Effects of weekly iron and folic acid supplements on malaria risk in nulliparous women in Burkina Faso: a periconceptional double-blind randomized controlled non-inferiority trial

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Summary: Weekly iron supplementation, given to young nulliparous women living in a malaria endemic area, neither improved iron status nor increased malaria risk, suggesting current iron recommendations may need re-visiting for these women.

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Running Head: Iron and malaria risk in young women;

Abstract

Background: Safety of iron supplementation for young women is uncertain in malaria endemic settings.

Methods: Double-blind randomized controlled non-inferiority trial in rural Burkina Faso.

Results: 1959 nulliparae assigned to weekly supplementation (60 mg iron and 2.8 mg folic acid) (n=980) or 2.8 mg folic acid (n=979) until first antenatal visit (ANC1), or 18 months if remaining non-pregnant. 315 women attended ANC1, and 916 remained non-pregnant. There was no difference at ANC1 in parasitemia prevalence (iron 53.4%, 95% CI 45.7:61.0; control 55.3%, 95% CI 47.3:62.9; prevalence ratio 0.97, 95% CI 0.79:1.18; P=0.82); anemia (adjusted effect 0.96, 95% CI 0.83:1.10; P=0.52); iron deficiency (adjusted risk ratio 0.84, 95% CI 0.46:1.54; P= 0.58); or plasma iron biomarkers. Outcomes in non-pregnant women were: parasitemia (iron 42.9%, 95% CI 38.3:47.5; control 39.2% , 95% CI 34.9:43.7, prevalence ratio 1.09, 95% CI 0.93:1.28; P=0.282); anemia (adjusted risk ratio 0.90, 95% CI 0.78:1.05; P= 0.17); iron deficiency (adjusted risk ratio 0.99, 95% CI 0.77:1.28; 0.96); with no iron biomarker differences.

Conclusions: Weekly iron supplementation did not increase malaria risk, improve iron status or reduce anemia in young, mostly adolescent menstruating women, nor in early pregnancy. WHO Guidelines for universal supplementation for young nulliparous women may need re-assessment.

Key Words: iron supplements; malaria; adolescents; randomized trial; Burkina Faso; Non-pregnant and pregnant

Trial Registration: ClinicalTrials.gov, number NCT01210040.

Introduction

Iron deficiency debilitates millions of young women in malaria endemic areas [1]. Iron supplementation is recommended where anemia is prevalent, but its safety and efficacy in malarious areas is uncertain. Reviews of daily or intermittent iron supplementation of non-pregnant women include few studies from malaria endemic settings [2, 3, 4], and in iron supplementation trials in pregnancy, malaria outcomes are mostly not reported [5]. Two trials showed no increased malaria risk at delivery with daily antenatal iron supplementation but malaria exposure was very low in one trial [6]. The other recruited mainly multigravidae [7], although in sub-Saharan Africa highest prevalence of *P.falciparum* malaria occurs during early pregnancy in primigravidae [8]. World Health Organization (WHO) guidelines on iron supplementation are applicable to both all-age pregnant women and all-age menstruating women. Safety of iron-folic acid supplementation in young nulliparous women before, and during their first pregnancy in malaria endemic areas has not been evaluated [9], and primigravidae are a higher malaria risk group than multigravidae.

As there had been no periconceptional trials of the safety and efficacy of iron supplementation in young nulliparous women living in malaria endemic areas, we carried out a double blind randomized controlled non-inferiority trial of malaria risk prior to, and during early pregnancy, in nulliparous women receiving weekly iron and folic acid supplementation over a period of up to eighteen months. The primary outcome was malaria parasitemia at first antenatal visit. Secondary outcomes assessed malaria in women who remained non-pregnant, and supplement efficacy in reducing iron deficiency. Malaria endemicity in this study area is typical of many settings in sub-Saharan Africa with perennial malaria transmission.

