, pg	REFERENCE	139 (83.7)	30.0	0.2	
	S&T60	131 (79.9)	29.6	0.2	-0.5 [-1.1, 0.1]
	S&T30	145 (87.9)	29.5	0.2	-0.5 [-1.1, 0.1]
Mean corpuscular haemoglobin concentration, g/L	REFERENCE	139 (83.7)	363.9	0.9	
	S&T60	131 (79.9)	362.9	0.9	-1.1 [-3.6, 1.4]
	S&T30	145 (87.9)	363.4	0.9	-0.6 [-2.1, 1.9]
Leukocyte count, $\times 10^9/L$	REFERENCE	139 (83.7)	8.1	0.2	
	S&T60	791 (31.9)	7.9	0.2	-0.2 [-0.6, 0.3]
	S&T30	145 (87.9)	7.7	0.2	-0.5 [-0.9, -0.0]
Lymphocytes, $\times 10^9/L$ †	REFERENCE	139 (83.7)	1.86	1.29	
	S&T60	131 (79.9)	1.88	1.30	1.01 [0.95, 1.07]
	S&T30	145 (87.9)	1.82	1.25	0.98 [0.93, 1.04]

Lymphocytes, %	REFERENCE	139 (83.7)	24.7	0.5	
	S&T60	131 (79.9)	25.4	0.5	0.7 [-0.7, 2.1]
	S&T30	145 (87.9)	25.5	0.5	0.7 [-0.6, 2.1]
Granulocytes, x10 ⁹ /L †	REFERENCE	139 (83.7)	5.38	1.34	
	S&T60	131 (79.9)	5.11	1.34	0.95 [0.89, 1.02]
	S&T30	145 (87.9)	5.00	1.37	0.93 [0.87, 1.00]
Plasma marker concentrations					
Hepcidin, µg/L *†	REFERENCE	140 (84.3)	6.26	3.44	
	S&T60	133 (81.1)	3.28	4.17	0.52 [0.37, 0.75]
	S&T30	147 (89.1)	2.32	4.74	0.37 [0.26, 0.52]
Ferritin, µg/L †	REFERENCE	139 (83.7)	34.56	1.87	
	S&T60	130 (79.3)	23.07	1.83	0.67 [0.58, 0.77]
	S&T30	145 (87.9)	21.42	1.74	0.62 [0.54, 0.71]
Transferrin, g/L	REFERENCE	139 (83.7)	3.1	0.1	
	S&T60	130 (79.3)	3.3	0.1	0.2 [0.0, 0.3]
	S&T30	146 (88.5)	3.4	0.1	0.3 [0.1, 0.4]
UIBC, µmol/L	REFERENCE	139 (83.7)	35.5	1.6	

	S&T60	13 (79.9)	42.6	1.6	7.1 [2.6, 11.6]
	S&T30	146 (88.5)	49.0	1.6	13.5 [9.1, 17.9]
Plasma iron, $\mu\text{mol/L}$	REFERENCE	140 (84.3)	32.7	1.3	
	S&T60	131 (79.9)	30.1	1.4	-2.7 [-6.3, 1.0]
	S&T30	146 (88.5)	25.2	1.3	-7.5 [-11.1, -3.9]
Soluble transferrin receptor, mg/L †	REFERENCE	140 (84.34)	3.25	1.39	
	S&T60	131 (79.88)	3.95	1.36	1.21 [1.13, 1.31]
	S&T30	146 (88.48)	4.03	1.36	1.24 [1.15, 1.33]
Ferritin index†	REFERENCE	139 (83.73)	2.15	1.53	
	S&T60	129 (78.66)	2.91	1.47	1.35 [1.23, 1.49]
	S&T30	145 (87.88)	3.08	1.48	1.43 [1.31, 1.58]
C-reactive protein, mg/L	REFERENCE	137 (82.5)	4.5	0.5	
	S&T60	129 (78.7)	4.2	0.5	-0.3 [-1.7, 1.0]
	S&T30	145 (87.9)	5.2	0.5	0.8 [-0.6, 2.1]
α 1-acid glycoprotein, g/L	REFERENCE	140 (84.3)	0.5	0.0	
	S&T60	131 (79.9)	0.4	0.0	-0.0 [-0.1, 0.0]
	S&T30	146 (88.5)	0.48	0.0	0.0 [-0.0, 0.1]

* Estimates obtained using Tobit regression on the natural-log transformed values, such that at corresponding transformed values, erythrocyte distribution width was left-censored at 11.5% and right-censored at 25%, and hepcidin concentration was left-censored at 0.049µg/L (limit of detection) and right-censored at 25µg/L with results exponentiated and presented in the table.

Values indicate mean (SE) or † geometric mean (GSD as geometric standard deviation). Exponentiation of log-transformed variables † yielded effect estimates that are expressed as relative differences between geometric means.

‡ SE = standard error obtained by the Delta method.

Supplementary Table 4: Trial outcome measures at Day 84 of intervention (per-protocol analysis), categorical variables

Outcome	Intervention Group	Prevalence, %	n/N	Effect [95% CI]
Anaemia (haemoglobin concentration <110g/L)				
By Medonic analyser	REFERENCE	45.3	63/139	Reference
	S&T60	57.3	75/131	11.93 [0.09, 23.77]
	S&T30	59.3	86/145	13.99 [2.48, 25.49]
By HemoCue field photometer	REFERENCE	26.4	37/140	Reference
	S&T60	28.0	37/132	1.60 [-8.98, 12.19]

	S&T30	32.0	47/147	5.54 [-4.95, 16.04]
Hepcidin concentration <2.5µg/L	REFERENCE	21.4	30/140	Reference
	S&T60	42.1	56/133	20.68 [9.88, 31.48]
	S&T30	52.4	77/147	30.95 [20.40, 41.51]
Mean corpuscular volume <85fL	REFERENCE	62.6	87/139	Reference
	S&T60	71.8	94/131	9.17 [-1.98, 20.31]
	S&T30	74.5	108/145	11.89 [1.17, 22.62]
Mean corpuscular haemoglobin <27pg	REFERENCE	7.2	10/139	Reference
	S&T60	10.7	14/131	3.49 [-3.32, 10.31]
	S&T30	12.4	18/145	5.22 [-1.65, 12.09]
Plasma ferritin concentration <15µg/L (not adjusted for inflammation)	REFERENCE	8.6	12/140	Reference
	S&T60	21.1	28/133	12.48 [4.14, 20.82]
	S&T30	21.8	32/147	13.20 [5.07, 21.32]
Iron deficiency (ferritin <15µg/L CRP <5mg/L) OR (ferritin <30µg/L CRP >5mg/L)	REFERENCE	19.3	27/140	Reference
	S&T60	30.5	40/131	11.2 [1.0, 21.5]
	S&T30	41.1	60/146	21.8 [11.5, 32.1]

Iron deficiency anaemia (Hb <110g/L ferritin < 15µg/L CRP <5mg/L) OR (Hb <110g/L ferritin < 30µg/L CRP >5mg/L & ferritin index >2)	REFERENCE	58.6	82/140	Reference
	S&T60	87.2	116/133	28.6 [18.7, 38.6]
	S&T30	89.1	131/147	30.5 [21.0, 40.1]
Plasma iron concentration <8.95µmol/L	REFERENCE	3.6	5/140	Reference
	S&T60	3.0	4/133	-0.56 [-4.79, 3.66]
	S&T30	6.8	10/147	3.23 [-1.87, 8.33]
Transferrin saturation (TSAT) <16%	REFERENCE	5.0	7/139	Reference
	S&T60	15.3	20/131	10.23 [3.08, 17.38]
	S&T30	19.9	29/146	14.83 [7.40, 22.25]
Iron-deficient erythropoiesis (sTfR concentration >4.4mg/L)	REFERENCE	14.3	20/140	Reference
	S&T60	38.2	50/131	23.88 [13.74, 34.02]
	S&T30	41.8	61/146	27.50 [17.62, 37.37]
Inflammation, CRP ≥5.0mg/L or AGP >1.0g/L	REFERENCE	33.6	46/137	Reference
	S&T60	23.3	30/129	-10.32 [-21.08, 0.43]
	S&T30	32.4	47/145	-1.16 [-12.14, 9.82]
Plasma ferritin <15µg/L and AGP <1.0g/L	REFERENCE	8.7	12/138	Reference

	S&T60	21.1	28/133	12.36 [3.98, 20.73]
	S&T30	22.1	32/145	13.37 [5.15, 21.60]
Plasma ferritin <15µg/L and CRP <5.0mg/L or AGP <1.0g/L	REFERENCE	10.6	10/94	Reference
	S&T60	21.4	22/103	10.72 [0.65, 20.80]
	S&T30	25.0	25/100	14.36 [3.83, 24.89]
Current or recent <i>P. falciparum</i> infection‡	REFERENCE	1.4	2/140	Reference
	S&T60	2.3	3/133	0.83 [-2.37, 4.03]
	S&T30	3.4	5/147	1.97 [-1.56, 5.50]

‡ As indicated by the presence in whole blood of histidine-rich protein II (HRP-II) antigen of *P. falciparum*

Supplementary table 5: Reported side effects, adverse and serious adverse events

Outcome	Intervention group	Observed counts per 1000 person-weeks	Cases/Person-weeks	Effect [95% CI]
Reported side effects				
Nausea	REFERENCE	11	21/1974	
	S&T60	14	27/1906	1.4 [0.64, 2.86]
	S&T30	7	14/2009	0.7 [0.29, 1.51]
Dizziness	REFERENCE	31	62/1974	
	S&T60	40	76/1906	1.3 [0.8, 2.0]
	S&T30	16	32/2009	0.5 [0.3, 0.9]
Constipation	REFERENCE	13	26/1974	
	S&T60	22	42/1906	1.7 [0.8, 3.4]
	S&T30	14	28/2009	1.1 [0.5, 2.3]
Black stool	REFERENCE	5	9/1974	
	S&T60	2	3/1906	0.3 [0.1, 1.4]
	S&T30	3	6/2009	0.7 [0.2, 2.0]
Stomach ache	REFERENCE	41	81/1974	
	S&T60	43	82/1906	1.0 [0.7, 1.6]
	S&T30	29	58/2009	0.7 [0.5, 1.1]
Fatigue	REFERENCE	11	21/1974	
	S&T60	16	31/1906	1.5 [0.7, 3.2]
	S&T30	8	16/2009	0.8 [0.3, 1.8]
Adverse events				
Cough, cold and chest pain	REFERENCE	11	20/1902	
	S&T60	10	19/1861	1.0 [0.5, 1.8]
	S&T30	9	18/1945	0.9 [0.5, 1.7]
Diarrhoea	REFERENCE	3	5/1902	
	S&T60	2	3/1861	0.6 [0.1, 2.6]
	S&T30	2	4/1945	0.8 [0.2, 2.9]
Fever	REFERENCE	3	5/1902	
	S&T60	2	4/1861	0.8 [0.2, 3.0]
	S&T30	5	9/1945	1.8 [0.6, 5.3]
General body pain #	REFERENCE	4	7/1902	
	S&T60	3	5/1861	0.7 [0.2, 2.5]
	S&T30	4	8/1945	1.1 [0.4, 3.4]
Headache #	REFERENCE	11	21/1902	
	S&T60	9	17/1861	0.8 [0.4, 1.6]
	S&T30	12	24/1945	1.1 [0.6, 2.1]
Heartburn #	REFERENCE	2	3/1902	

	S&T60	2	3/1861	1.0 [0.2, 5.7]
	S&T30	6	11/1945	3.6 [0.9, 14.8]
Lower abdominal pain #	REFERENCE	20	38/1904	
	S&T60	16	30/1861	0.8 [0.5, 1.3]
	S&T30	15	30/1945	0.8 [0.5, 1.3]
Toothache	REFERENCE	6	11/1902	
	S&T60	2	4/1861	0.4 [0.1, 1.2]
	S&T30	3	6/1945	0.5 [0.2, 1.4]
Urinary tract infection or dysuria	REFERENCE	9	17/1902	
	S&T60	10	18/1861	1.1 [0.6, 2.1]
	S&T30	9	17/1945	1.0 [0.5, 1.9]
Vomiting	REFERENCE	1	2/1902	
	S&T60	2	3/1861	1.5 [0.3, 9.2]
	S&T30	1	2/1945	1.0 [0.1, 6.9]
Gastritis #	REFERENCE	2	3/1902	
	S&T60	4	7/1861	2.4 [0.6, 9.8]
	S&T30	2	4/1945	1.3 [0.3, 6.2]
Nausea	REFERENCE	1	1/1902	
	S&T60	1	1/1861	1.0 [0.1, 16.4]
	S&T30	1	2/1945	2.0 [0.2, 21.6]
Others	REFERENCE	18	34/1902	
	S&T60	19	35/1861	1.1 [0.7, 1.7]
	S&T30	20	40/1945	1.2 [0.7, 1.8]
Serious adverse events^a				
Death	REFERENCE	0	NA	
	S&T60	0	NA	NA
	S&T30	0	NA	NA
Life-threatening	REFERENCE	0	NA	
	S&T60	0	NA	NA
	S&T30	0	NA	NA
Hospitalisation Required or Prolonged	REFERENCE	3	1/1904	
	S&T60	10	3/1861	1.5 [0.2, 12.3]
	S&T30	0	0/3255	0.0 [0.0]
Congenital anomaly/birth defect	REFERENCE	0	NA	
	S&T60	0	NA	NA
	S&T30	0	NA	NA
Miscarriage	REFERENCE	20	4/1904	
	S&T60	31	5/1861	1.5 [0.2, 12.3]
	S&T30	4	1/1945	0.2 [0.0, 2.5]

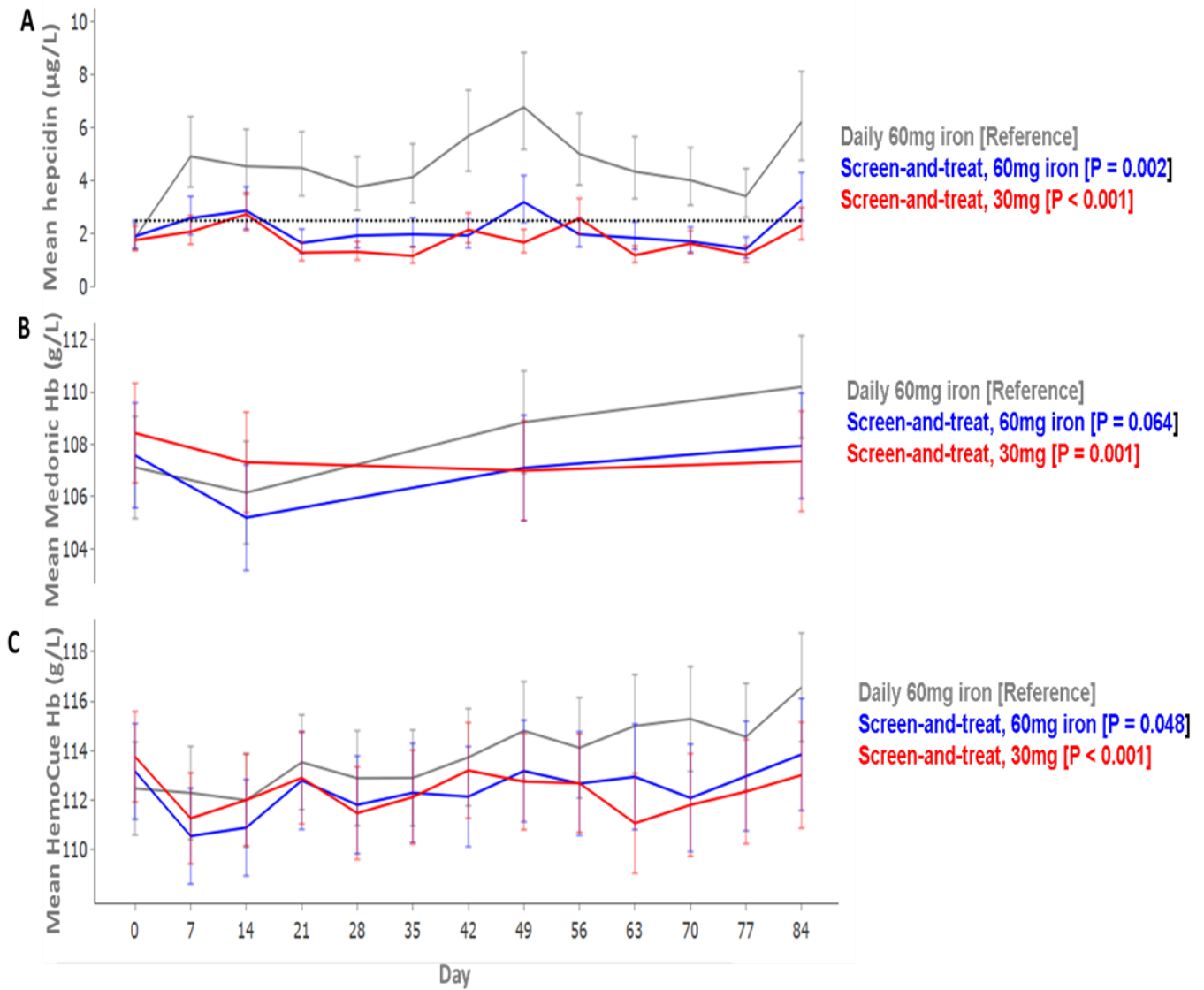
Stillbirth #	REFERENCE	13	4/1904	
	S&T60	20	6/1861	1.6 [0.4, 5.6]
	S&T30	15	5/1945	1.2 [0.3, 4.5]

used Negative Binomial regression, otherwise Poisson regression used

α Calculated using: observed counts per 10,000 person weeks

Supplementary Figure 3

Variation in plasma hepcidin through the course of the intervention and change over time of hepcidin and haemoglobin concentration, by intervention group (per protocol analysis)

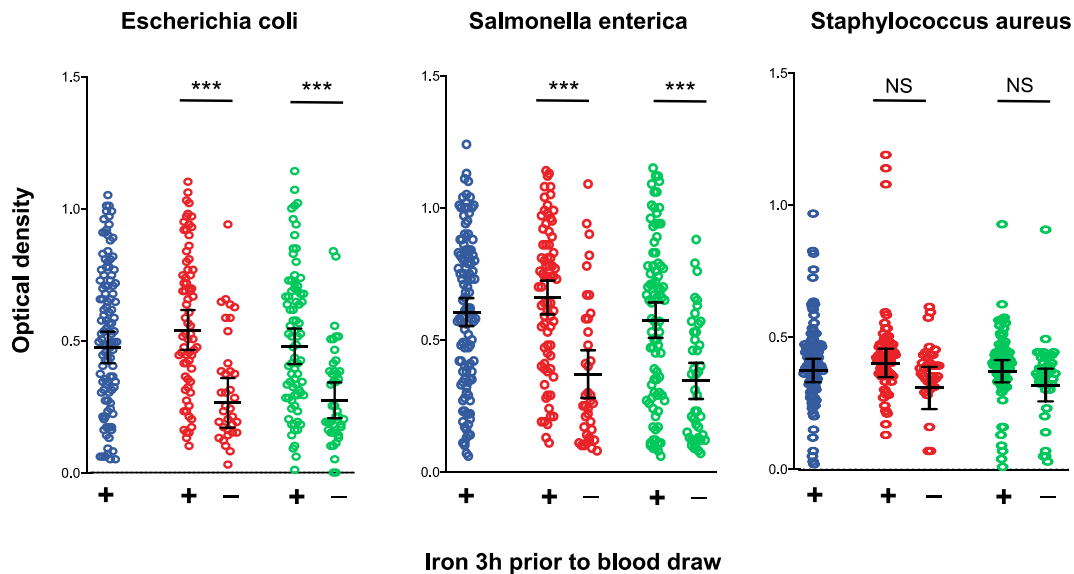


Legend:

Hepcidin concentration (**A**) was analysed by Tobit regression to account for right-censored values (see text). Mean values and 95%CI. P-values for time × intervention interaction effects: S&T60 versus REF, $P = 0.002$ and S&T30 versus REF, $P < 0.001$. Haemoglobin concentration measured by Medonic analyser (**B**): S&T60 versus REF, $P = 0.064$ and S&T30 versus REF, $P = 0.001$. Haemoglobin concentration measured by HemoCue photometer (**C**): S&T60 versus REF, $P = 0.048$ and S&T30 versus REF, $P < 0.001$.

Supplementary Figure 4

Sentinel bacterial growth rates at Day 84 according to whether pregnant women received iron or placebo 3h prior to blood draw



Legend:

Ex vivo bacterial growth rates in serum from Day 84. Blue = REF, red = S&T60, green = S&T30. On this final day of the study pregnant women received multiple micronutrients with iron (+) or without iron (-) according to their hepcidin level measured 7 days previously except in REF who all received multiple micronutrients with iron as per the protocol. *** = $P < 0.001$, NS = not significant. In the women receiving iron there was no difference in mean growth between the three intervention groups.

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1. Bah A, Wegmuller R, Cerami C, Kendall L, Pasricha SR, Moore SE, Prentice AM. A double blind randomised controlled trial comparing standard dose of iron supplementation for pregnant women with two screen-and-treat approaches using hepcidin as a biomarker for ready and safe to receive iron. *BMC Pregnancy Childbirth*. **16**; 157 (2016).
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Chapter 6: Summary discussion

6.1 Current approaches to combatting ID and anaemia in pregnancy

The World Health Organization (WHO) currently recommends daily supplementation with 30 - 60mg elemental iron (as ferrous salt) throughout pregnancy, starting as early in pregnancy as possible, with a preferred daily dose of 60mg of elemental iron in settings where anaemia in pregnant women is a severe public health problem (prevalence of 40% or higher) [132].