Methods

This periconceptional trial compared 2 cohorts supplemented with iron and folic acid versus folic acid alone - non-pregnant women and women who experienced pregnancy (additional details in Supplement 1). The study received ethical approval in Burkina Faso (Comité d'Ethique pour la

Recherche en Santé), England (Liverpool School of Tropical Medicine), and Belgium (University Hospital, Antwerp). All women in the trial provided written informed consent.

The study was conducted (April 2011 - January 2014) in the Nanoro Health and Demographic Surveillance System area (DHSS) where malaria is hyperendemic, with highest transmission between June and December [10]. The iron regimen used followed World Health Organization guidelines updated in 2016 [3, 11], and satisfied recommended nutrient intakes for non-pregnant women assuming 10-25% iron absorption, with potential for good compliance and reduced side effects compared to daily use.

Sample

Eligible participants were nulliparous, non-pregnant residents in 30 villages within the DHSS area. Potential participants aged 15-24 years identified through the DHSS database were approached by female field assistants (FFA) for pre-screening, (additional details on informed consent procedures in Supplement 1). We excluded women with possible or confirmed pregnancy, chronic disease (eg sickle cell), or illness requiring hospitalization. In this area human immunodeficiency virus prevalence was 1.2% among women 15–49 years and 0.76% among pregnant women [12]. At enrolment demographic data, history of illness, obstetric history, last menstrual period, age at menarche and sexual activity were recorded. A study clinician performed a general clinical examination, duplicate anthropometric measurements and axillary temperature. We collected venous blood for plasma ferritin, serum transferrin receptor (TfR), hepcidin, and C-reactive protein measurements (CRP). Plasma was stored at minus 80^0 C (additional laboratory details supplement 3). All participants received a long-lasting insecticidal net (LLIN) and single doses of albendazole (400mg) and praziquantel.

Recruitment continued until the target number was reached. We anticipated 34% malaria prevalence in controls at first antenatal visit (ANC1), [13]. 390 women in intervention and control groups

would provide 90% power to detect a non-inferiority margin of 10% of malaria prevalence between trial arms. The number of non-pregnant nulliparous women aged 15 to 24 years necessary to reach the required number of pregnant women was estimated from the proportion of nulliparous women (0.5) of child-bearing age (0.23), aged 15-24 years (0.33), which provides 1,973 potentially eligible women for a population of 52,000 in the DHSS area (Figure 1).

Randomization and Blinding

Participants were individually randomized to receive weekly one of identical red colored vegetable cellulose (hypromellose) capsules (disintegration time <30 minutes, mean 9.5 minutes), containing either ferrous gluconate (60 mg elemental iron, 479 mg gluconate) and folic acid 2.8 mg, or folic acid alone. A block allocation sequence was used with randomly determined block lengths. Four containers (4x20 capsules) were assigned the same randomization code and were allocated to each participant. The FFA kept one container per participant, obtaining a replacement as required. Supplements were not kept by participants. Supplement codes, unknown to investigators and maintained independently by the sponsor, were revealed only after data base lock and completion of data analysis. Women received a card with a unique study number corresponding to the randomization list. Supplements were stored at 20 -25^{0}C and at ambient temperature while with FFAs.

Follow-up

FFAs provided supplements during weekly home visits and ingestion was directly observed. Participants not located for two consecutive attempts but then re-contacted, were reported as temporarily absent. In cases of fever (T°≥37.5°C) or history of fever in the previous 48 hours the FFA performed a malaria Rapid Diagnostic Test (RDT, (Bioline SD, Malaria Antigen P.f) and if positive, collected a blood sample for a thick film. RDT positives were treated with artesunate-amodiaquine following national guidelines. If no menses were reported at 5 consecutive weekly

visits, a urine pregnancy test was done for human chorionic gonadotropin, immediately or at the next weekly visit. Supplement safety and tolerability were evaluated by recording and grading adverse events (AE). There were two levels of recording: weekly FFA symptom using a checklist, and AE through passive follow-up at health facilities. Serious adverse events (SAEs), including deaths, were collected by active (weekly) and passive surveillance and reported according to available information from FFAs and health center staff. SAE classification used all available clinical evidence and was not bound by stringent laboratory or diagnostic criteria. If a woman died outside hospital, verbal autopsies were done by the DHSS team following a standardized protocol.