6.2 Evidence for efficacy of current approaches

Universal antenatal iron supplementation provides maternal and neonatal health benefits in iron-deficient pregnant women, with the balance between benefits and risks probably being more favourable in low-income countries than in high-income countries. The maternal health benefits include: reduction in the risk of anaemia at term, reduction in risk of pre-term delivery and reduced risk of transfusion being required by the mother at term, and a reduction of the risk of maternal haemorrhage thus increasing the chances of survival [153]. The neonatal health benefits would include: increased birthweight by as much as 150g [154]), decreased risk of low birthweight by 19%, increased gestational duration leading to decreased risk of prematurity, increased neonatal length, and improved neonatal iron stores at one month postpartum [153]. There is evidence to suggest that supplementation with iron and folic acid for pregnant women in low and middle-income countries should be expanded with other micronutrients as multiple micronutrient deficiencies are common among women of reproductive age and can impair the utilisation of iron [149].

6.3 Limitations of current approaches

However, there are uncertainties about the appropriate dosage regimen, particularly the optimal intervals and doses for iron supplementation. As compliance or adherence is known to be low in least developed countries [142, 155], reducing the intake of supplemental iron can avoid self-limiting and dose-dependent adverse effects in the gastrointestinal tract (constipation, nausea, vomiting, and epigastric discomfort) that limit adherence [156]. There are also concerns, however, that iron supplementation can predispose to maternal *Plasmodium* infection [138], bacteraemia [157], haemoconcentration (haemoglobin concentration exceeding 130g/L) [7], gestational diabetes [158, 159] and preeclampsia [134, 160].

6.4 Alternative approaches for iron supplementation

There is now some evidence that smaller doses of 30mg iron daily could achieve similar results as the daily 60mg iron [49]. A Cochrane review on the treatments of iron deficiency anaemia in pregnancy [147] indicated that daily low dose iron supplementation may be effective at treating anaemia in pregnancy with fewer gastrointestinal side effects compared with higher doses. Weekly Iron-Folic Acid Supplementation (WIFS) is an approach that can be effective for ensuring adequate iron status of women, particularly before pregnancy and during the first trimester in communities where food-based strategies are not yet fully implemented or effective [161]. Although efficacy in reducing anaemia is low it may be a feasible alternative to daily iron supplementation among those pregnant women who are not anaemic and have adequate antenatal care [162].

Iron deficiency is known to also occur as a result of the intake of diets that are monotonous, low in animal food sources, and primarily based on unrefined cereals, grains and legume seeds. Although these foods have reasonable iron content, they also contain high concentrations of phytate that hinder the absorption and utilisation of the iron [153].

Therefore, food fortification has been shown to reduce anaemia among women and children [163] and in countries with flour fortification, each year of flour fortification is associated with a 2.4% decrease in anaemia prevalence among non-pregnant women [164].

6.5 Could a screen-and-treat approach be better than a universal daily iron supplementation?

We hypothesised that a hepcidin-guided screen-and-treat approach to iron supplementation would be non-inferior to the WHO-recommended universal daily supplementation and evaluated this in a RCT with haemoglobin concentration at the end of the intervention as the primary endpoint.

6.6 Developing the diagnostic threshold

In the first study, we set out to define a hepcidin cut off value based on the analysis of plasma from 395 pregnant women participating in the ENID study [165] with samples available for 3 time points (14 weeks, 20 weeks and 30 weeks gestation). We established $<2.5\mu\text{g/L}$ as a threshold for 'ready and safe' to receive iron [166]. We noted in this study that hepcidin is a good indicator for detecting iron deficiency in pregnancy and believed that the diagnostic cut off was valid and worked well as the sensitivity for detection was greater than 80%. Although hepcidin does decrease from the second and third trimester and correlates well with iron deficiency in pregnancy, it can become undetectable by the third trimester [167] and it is known that undetectable serum hepcidin does enable dietary iron transfer from mother to foetus [123]. However, we observed in our study that a reduction in iron stores (ferritin, sTfR and total body iron) that occurred between 20 and 30 weeks of pregnancy was not supported by a further reduction in hepcidin concentration. This may suggest that other factors other than iron deficiency alone may have played a role in the smaller reduction of hepcidin concentration.

6.7 Testing the screen-and-treat approach

In the second study, we identified 527 pregnant women with gestational age 14–22 weeks and enrolled/randomised 498 participants to the 3 arms from June 2014 to March 2016 in rural Gambia. The prevalence of anaemia among pregnant women in this setting is high (over 50%) and this is

consistent with national prevalence of 67.9% [168]. The screen-and-treat threshold resulted in a 50% reduction in iron administration and, in this respect, was highly successful in setting up a meaningful trial. In the per protocol analysis, the mean haemoglobin concentration difference was assessed at Day 84 and the lower confidence interval used to determine non-inferiority. The data from the trial indicated that hepcidin-guided screen-and-treat approaches with either 60mg or 30mg iron as ferrous fumarate using mean haemoglobin difference remained marginally within the non-inferiority margin of -5.0g/L, but performed worse than the daily supplementation (screen-and-treat 60 (-4.6g/L) and for screen-and-treat 30 (-5.0g/L)). The results of the intention to treat analysis were similar. Whilst the prevalence of anaemia (haemoglobin <110g/L) was reduced from 58.2% to 45.3% in the Reference group, the prevalence increased in both the screen-and-treat 60 and screen-and-treat 30 groups (52.2% to 57.3%) and (52.8% to 59.3%), respectively.

Additionally, improvement in haemoglobin was low as the effectiveness of the supplementation yielded a mere 3.3g/L, and a 13% reduction in anaemia prevalence in the Reference group. The modest intervention effect on haemoglobin has been shown by others previously [7, 153].

The most significant contributor to the onset of anaemia worldwide is iron deficiency [1] and WHO estimates that iron deficiency anaemia affects almost half of the world's pregnant women and pre-school children with a prevalence of over 65% in Africa and Asia, and that it causes (directly or indirectly) one fourth of all maternal deaths [2]. Our results clearly show that mean measures of iron deficiency (hepcidin, ferritin) were significantly worse in the screen-and-treat groups than the Reference group. Other iron markers such as plasma iron, transferrin, soluble transferrin receptor also show similar results while unsaturated iron binding capacity were higher. The corresponding prevalence of iron deficiency determined by ferritin, transferrin saturation, soluble transferrin receptor, increased in the screen-and-treat groups. Prevalence of iron deficiency anaemia was also higher in the screen-and-treat groups.

Although the hepcidin-guided approach was able to halve the iron exposure in the two screen-and-treat groups, where screen-and-treat 60 group received 45.2% weeks of iron in the intervention period and screen-and-treat 30 received 52.6%, our data show that for hepcidin as a marker of 'ready to receive' iron, prevalence was higher in the screen-and-treat 60 group (41.7%), screen-and-treat group 30 (52.4%), than the Reference group (21.4%). Apart from our results showing that the approaches were able to half the amount of iron received by pregnant women, there were no advantages for the screen and treat approaches. The approaches may not have worked as anaemia has many causes which are not amenable to iron therapy. In Africa, only 44% of anaemia cases are attributed to iron deficiency and are amenable to iron supplementation. A higher threshold (above 2.5µg/L) may have increased the efficacy but this would have increased the amount of iron given to the pregnant women in the screen-and-treat groups, and reducing the overall iron given to those who did not need them was one of our objectives. On the other hand, a study among non-pregnant Indian women has shown a cut off $\leq 4.5\mu\text{g/L}$ was associated with higher diagnostic likelihood for IDA [169]. Although there were no differences between the groups for inflammation (CRP and AGP), and for illnesses, our threshold could have failed to fully capture the effect of low grade inflammation as seen by our group in Gambian children [170].

Adherence in our study was high, exceeding 80% in all three groups as seen elsewhere [171]. This was however achieved as the women were aware that their intake of the capsules were being monitored. However, other studies have shown that adherence could be low in areas where the rate of iron-folic acid supplementation during pregnancy is relatively low, there is poor counselling on the use of iron and folic acid intake, low promotion of its benefits, inadequate encouragement for early antenatal care attendance and inadequate general health promotion on anaemia prevention [172].

Much has been made of the negative contribution of iron and folic acid supplementation on the occurrence of illnesses and side effects during pregnancy. However, contrary to the belief that women stop taking iron tablets mainly due to negative side effects, only about one-third of women

reported that they experienced negative side effects in this study. The major barrier to effective supplementation programmes is inadequate supply, counselling and distribution of iron tablets, difficult access and poor utilization of prenatal health care services, beliefs against consuming medications during pregnancy, and in most countries, fears that taking too much iron may cause too much blood or a big baby, making delivery more difficult [3]. Our data show that reported illnesses and side effects were similar between the Reference daily supplemented group and the screen-and-treat 60 and slightly lower in the screen-and-treat 30 group. The reduction in the episodes of side effects reported in the screen-and-treat 30 group may have been as a result of them receiving reduced doses of iron.

The frequency of adverse events (respiratory infections, diarrhoea, fever, general body pain, urinary tract infection, vomiting, nausea, headache, toothache, heart burn) and serious adverse events (death, life threatening, prolong hospitalisation, congenital anomaly/birth defect, miscarriage, stillbirth) were similar between the groups. There were no reported deaths or life-threatening situations.

To further assess safety of the iron administration, *ex vivo* growth of three bacteria (*Escherichia coli*, *Salmonella enterica* and *Staphylococcus aureus*) were done. Growth was seen to be faster after the commencement of the study but there were no differences between the treatment groups on Days 14 and 49. However, on Day 84 when supplements were given 3 hours before blood draw, the Reference group supported a faster growth than the screen-and-treat 30 group for *E. coli* and *S. aureus*, and for the screen-and-treat 60 only for *E. coli*. We found no difference in bacterial growth across intervention groups among women who received iron 3 hours prior to the blood draw, and significantly lower growth in those in the screen-and-treat groups who did not receive iron.

Further more, we asses growth of malaria parasites in fresh RBCs and found growth to be suppressed at baseline (compared to the non-anaemic controls used in the assay). Growth was then greatly stimulated at Day 14 and gradually declined to Day 84. Although *Ex vivo* malarial

parasite growth in erythrocytes were increased by iron administration, growth did not differ by treatment group.

6.8 Potential explanation for why screen-and-treat did not work

Anaemia can be caused by other factors (including inflammation) besides iron deficiency.

Inflammation has been shown to upregulate hepcidin which can block iron absorption leading to a poor response to iron treatment [121]. On the other hand, iron interventions have been shown to prevent 20 – 50% of the prevalence of anaemia in pregnant women [173, 174]. Some of the other potential explanations are contained in the limitations of the study below.

Although the two screen-and-treat approaches were found to be non-inferior to the daily iron supplementation recommended by WHO, the daily iron supplementation performed better with regards to anaemia and other iron markers including hepcidin, ferritin, transferrin saturation and soluble transferrin receptor. We were therefore unable to demonstrate support for screen-and-treat approach to iron supplementation based on hepcidin concentration $<2.5\mu\text{g/L}$ in pregnant Gambian women.

6.9 Study limitations

One of the limitations of the main study is the quantification of hepcidin as a single measure to allow for the availability of the hepcidin results within 24 hours after blood is collected. When hepcidin results were not available for a particular week, results of the previous week were used. This was done due to cost and the need for the availability of the results to enable allocation of treatment.

The availability of an affordable and reliable hepcidin-based PoC test that was rapid enough to inform our iron treatment before an individual's iron status significantly alters [175] would have enabled us screen more frequently.

In the determination of hepcidin cut off $<2.5\mu\text{g/L}$, we prioritised sensitivity over specificity as a trade-off in order not to miss pregnant women who needed iron and were ready to utilise it. Ideally, we could have prioritised both.

Gestational age at enrolment was assessed by means of reported first date of last menstrual period and by fundal height estimation conducted by experience midwives. The use of the above methods may not be as accurate as say estimation using ultra sound. This has sometimes led to the under-estimation of gestational age and this may have resulted to the delivery by women before they finished the study. Although this did not affect the results, the highest contributor to the dropout rate was women delivering during the study period as seen in the CONSORT diagram.

We could have included a fourth arm of 30mg iron daily to assess the outcome of reduced iron use (30mg iron daily) compared to 60mg daily. The 30mg daily may have been equally efficacious compared to the 60mg daily in this setting. Reveiz et al [147] concluded that daily low-dose iron supplements may be effective at treating anaemia in pregnancy with fewer gastrointestinal side effects compared with higher doses.

6.10 Public health implications and policy recommendations

Our study (Paper II-chapter 4) demonstrated that hepcidin performs well as a diagnostic test for iron deficiency in pregnant Gambian women and has enabled us to propose putative cut offs for when the pregnant woman is ready and it is safe for her to receive iron. Kanuri et al [169] found hepcidin to be a valuable diagnostic tool for IDA among Indian women. These results should however be validated with a large-scale trial to increase the degree of certainty as well as looking at the cost implication in order to support the use of hepcidin as an assessment indicator for iron deficiency that will complement the use of ferritin as a conventional marker.

Our main trial (Paper III-chapter 5) has shown that it is possible to replace iron and folic acid with a multiple micronutrient supplement containing 15 vitamins and minerals into the routine national supplementation services in The Gambia.

Women receiving daily 60mg iron are less likely to be anaemic and iron deficient and therefore the WHO recommendation of daily iron folic acid supplementation of pregnant women where anaemia is a public health problem should be maintained.

6.11 Future research needs

On the basis of our findings, coupled with available evidence that current approaches and interventions to combat iron deficiency and anaemia in pregnancy still have somewhat limited efficacy, future research may look into:

- i. Testing the efficacy of 30mg iron daily (lower dose with increased bioavailability) against 60mg daily in developing countries as demonstrated by Milman et al, 2014 (for 25mg iron) among pregnant women in advanced country setting [176]. This may be beneficial to pregnant women who are iron replete and may not need or will not benefit from consuming 60mg elemental iron daily. As shown from our data that the risk of self-reported illnesses and side effects was lower in the reduced dose of 30mg (screen-and-treat) than the reference daily 60mg supplementation, goes to show that a reduced dose may be beneficial for pregnant women with replete iron status.
- ii. The suggestions that the optimal pregnancy outcomes in terms of birth weight and pre-term labour occur at a mid-pregnancy haemoglobin of between 95 and 105g/l [85], which is actually lower than the current WHO definition of anaemia (haemoglobin cut off of <110g/L). Per our data, the prevalence of anaemia was 58.2% for the Reference arm at baseline (haemoglobin cut off <110g/L). We noted that, this could have been 41.0% (a difference of 17.2%) if the cut off was to be haemoglobin <105g/L from the second trimester as suggested by the CDC [177, 178]. It would be important to investigate as to how much of this lower prevalence is actually due to ID and therefore iron supplementation of this may be more efficacious.
- iii. Even though we have not seen significant differences in inflammation markers (CRP and AGP) and reported illnesses, we may have failed to capture low-grade inflammation as seen

in children [20]. Therefore, it may be beneficial for future research to investigate the role of infections and inflammation on hepcidin variation in pregnancy.

- iv. The development and validation of a low cost hepcidin-based PoC test kit for iron deficiency in pregnancy with reference standards using ferritin, sTfR and TSAT. This is important as the physiological adaptation to iron needs and the lower loss of iron due to the cessation of menses during pregnancy, may potentially enhance the vulnerability to high iron intakes in iron-replete individuals [179], and therefore the need to accurately assess ID. Serum hepcidin as a key regulator of iron homeostasis, is an important biomarker because its levels determines how well oral iron is absorbed, with low hepcidin levels indicating both a requirement for iron and the body's ability to utilise it [119, 180]. Serum ferritin is one of the few biochemical indices of which low levels reflect depleted iron stores [94, 95] but it is known to be raised by infection and inflammation as it is an acute phase protein and thus has very high false negative rates in least developed countries [96]. Similar problems also arise with the other commonly used iron status indicators as summarised in **Table 2** above.

6.12 How well was the overall aim of the study met?

The overall aim was to find a better and safer way to administer iron supplementation to pregnant women through screen-and-treat approaches among rural Gambian women. We established a hepcidin threshold to guide the screen-and-treat approaches. However, the hepcidin-guided screen-and-treat approaches were not as efficacious in combatting anaemia, ID and IDA as the 60mg WHO recommended daily iron supplementation for where anaemia in pregnant women is a severe public health problem, and the approaches had no added advantage than universal daily iron supplementation in terms of adherence, side effects or safety outcomes. We therefore suggest that the current WHO policy for iron supplementation for pregnant women be continued in this setting. Therefore, the objective of setting up a study for the PhD to test the efficacy of a screen-and-treat approach to combat ID and anaemia was successfully achieved.

7 Appendices

Appendix 1: Ethics approval, Gambia

The Gambia Government/MRC Joint

ETHICS COMMITTEE

C/o MRC Unit: The Gambia, Fajara
P.O. Box 273, Banjul
The Gambia, West Africa
Fax: +220 – 4495919 or 4496513
Tel: +220 – 4495442-6 Ext. 2308
Email: ethics@mrc.gm

26 December 2013

Mr Amat Bah
MRC International Nutrition Group
MRC Unit, The Gambia
Keneba Field Station

Dear Mr Bah

SCC 1357v3, A double blind randomised controlled trial comparing standard dose of iron supplementation for pregnant women with two screen-and-treat approaches using hepcidin as a bio marker for ready and safe to receive iron

Thank you for submitting your proposal dated 12 December 2013 for consideration by the Gambia Government/MRC Joint Ethics Committee at its meeting held on 19 December 2013.

We are pleased to approve your project proposal.

With best wishes

Yours sincerely


Mr Malamin Sonko
Chairman, Gambia Government/MRC Joint Ethics Committee

Additional documents submitted for review:-

- Informed Consent Document, version 1.0 – 18 November 2013
- Protocol, version 1.0 – 12 December 2013
- CV-Amat Bah

The Gambia Government/MRC Joint Ethics Committee:

Mr Malamin Sonko, Chairman
Professor Ousman Nyan, Scientific Advisor
Ms Naffie Jobe, Secretary
Mrs Tulai Jawara-Ceesay
Dr Ahmadou Lamin Samateh
Dr Roddie Cole

Professor Tumani Corrah
Dr Stephen Howie
Dr Kalifa Bojang
Dr Ramatoulie Njie
Dr Adama Demba
Dr Siga Fatima Jagne

Scientific Coordinating Committee

**MRC Unit: The Gambia, Fajara
PO Box 273 Banjul, The Gambia
West Africa**

Switchboard (+220) 4495442/6 Ext 2308

Fax (+220) 4495919/4496513

E-mail: scc@mrc.gm

Intranet: <http://mrcportal/Committees/SCC/SitePages/Home.aspx>

Webpage: <https://mrcportal.mrc.gm/Committees/SCC/SitePages/Home.aspx>



12 December 2013

Mr Amat Bah
MRC International Nutrition Group
Keneba Field Station

Dear Mr Bah

SCC 1357v3, A double blind randomised controlled trial comparing standard dose of iron supplementation for pregnant women with two screen-and-treat approaches using hepcidin as a bio marker for ready and safe to receive iron

Thank you for submitting your revised proposal dated 12 December 2013 addressing the issues raised by the SCC at its meeting held on 5 December 2013.

I am pleased to approve your project proposal which will be forwarded to the Ethics Committee for consideration at its meeting on 19 December 2013.

With best wishes

Yours sincerely



Professor Umberto D'Alessandro
Chairman, Scientific Coordinating Committee

Additional documents submitted for review:-

- Informed Consent Document, version 1.0 – 18 November 2013
- Protocol, version 1.0 – 12 December 2013
- CV-Amat Bah

Appendix 2: Ethics approval from The LSHTM

London School of Hygiene & Tropical Medicine

Keppel Street, London WC1E 7HT
United Kingdom
Switchboard: +44 (0)20 7636 8636

www.lshtm.ac.uk

LONDON
SCHOOL of
HYGIENE
& TROPICAL
MEDICINE



Observational / Interventions Research Ethics Committee

Mr Amat Bah
Research Degree Student
EPH
LSHTM

2 June 2014

Dear Mr. Bah,

Submission Title: A double blind randomised controlled trial comparing standard dose of iron supplementation for pregnant women with two screen-and-treat approaches using hepcidin as a bio marker for ready and safe to receive iron

LSHTM Ethics Ref: 7168

Thank you for your letter of 22 May 2014, responding to the Interventions Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Conditions of the favourable opinion

Approval is dependent on local ethical approval having been received, where relevant.

Approved documents

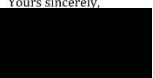
The final list of documents reviewed and approved by the Committee is as follows:

Document Type	File Name	Date	Version
Protocol / Proposal	SCC 1357_Bah_Protocol_12Dec13, clean.doc	22/01/2014	1.0
Information Sheet	SCC 1357v2_Bah_ICD (adult)_12Dec13, clean.doc	22/01/2014	1.0

After ethical review

Any subsequent changes to the application must be submitted to the Committee via an Amendment form on the ethics online applications website. The Principal Investigator is reminded that all studies are also required to notify the ethics committee of any serious adverse events which occur during the project via an Adverse Event form on the ethics online applications website. An annual report form is required on the anniversary of the approval of the study and should be submitted during the lifetime of the study on the ethics online applications website. At the end of the study, please notify the committee via an End of Study form on the ethics online applications website. Ethics online applications website link: <http://leo.lshtm.ac.uk>

Yours sincerely,


Professor John DH Porter
Chair

ethics@lshtm.ac.uk
<http://www.lshtm.ac.uk/ethics/>

Improving health worldwide

Appendix 3: Participant information sheet and consent form

PARTICIPANT INFORMATION SHEET



Version 2.0

Date 14.11.2014

Study Title : **A double blind randomised controlled trial comparing standard dose of iron supplementation for pregnant women with two screen-and-treat approaches using hepcidin as a biomarker for ready and safe to receive iron.**

SCC:	1357	Protocol:	
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Sponsor: MRC-ING

What is informed consent?

You are invited to take part in a research study. Participating in a research study is not the same as getting regular medical care. The purpose of regular medical care is to improve one's health. The purpose of a research study is to gather information that may be useful in future for the whole population. It is your choice to take part and you can stop any time.

Before you decide you need to understand all information about this study and what it will involve. Please take time to read the following information or get the information explained to you in your language. Listen carefully and feel free to ask if there is anything that you do not understand. Ask for it to be explained until you are satisfied. You may also wish to consult your spouse, family members or others before deciding to take part in the study.

If you decide to join the study, you will need to sign or thumbprint a consent form saying you agree to be in the study. You will receive a copy of this.

Why is this study being done?

Anaemia in pregnancy in The Gambia is a major public health problem with over 70% of pregnant women affected. WHO recommends iron supplementation for all pregnant women using 60 mg iron and 400 ug folic acid daily (red tablets given at antenatal clinics) to prevent and manage anaemia. This is known to reduce anaemia, however, recent studies have indicated that giving iron to those who do not need it can pose risks to pregnant women. The aim of this study is to evaluate a screen-and-treat approach (who needs or does not need iron and at what time) using hepcidin (a body hormone) to assess this. We believe that proper assessment and the giving of iron at a lower dose will improve safety and tolerability.

The results of the study will be made available to your community.

What is the new vaccine/drug?

What we are using for this study is not a new drug but a nutritional supplement that has been recommended and is being used for the prevention of anaemia.

What does this study involve?

Once you are enrolled in this study you will be registered. You will be asked to provide a 7 ml venous blood (Day 0 below). You will then be assigned by chance to one of 3 study groups to either receive: a) a multiple micronutrient supplement with 60 mg iron daily or b) multiple micronutrient supplement with 60 mg iron when hepcidin analysis indicate it is safe to be given iron or c) multiple micronutrient supplement with 30 mg iron when hepcidin analysis indicate it is safe to be given iron. Please note that, your participation in this study will in no way affect your attendance of Government antenatal services.

As a participant of this study, field workers will be inviting you every week to screen you using a finger prick blood sample and to provide you with a 7 day supply of your supplements. You will also be provided with a long lasting insecticide-treated bed net.

In order to facilitate analysis in this study you will be asked to provide 7 ml venous blood at 4 different times (Day 0, 14, 49 and day 84) within the 12 weeks period of the study.

You will be tested for malaria every week (using the finger prick blood sample as described above) and, if at any day you are found to have malaria, you will be asked for a further 2 ml of blood. This will allow us to conduct some further tests in relation to iron and malaria.

In case the investigator discovers you are sick and decides that you cannot participate in the study because of that, you will receive immediate care at the study site and then be referred to the appropriate health facility.

If the research study needs to be stopped, you will be informed and you will have your normal medical care.

What will happen to the samples taken in this study?

The blood samples collected will be analysed in Keneba to get answers but part of it will be stored for further analysis. Part of the stored blood will also be used for infection and genetic analysis. Some of the blood samples will be transferred to a laboratory overseas for analysis because we don't have the equipment required for measuring all of the factors we are investigating in The Gambia.

What harm or discomfort can you expect in the study?

There will be minimum discomfort during the collection of finger prick and venous blood samples. The risk of iron over dosage is minimised as the reference arm of this study is a standard government practice and the intervention is an overall lower dosage.

What benefits can you expect in the study?

Benefits will include study participants having access to basic medical services on top of what is provided by the RCH. Participants will also benefit from weekly monitoring by qualified field workers where anaemia can be detected. Malaria can also be diagnosed and managed immediately. Participants will be followed-up by the research team even after the 12 weeks intervention to assess their pregnancy outcome and the health of their babies.

Will you be compensated for participating in the study?

You will not get paid for participation, but you will get either transport by MRC or get the costs for the transport reimbursed.

Are there other products or treatment?

No.

What happens if you refuse to participate in the study or change your mind later?

You are free to participate or not in the study and you have the right to stop participating at anytime without giving a reason. This will not affect the medical care that you would normally receive.

In case you decide to withdraw your participation during the study, we will not work on your samples without your permission, but any information already generated from the samples will be kept. The study doctor may also ask for tests for your safety.

Should any new information become available during the study that may affect your participation, you will be informed as soon as possible.

If you are injured in the study what compensation will be available?

We will be responsible to provide for treatment caused by the research study. If you have an unwanted reaction, we will treat you or refer you as needed.

If medical treatment is required as an emergency, please refer to your health centre or clinic and contact the field worker who gave his/her telephone number to you or contact Mr. Amat Bah, 9901696 or Dr. Rita Wegmuller, 9963991.

How will personal records remain confidential and who will have access to it?

All information that is collected about you in the course of the study will be kept strictly confidential. Your personal information will only be available to the study team members and might be seen by some rightful persons from the Ethics Committee, Government authorities and sponsor.

Who should you contact if you have questions?

If you have any queries regarding the study you can contact Mr. Amat Bah, 9901696 or Dr. Rita Wegmuller, 9963991, and you can always call the personal numbers of the study staff given to you. If you have any concerns you can also contact staff at your health centre or clinic.

Please feel free to ask any question you might have about the research study.

Who has reviewed this study?

This study has been reviewed and approved by a panel of scientists at the Medical Research Council and the Gambia Government/MRC Joint Ethics Committee, which consists of scientists and lay persons to protect your rights and wellbeing.

CONSENT FORM

Participant Identification Number: |_|_|_|_|_|_|_|_|_|_|_|_|_|_|_|_|_|_|_|_|_|_|_|_|

(Printed name of participant)

- I have read the written information **OR**
- I have had the information explained to me by study personnel in a language that I understand and I confirm that my choice to participate is entirely voluntarily, confirm that I have had the opportunity to ask questions about this study and I am satisfied with the answers and explanations that have been provided, understand that I grant access to data about me to authorised persons described in the information sheet, have received time to consider to take part in this study, agree to take part in this study.

Tick as appropriate

I agree to further research on my samples as described in the information sheet Yes No

Participant's signature/
thumbprint*

Date (dd/mmm/yyyy) Time (24hr)

Printed name of impartial
witness*

Signature of impartial
witness*

Date (dd/mmm/yyyy) Time (24hr)

Printed name of person
obtaining consent

I attest that I have explained the study information accurately in _____ and was understood to the best of my knowledge by the participant and that he/she has freely given consent to participate *in the presence of the above named impartial witness (where applicable).

Signature of person
obtaining consent

Date (dd/mmm/yyyy) Time (24hr)

** Only required if the participant is unable to read or write.*
A copy of this informed consent document has been provided to the participant.

Appendix 4: Investigational products or intervention

We administered three investigational products as nutrition supplements:

UNIMMAP with 60 mg iron

UNIMMAP with 30 mg iron

UNIMMAP with 0 mg iron.

Description of product or intervention

The nutritional supplement used in this trial is the UNICEF/WHO/UNU international multiple micronutrient preparation (UNIMMAP). All formulation also contain 400 ug folic acid and 14 other micronutrients (Table-----). The UNIMMAP supplement has been used safely in other pregnancy trials [181].

Formulation, packaging and labelling

The formulation was produced by DSM South Africa under GMP conditions where was dosed into gelatin capsules, packed in tubs. The labelling included a statement that 'trial medications are only for use of trial participant'.

Table ---: Intervention product - Formulation based on UNU/UNICEF/WHO supplement called UNIMMAP

Micronutrients	Dose/day
Vitamin A (ug RE)	800
Vitamin D (IU)	200

Vitamin E (mg)	10
Thiamine (mg)	1.4
Riboflavin (mg)	1.4
Niacin (mg)	18
Folic acid (ug)	400
Vitamin B6 (mg)	1.9
Vitamin B12 (ug)	2.6
Vitamin C (mg)	70
Zinc (mg)	15
Iron (mg)	60 or 30 or 0 (placebo)
Iodine (ug)	150
Selenium (ug)	65
Copper (mg)	2

Product storage and stability

We stored the products under controlled conditions (in an air-conditioned storage house at around 20°C) at the MRC Keneba. The product is stable for 18 months if kept under these conditions.

Dosage, preparation and administration of investigational product or intervention

Each participant received 1 daily dose of the supplement which corresponds to 1 capsule per day. Each week field workers visited the study participants and distributed the respective weekly supply (7 capsules) to each participant. The participants were instructed to take 1 capsule a day with a bit of water or a drink. Each time the field workers distribute the new weekly supply of capsules they accounted for the number of capsules consumed/not consumed from the previous week in order to check for compliance.

Appendix 5: Safety considerations and oversight

This trial was overseen by a Data Safety Monitoring Board (DSMB); chaired by Dr Jay Berkley, KEMRI Wellcome Trust, Kilifi, Kenya): to safeguard the interests of trial's participants, investigators and sponsor; to assess the safety and efficacy of the trial's intervention, and to monitor the trial's overall conduct, and protect its validity and credibility. The DSMB was assisted by a Trial monitor and a Trial Steering Committee (TSC).

The DSMB undertook interim review of the trial's progress by:

- assessing data quality, recruitment and losses to follow-up
- monitoring compliance with the protocol by participants and investigators
- monitoring evidence for treatment differences in the main outcome measures and for treatment harm
- recommending action whether the trial should continue to recruit or follow-up
- recommending or advising on any major changes or modifications to the protocol
- suggesting additional data analyses
- assessing the impact and relevance of any external evidence provided

The DSMB was additionally responsible for reviewing all Serious Adverse Events (SAE) defined below.

Methods and timing for assessing, recording, and analysing safety parameters

We conducted the trial according to Good Clinical Practice (GCP) principles (ref: MRC DMID Protocol Template_Att1_V3_Protocol Template_SOP-CTS004) (Declaration of Helsinki, Adopted

by: 64th WMA General Assembly, Fortaleza, Brazil, October 2013). The DSMB determined how they were to monitor the data and safety interest of the participants. The DSMB also determined how and the frequency of its meetings.

Adverse events

An adverse event (AE) was defined as any untoward or unfavourable medical occurrence in a human subject, including signs and symptoms which are temporally associated with the research procedure or trial intervention, whether or not considered related to the subject's participation in the research. Participants were monitored for AEs on each scheduled follow up day. All symptoms or signs reported or observed were assessed by the study Field Assistant and recorded. If help is needed, was sought from the study nurse.

Serious adverse events (SAEs)

A **SAE** was defined as any AE that was life-threatening or results in death or requires hospitalisation or prolongation of hospitalisation, was a persistent or significant disability/ incapacity or a congenital anomaly/birth defect. Reported maternal deaths, miscarriages, stillbirths were recorded as SAEs. All SAEs were investigated by the trial physician.

Assessment of intensity

The trial nurses with the support of the PI and other member of the Keneba clinical team, assessed the severity or intensity of the AEs and laboratory changes as follows and document them into the AE form:

Grade	Description
1 Mild	Awareness of sign or symptom, but easily tolerated
2 Moderate	Enough discomfort to cause interference with usual activity

3	Severe	Incapacitating with inability to work or do usual activity
4	Life-threatening	This grade was considered as SAE

The term “severe” was often used to describe the intensity (severity) of a specific event (as in mild, moderate, or severe myocardial infarction); the event itself, however, may be of relatively minor medical significance (such as severe headache). This is not the same as “serious”, which was based on the outcome or criteria defined under the SAE definition. An event was considered serious without being severe if it conforms to the seriousness criteria; similarly, severe events that did not conform to the criteria were not necessarily serious. Seriousness (not severity) serves as a guide for defining regulatory reporting obligations.

Assessment of causality

Every effort was made by the PI and team to explain each AE and assess its causal relationship to administration of the trial intervention. This explanation was based on the type of event, the relationship of the event to the time of trial intervention, and the natural history of the underlying diseases, concomitant therapy, etc. The results were documented on the CRF. The relationship of an AE to the investigational product was assessed according to the following definitions:

Not related

- No temporal relationship to trial intervention; *and*
- Event could be explained by alternate aetiology (clinical state, environmental or other interventions).

Unlikely related

- Temporal relationship to trial intervention improbable (but not impossible);
but

- Disease or other products provide plausible explanations;

Possibly Related

- Reasonable temporal relationship to trial intervention; but
- Event could also be explained by alternate aetiology (clinical state, environmental or other interventions);

Probably Related

- Reasonable temporal relationship to trial intervention; and
- Event could not be explained by alternate aetiology (clinical state, environment, or other interventions);

Definitely Related

- Reasonable temporal relationship to trial intervention; and
- Event could not be explained by alternate aetiology (clinical state, environmental or other interventions); or
- Event could be confirmed with a positive re-challenge test, where applicable.

The participants were instructed to contact the field assistant or a member of the study team, should the participant manifest any signs or symptoms they perceive as severe during the period extending from performance of the first trial procedure to the end of the study.

All findings observed or reported from the day of the first administration of the trial intervention were recorded on the CRF by the team. Whenever possible, AEs were documented in terms of a diagnosis or syndrome rather than multiple symptoms that are clear manifestations of the same

diagnosis/syndrome. In case signs and symptoms are reported by the participants, a medical diagnosis was obtained by the nurses and PI. If a diagnosis cannot be obtained then each sign or symptom was recorded as separate events.

The action taken (e.g. discontinuation of investigational product, withdrawal of the participant from the trial, requirement of concomitant medication or treatment, others) was recorded on the appropriate section of the CRF. If hospitalisation or its prolongation was required this was reported as a SAE.

All AEs were followed until resolution of the event and/or the end of the trial. The outcome was assessed as follows:

- Resolved
- Resolved with sequelae
- Ongoing
- Death
- Lost to follow up

Treatment for any AE and SAE was recorded on the appropriate section of the CRF.

Reporting procedures

The PI reported all SAEs without filtration, whether or not related to the trial intervention, within 24 hours of becoming aware of the event to the DSMB and the Sponsor. Plan was that if the SAE was related to the trial intervention, the Ethics Committee be notified according to their procedures.

The minimum information required for this initial SAE report was:

- Trial number and (short) title
- Participant's ID
- Date and time of onset
- Description of the event (clinical history, associated signs and symptoms)
- Intervention product administered

The PI was not to wait for additional information to fully document the event before notifying. The report was then followed by submission of a completed SAE Report Form as soon as possible, detailing relevant aspects of the SAE in question. We reported all actions taken by the PI and the outcome of the event.

For documentation of the SAE, any actions taken, outcome and follow-up, the SAE Report Forms was used. All follow-up activities were reported, where necessary on one or more consecutive SAE report forms in a timely manner. All fields with additional or changed information were completed and the report form forwarded to the DSMB within 5 calendar days after receipt of the new information. We obtained hospital case records and autopsy reports including verbal autopsies, where applicable.

Withdrawal of participants

A study participant was discontinued from participation in the study if:

- Any clinical significant adverse event (AE), laboratory abnormality, intercurrent
- illness, or other medical condition or situation occurs such that continued
- participation in the study would not be in the best interest of the participant
- Development of any exclusion criteria

Discontinuation criteria

Participant's premature termination

A participant had the right to stop participating in the study at any time without giving a reason and this did not affect the medical care that would normally be received. The trial team or the DSMB withdrew participants from the study when deemed necessary at any time taking in to consideration the reasons mentioned below. We documented all the reasons for a participant's premature termination on the appropriate page of the CRF and specified which of the following possible reasons were responsible for the premature termination:

- Serious Adverse Event
- Adverse Event
- Participant's consent withdrawal
- Development of withdrawal criterion
- Migrated/moved from the study area

- Lost to follow-up

A 'lost to follow-up' was any participant who completed all protocol specific procedures up to the administration of the investigational product or intervention, but was then lost during the study period to any further follow-up, with no safety information and no efficacy endpoint data ever available

In case the participant decided to withdraw participation or consent during the study, we did not work on that participant's samples without permission, but any information that was already generated from the samples was kept and used. The study physician was responsible for asking for tests for the participant's safety. The PI had the responsibility to inquire about the reason for any withdrawal and follow-up with the participant regarding any unresolved AEs.

We did not collect any specific data for withdrawn participants. We did not replace subjects as our sample size calculation took into account a dropout rate of 15%.

Study discontinuation

The rules for study termination or discontinuation were set by the DSMB in the DSMB Charter.

Appendix 6: Statistical Analysis Plan (SAP)

A randomised controlled trial comparing two screen-and-treat iron supplementation based on plasma hepcidin concentration with a daily universal iron supplementation in pregnant Gambian women

Brief title: Hpcidin and anaemia in pregnancy (HAPn)

Statistical Analysis Plan(SAP)

Version: 1.0

Date: 4 July 2017

Prepared by: Mr Amat Bah, PhD student and Principal Investigator

Approved by: Prof. Andrew M. Prentice, Supervisor

Dr Hans Verhoef, Statistical adviser to student

Abbreviations

AE	Adverse Event
APGAR	Appearance, Pulse, Grimace, Activity, Respiration
AUC ^{ROC}	Area Under the Curve
EDTA	Ethelenediamine tetraacidic acid
ENID	Early Nutrition and Immune Development
HAPn	Hepcidin and anaemia in pregnancy
IQR	Inter Quartile Range
ITT	Intent To Treat
LMP	Last Menstrual Period
MCV	Mean Corpuscular Volume
mITT	Modified Intent To Treat
PI	Principal Investigator
PP	Per Protocol
RDT	Rapid Diagnostic Test
ROC	Receiver Operating Characteristics
SAE	Serious Adverse Event
SD	Standard Diviation
SOP	Standard Operating Procedure
TSAT	Transferrin Saturation
UNIMMAP	UNU/UNICEF/WHO international multiple micronutrient preparation
WHO	World Health Organisation

Overview

Anaemia affects all population groups but those at greatest risk are pregnant women and children. For women, anaemia is associated with poor pregnancy and birth outcomes including premature delivery, low birth weight and increased perinatal mortality. The most significant contributor to the onset of anaemia is iron deficiency[1]. WHO recommends iron supplementation for all pregnant women (60mg/d iron and 400ug/d folic acid) living in areas where anaemia rates exceed 40%. There are concerns that such universal iron supplementation can increase risks of haemoconcentration, gestational diabetes and pre-eclampsia to pregnant women[130, 134]. Therefore, there is a need to explore screen-and-treat options to minimise iron exposure during pregnancy using an overall lower dosage of iron that would achieve non-inferior benefits as the WHO recommendation. There is some evidence that smaller doses of 30mg iron daily could achieve similar results as the daily 60mg iron[49].

Furthermore, WHO recommends supplementation with daily doses between 30–60mg iron for pregnant women[132]. Evidence also suggest that the use of multiple micronutrient supplements with three or more micronutrients is associated with a 39% risk reduction in maternal anaemia compared with placebo or with two micronutrients or fewer (relative risk 0.61, 95% CI 0.52—0.71). Multiple micronutrient supplementation is also known to result in a decrease in the risk of low-birth weight babies (0.83, 0.76—0.91) and small-for-gestational-age babies (0.92, 0.86—0.99)[148]. Therefore, this trial used the UNICEF/WHO/UNU international multiple micronutrient preparation (UNIMMAP) containing either 60mg or 30mg or 0mg iron per day.

However, there is a lack of agreement on how to best assess iron deficiency in the presence of infection-induced inflammation. Hepcidin, a peptide hormone, is believed to have the potential of being an ideal index for 'safe and ready to receive' iron. In this trial, we used a predetermined cut-off value for hepcidin concentration of $<2.5\mu\text{g/L}$ as a threshold to decide on whether or not to receive iron. This cut-off value is based on the analysis of sera from 395 pregnant women participating in the ENID study[165] with samples available for 3 time points (14 weeks, 20 weeks and 30 weeks gestation). A receiver operating characteristics (ROC)-curve was generated to measure the area under the curve (AUC^{ROC}). Method described elsewhere[117].

This study aims to evaluate the hypothesis that a screen-and-treat approach to iron supplementation below the pre-determined hepcidin cut-off value ($<2.5\mu\text{g/L}$), is non-inferior to the reference arm (WHO-recommended universal iron supplementation) in preventing anaemia and iron deficiency at a lower dose and hence improve safety and tolerability after 12 weeks intervention with haemoglobin concentration as the primary endpoint.

Objectives:

Primary objectives:

1. To evaluate if a screen-and-treat supplementation strategy (i.e., weekly screening of plasma hepcidin concentration for 12 weeks, each time succeeded by daily supplementation for 7 days using micronutrients with or without 60mg iron as ferrous fumarate, depending on plasma hepcidin concentration) is non-inferior to daily universal supplementation (i.e. micronutrients including 60mg iron as ferrous fumarate) regarding haemoglobin concentration at the end of 12 weeks of intervention;
2. To evaluate if a screen-and-treat supplementation strategy (as above, with 30mg iron instead of 60mg iron) is non-inferior to daily universal supplementation (i.e. micronutrients including 60mg

iron as ferrous fumarate) regarding haemoglobin concentration at the end of 12 weeks of intervention;

Secondary objectives:

3. To compare screen-and-treat supplementation strategies with daily supplementation with regards to anaemia;
4. To compare screen-and-treat supplementation strategies with daily supplementation with regards to iron status;
5. To compare screen-and-treat supplementation strategies with daily supplementation regarding the number of events per week of self-reported side-effects, adverse or serious adverse events;
6. To compare screen-and-treat supplementation strategies with daily supplementation regarding adherence or compliance;
7. To compare exposure to supplemental iron in the two screen-and-treat groups.

Population and sample

Participants for the study are women of Jarra West and Kiang East (rural Gambia), identified by nurse midwives as they visited the Reproductive and Child Health clinics to register and book their pregnancies. They were pregnant women in the age range 18–45 years with gestational age of 14–22 weeks as assessed by the reported first date of last menstrual period (LMP) and by fundal height assessment.

Pregnant women were ineligible for randomisation and excluded if: unlikely to be resident in the study area for the entire duration of the intervention period; severely anaemic (haemoglobin concentration <70g/L); seriously ill (infectious disease of clinical significance) or suffering from a chronic disease; or have pregnancy complications (e.g. pre-eclampsia); or if already participating in another study.

Randomisation

Recruited women were randomly allocated, using computer-generated numbers to one of 3 intervention arms 'X', 'Y' and 'Z' (representing the treatment arm 'A', 'B' and 'C' below), based on a stratified permuted block design with a 1:1:1 allocation ratio, balanced by the haemoglobin concentration and gestational age at baseline. To achieve this, at each day of recruitment, subjects were categorised into four strata formed by cross-classification by haemoglobin class (above and below the median haemoglobin of the respective day) and gestational age (14–18 weeks, 19–22

weeks). In each of the 4 classes, the women were randomly assigned to the 3 treatment arms using a predetermined block randomisation. To minimise biases the study was double blinded

Interventions

The groups/treatment arms received the following interventions:

- A) Daily supplementation with UNU/UNICEF/WHO international multiple micronutrient preparation (UNIMMAP) capsules containing 60mg iron as ferrous fumarate (reference treatment) for 12 weeks;
- B) Weekly screening of plasma hepcidin concentration for 12 weeks, each time succeeded by daily supplementation for 7 days with UNIMMAP containing (60mg iron as ferrous fumarate) or placebo (no iron), depending on plasma hepcidin concentration being $<2.5\mu\text{g/L}$ or $\geq 2.5\mu\text{g/L}$, respectively;
- C) Screen-and-treat supplementation as in group b), but with UNIMMAP containing an iron dose of 30mg/day instead of 60mg/day.

Field procedures

Collection of data and samples started 16th June 2014 and ended 3rd March 2016, and this is schematically shown in **Figure 1**. We identified and screened pregnant women at first antenatal care visits at two health facilities. During screening: we obtained prior informed consent; collected demographic information; and provided a long-lasting insecticide-treated mosquito net to each participant. We conducted a medical examination (including assessment of gestational age by last menstrual period (LMP) and fundal height measurement), and collected a 5-7mL venous blood sample in EDTA tubes. For women who were excluded, we recorded reasons for not randomised when possible.

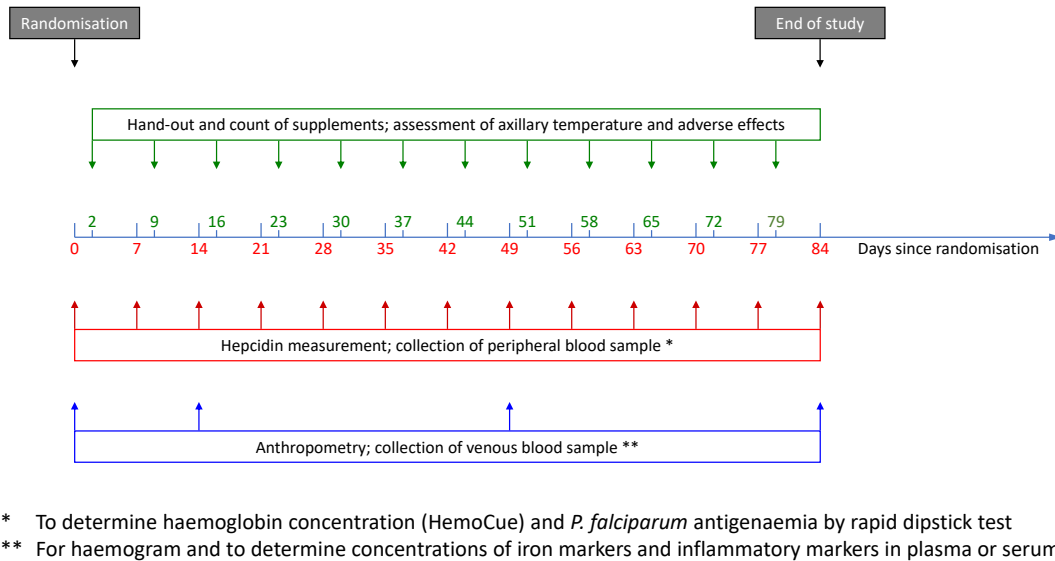
We used the blood samples to determine haemoglobin concentrations in the field by photometer (HemoCue) and to assay the presence of *P. falciparum* antigens by rapid diagnostic tests (RDTs; SD Bioline Malaria Ag P.f.). If the result of the rapid diagnostic test was positive, we prepared a blood slide for microscopic examination. At the laboratory in Keneba, we produced a haemogram (haemoglobin concentration, blood cell counts, mean corpuscular volume, etc.) using an automated blood analyser (Medonic M Series). We measured serum hepcidin concentration by ELISA (Hepcidin-25 (human) EIA Kit, Bachem) using a Thermofisher Scientific Multiskan FC Microplate

Photometer with a detection range 0.049 - 25.0ug/L). We assessed in vitro growth of *P. falciparum* in washed red blood cells. We stored plasma at -20°C for subsequent determination of plasma markers (concentrations of soluble transferrin receptor, iron, etc).

The intervention started at Day 0 (day of recruitment and randomisation) and continued for 84 days or until delivery, whichever came first. At Day 2 and thereafter weekly, each participating woman was seen weekly by fieldworkers, who gave a 1-week supply of supplements, counted supplements remaining from the previous week, measured axillary temperature, and recorded self-reported side effects that occurred in the preceding week on a standardised form (**Figure 1**). At Days 14, 49 and 84, we collected additional venous blood for assessments and storage of plasma samples as described for baseline. At Day 7 and thereafter weekly (except for the dates when venous blood was collected), field staff collected peripheral blood samples by finger prick. At each time point, we assessed *P falciparum* infection by rapid dipstick test, and we measured hepcidin concentrations in plasma samples within 2 days of blood collection. Depending on hepcidin concentrations being $<2.5\mu\text{g/L}$ or $\geq 2.5\mu\text{g/L}$, the woman received a subsequent 1-week supplementation cycle with or without iron, respectively.

At delivery, we recorded the place of delivery, delivery mode, complications, birth weight and APGAR (appearance, pulse, grimace, activity, respiration) score.

For adverse events/serious adverse events, we recorded a description, duration and relation to intervention. Where possible, we recorded reasons for being lost to follow up.



FIGURE

1. Collection of samples and data within the HAPn study

Data cleaning

The PI and study team will inspect the data using descriptive statistics and histograms to detect missing values, incorrectly entered values and impossible outliers. Data will be corrected if needed and if possible using source data available from the field and laboratory.

Blind review

In a preliminary, blind review of the data, we will calculate descriptive statistics at baseline and at the end of intervention by group (A, B, C). This review will help in the finalisation of the statistical plan, and in the identification of imbalances in baseline factors that are prognostic for outcomes.

Data locking

Once the data is cleaned to the satisfaction of the PI, his supervisors and the trial statistician, the data will be locked per Medical Research Council procedure (SOP-DMA-019) to prevent any further interference with the data set. If there is need to unlock the database later, provision of the SOP must be used.

Missing values and outliers

All missing values and outliers present after the locking of the data shall be maintained. One set of analysis will be performed with the missing values for per protocol analysis. In a copy of this dataset, missing values will be replaced by multiple imputations to allow intention-to-treat (ITT) analysis. Both analyses will be reported.

ITT analysis generally leads to intervention effects being underestimated, which we consider undesirable to assess effects on adverse events. Thus, emphasis in the interpretation of effects on adverse events will be on the per protocol analysis.

We will use a modified ITT (mITT) analysis, i.e. excluding participants who were lost to follow-up or withdrawn before the first dose of supplementation was received. We will use Multivariate Imputation by Chained Equations using Stata 14), with replacement of missing data under a missing-at-random assumption by multiple imputation. We will log-transform variables as necessary to normalize distributions. To ensure convergence to a stationary distribution, we will use a burnin of 1000. We shall also test different number of imputations that ensures the Monte Carlo error estimates follow the practical guidelines from White et al. [182]. A list of variables used in the imputation model will be submitted as Supplementary material. For binary outcomes, multiple imputations will yield an integer number of cases per iteration, but pooled estimates from multiple iterations may result in these numbers to be estimated in non-integer values. Because this precludes computation of confidence intervals for differences in proportions, we will instead calculate differences in means under the assumption that binary outcome variables have a Bernoulli distribution.

Definitions

1. *Anaemia*: haemoglobin concentration <110g/L.
2. *Iron status*: plasma ferritin concentration at Day 84, adjusted for the degree of inflammation and among non-inflamed.
3. *Iron deficiency anaemia (IDA)*: iron deficiency in the presence of anaemia
4. *Iron receptivity*: ability or readiness to absorb and utilise iron by the body, as indicated by plasma hepcidin concentration;
5. *Tissue iron deficiency*: iron deficiency due to impaired physiological systems for transporting iron to target tissues, as indicated by plasma soluble transferrin receptor concentration, with adjustment for the degree of inflammation.

Participant flow

We will produce a flowchart describing the progress through various phases of the trial (i.e. enrolment, intervention allocation, follow-up, and data analysis) of the three intervention groups as per the CONSORT guidelines (**Figure 2**).

We will report:

- Number of participants identified or screened
- Number of participants consented
- Number of participants enrolled
- Number of participants randomised to the 3 study arms
- Number lost to follow-up and reasons
- Number of births that occurred before the end of the 84-day intervention period for each of the study arms
- Summary of Adverse Events (AEs)
- Summary of Serious Adverse Events (SAEs)
- Number that completed study
- Number analysed

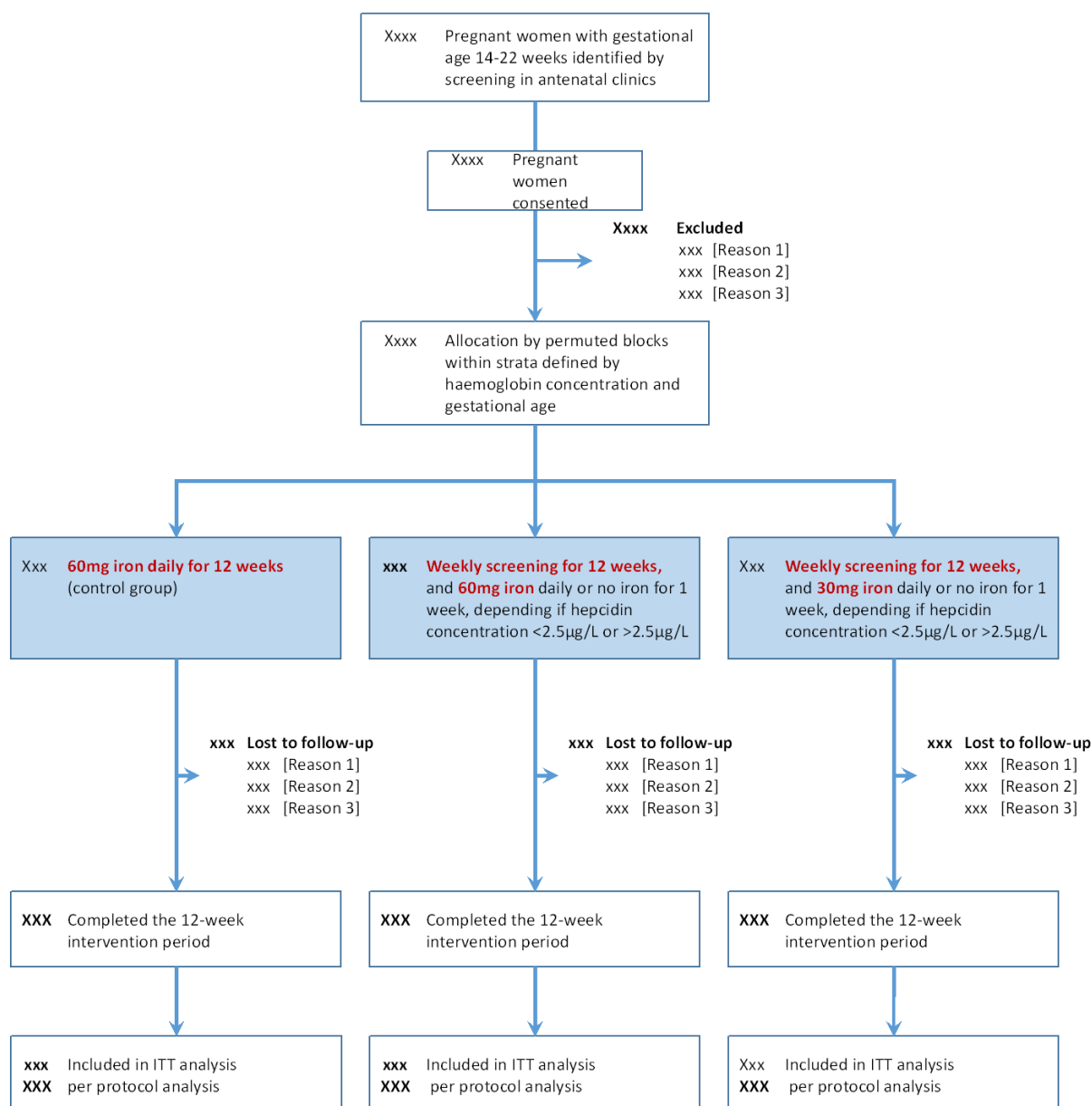


Figure 2: Flowchart

Description of baseline characteristics

We will report in a table describing the following baseline characteristics by intervention group: maternal age (continuous variable), gestational age (continuous variable), parity, gravida (both continuous and categorical), weight, height (continuous), haemogram markers (haemoglobin, mean corpuscular volume, mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration, red blood cells, red blood cell distribution width, haematocrit, white blood cells); (both continuous and categorical), genotype (categorical), iron and inflammation markers (both continuous and categorical)

ethnicity categorical), *Plasmodium* infection (binary), and provision of impregnated mosquito nets (binary).

For all groups, we will report sample size. For ferritin concentrations, we will also report values adjusted for C-reactive protein and α_1 -acid glycoprotein. Ferritin when there is inflammation CRP >5mg/L and AGP \geq 1g/L. For normally distributed variables, we will report group means (SD). For variables with a lognormal distribution, we will report geometric means (geometric SD). For continued variables that are not normally distributed, or that cannot be normalised by log-transformation, we will report medians with corresponding 25th- and 75th-centiles that indicate the limits of the IQR.

Description of outcomes

We will report the following outcomes by intervention group, (with descriptive parameters as described above):

1. Haemogram markers (haemoglobin concentration, erythrocyte count, MCV);
2. Iron markers (plasma concentrations of ferritin, iron, soluble transferrin receptors, total iron binding capacity, unsaturated iron binding capacity, transferrin);
3. Inflammation markers (C-reactive protein, α_1 -acid glycoprotein; leukocyte count)
4. Serum hepcidin concentration;
5. Anthropometry
6. Birth weight.

For continuous outcome variables measured at the end of the intervention, we will compare pairs of intervention groups by estimating the difference in means assuming a t-distribution of the outcome. A logarithmic transformation will be applied to log-normally distributed variables.

The analysis for the primary end point (haemoglobin at Day 84) will be based on a test for non-inferiority with a per-protocol analysis. As per acceptable practice, a modified intent-to-treat analysis will also be performed on the randomised population.

Primary analysis:

The primary non-inferiority endpoint is pregnancy-adjusted haemoglobin concentration at Day 84. Groups will be compared using linear regression analysis, with intervention entered as a dummy-coded categorical variable and using the control arm (universal daily supplementation) as the reference group. To indicate non-inferiority, the lower limit of the 95.0% confidence interval for the difference in mean haemoglobin concentration between either of the screen-and-treat arms and the reference arm shall be above -5.0 g/L.

Secondary analyses

Groups will be compared using linear regression analysis as described below for secondary outcomes measured at Day 84 (plasma concentrations of ferritin, soluble transferrin receptor and total transferrin saturation (TSAT), with adjustments for inflammation (where appropriate) as described below.

Thus, we will use linear regression analysis indicated by concentrations of C-reactive protein and α_1 -acid glycoprotein, with arbitrarily selected reference values of 5 μ g/L and 1mg/L, respectively. Results will be reported for adjusted ferritin concentration as a continuous variable and dichotomised as iron deficient or iron replete (<15 μ g/L and \geq 15 μ g/L, respectively; WHO 2011)[183]. In addition, we will conduct a stratified analysis by using unadjusted ferritin concentration <15 μ g/L but restricting analysis to those without inflammation.

We will use mixed-effect linear regression models to compare intervention groups regarding the development over time of continuous outcomes (haemoglobin concentration, plasma concentrations of ferritin, soluble transferrin receptor, transferrin, total iron saturation), with adjustment for baseline values. The models will include main terms for time and intervention group, and their product term to assess changes in the intervention effect over time. In these analyses, outcome variables will be log-transformed as appropriate.

The hepcidin values may possibly be censored due to the range of the competitive ELISA (Bachem Hepcidin-25; now marketed by Peninsula Laboratories International) which was used in quantifying the plasma levels. Hence, in such an eventuality, we would use a Tobit model to investigate any differences in plasma hepcidin levels between groups, otherwise it would be assessed as other continuous variables.

Safety analysis

We will perform analysis using rate and rate ratios for the side effects (morbidity assessment), AEs and SAEs. Simple comparison of rates of side effects, AEs and SAEs would be used to assess differences between groups. We shall use Poisson regression to determine the number of events per person weeks. In the event of over-dispersion, we shall use a negative binomial model instead. We will only perform crude comparisons as we believe the randomisation would adequately address most confounding. However, we expect that the observation period of the women in the study would vary and would thus be a possible confounder for this analysis. We shall address any imbalance in the observation periods of the women by using an exposure variable for the regression models. Comparisons between groups Y and Z vs. group X would be reported as rate ratios.

We will analyse *P falciparum* growth in serum (difference between *in vitro* growth rates at Days 0, 14, 49 and 84) to determine relationship to study arms and also Ex vivo growth of sentinel bacteria (difference between *in vitro* growth rates at Days 0, 14, 49 and 84)

Adherence assessment

We will conceive adherence as the extent to which the participant's history of supplementation coincided with the prescribed supplementation. We will estimate adherence as the number of days that supplements were consumed (as indicated by tablet count) divided by the number of days of follow-up (minus 2 days to account for the first two days after randomisation, when supplementation was put on hold depending on the results of the first hepcidin concentration assessment). In this assessment, women who completed the intervention as scheduled; left the study prematurely due to refusals; left due to medical or unknown reasons; or who were withdrawn because of poor compliance, contributed follow-up time until the scheduled end of intervention (i.e., 85 days, including Day 0). For women who left the study prematurely for reasons that we considered unrelated or unlikely to be related to supplementation use (i.e., delivery or emigration), we will calculate follow-up time as the time until leaving the study. Summary measures (e.g., mean, percentiles) of adherence calculated for individual women are biased because of differences between women in follow-up time. Thus, we will estimate group adherence as the average number of days that supplements were consumed divided by the average days of follow-up time. Thus defined, group adherence is essentially an average of individuals' adherence weighted by observation time.

Exposure to supplemental iron

For each of the groups that received screen-and-treat supplementation, we calculated the reduction in prescribed iron supplements due to screen-and-treat approach as the percentage of supplements prescribed that contained no iron.

Further exploratory analysis

- Primary endpoints adjusted for C-reactive protein, Alpha 1-acid glycoprotein and malaria (we decided apriori that the primary endpoints will not be adjusted for the above. However, we wish as part of an exploratory analysis to adjust for them)
- Ferritin
- Soluble transferrin receptor
- Transferrin saturation (TSAT)

Appendix 7: Pictures from the HAPn trial (permission for reproducing the photographs was ganted)



Community and radio sensitisations in the HAPn project areas of Kiang East and Jarra West



Weekly clinic sessions on a typical venous blood collection day



HIGH
 THE GAMBIA
 REPRODUCTIVE AND CHILD HEALTH (RCH)
 SERVICES, MATERNAL RECORD

Name: *Musa* Sex: *M* BIODOC
 Date of Birth: *1988*

Measurement	Quantity	Concentration
Haemoglobin (g/dl)	100	160
Urea Nitrogen (mg/dl)	200	9 mg/dl
Urea Nitrogen (mg/dl)	200	8
Prothrombin Time (sec)	21.4	0.5
Prothrombin Time (sec)	21.6	0.5
Prothrombin Time (sec)	21.8	0.5
Prothrombin Time (sec)	22.0	0.5
Prothrombin Time (sec)	22.2	0.5
Prothrombin Time (sec)	22.4	0.5
Prothrombin Time (sec)	22.6	0.5
Prothrombin Time (sec)	22.8	0.5
Prothrombin Time (sec)	23.0	0.5
Prothrombin Time (sec)	23.2	0.5
Prothrombin Time (sec)	23.4	0.5
Prothrombin Time (sec)	23.6	0.5
Prothrombin Time (sec)	23.8	0.5
Prothrombin Time (sec)	24.0	0.5
Prothrombin Time (sec)	24.2	0.5
Prothrombin Time (sec)	24.4	0.5
Prothrombin Time (sec)	24.6	0.5
Prothrombin Time (sec)	24.8	0.5
Prothrombin Time (sec)	25.0	0.5
Prothrombin Time (sec)	25.2	0.5
Prothrombin Time (sec)	25.4	0.5
Prothrombin Time (sec)	25.6	0.5
Prothrombin Time (sec)	25.8	0.5
Prothrombin Time (sec)	26.0	0.5
Prothrombin Time (sec)	26.2	0.5
Prothrombin Time (sec)	26.4	0.5
Prothrombin Time (sec)	26.6	0.5
Prothrombin Time (sec)	26.8	0.5
Prothrombin Time (sec)	27.0	0.5
Prothrombin Time (sec)	27.2	0.5
Prothrombin Time (sec)	27.4	0.5
Prothrombin Time (sec)	27.6	0.5
Prothrombin Time (sec)	27.8	0.5
Prothrombin Time (sec)	28.0	0.5
Prothrombin Time (sec)	28.2	0.5
Prothrombin Time (sec)	28.4	0.5
Prothrombin Time (sec)	28.6	0.5
Prothrombin Time (sec)	28.8	0.5
Prothrombin Time (sec)	29.0	0.5
Prothrombin Time (sec)	29.2	0.5
Prothrombin Time (sec)	29.4	0.5
Prothrombin Time (sec)	29.6	0.5
Prothrombin Time (sec)	29.8	0.5
Prothrombin Time (sec)	30.0	0.5



A participant who completed the study and had twins. The HIGH/HAPn award winning team

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