At the end of the first malaria transmission season finger prick samples were taken from all non-pregnant participants for an interim safety analysis of malaria risk (microscopy and RDT). Positive RDTs were treated following national guidelines (additional details in procedures, Supplement 1).

Weekly supplements continued for 18 months, when women who remained non-pregnant were referred for end assessment. This included: medical history, clinical examination, anthropometry and axillary temperature. A venous blood sample was collected for malaria smear, hemoglobin (Hemocue AB), plasma ferritin, TfR, hepcidin, and CRP measurements, and for storage on filter paper. If febrile (≥37.5°C) or experiencing fever in the previous 48 hours, a RDT was performed and positives treated. Urine pregnancy tests were repeated when indicated. On trial completion anemic non-pregnant women (Hb <12 g/dl) received iron and folic acid tablets daily for one month, and if severely anemic (Hb <8g/dl) referred to hospital.

Women becoming pregnant within the follow up period were referred to Nanoro hospital for ANC1 with a study nurse/doctor at 13-16 weeks gestation according to the last menstrual period. The weekly supplement was withdrawn and hematinics provided according to national policy (60 mg iron, 400µg folic acid daily), although weekly follow-up continued. All women received the first dose of intermittent preventive treatment with sulfadoxine-pyrimethamine (IPTp-SP) if gestational

age was >13 weeks. Women ≤13 weeks gestation, if RDT positive, were treated with oral quinine. At ANC1 procedures included: routine antenatal care, venous blood collection for RDT, malaria film, hemoglobin, plasma ferritin, TfR, hepcidin, zinc protoporphyrin (ZPP) and CRP measurements. Ultrasound was performed to confirm pregnancy, determine fetal location, number and viability, and gestational age. Following ANC1 women with moderate anemia (Hb 7.0-9.9 g/dl) received twice the recommended daily supplement during one month, and if severely anemic (Hb <7 g/dl), were referred to Nanoro hospital. Women followed routine ANC visits where they received a second IPTp dose. Routine ANC and unscheduled health center visits were recorded on study questionnaires by study clinicians/nurses who collected data in health centers regularly (additional details in Methods Supplement 1). Procedures were similar at the second study visit (ANC2) (33-36 weeks gestation) when women were encouraged to deliver at Nanoro Hospital or the nearest Health Center, where free obstetric care was provided by the study. Study nurses examined babies for congenital malformations within 24-48 hrs of delivery and information on infant survival was obtained by FFA who visited or telephoned families.

Laboratory Measurements

Blood samples were transported to the laboratory within 3 hours, centrifuged and aliquoted. Blood films were Giemsa stained and read for malaria and parasite density (additional details laboratory procedures, Supplement 1). Hemoglobin was measured (Sysmex automated analyser) on fresh whole blood, and ZPP by fluorometry (Aviv Biomedical). Plasma ferritin and TfR were measured using mean values from duplicate ELISA samples (Spectro Ferritin S-22 and TFC 94 TfR, RAMCO Inc). CRP was measured by ELISA (EU59131IBL, GmbH). Intra-assay coefficients of variation (CVs) were all < 10%. Plasma hepcidin was measured by competitive ELISA at an International Reference Laboratory (laboratory procedures, Supplement 1).

Outcomes

The primary outcome was *Plasmodium* parasitemia prevalence at ANC1. Pre-specified secondary outcomes at ANC1 were prevalence of: RDT positivity with or without fever ($\geq 37.5^{o}$); clinical malaria (fever or history of fever in previous 48 hours with parasitemia), iron deficiency, anemia; adverse pregnancy outcomes, ie miscarriage, stillbirth, perinatal or neonatal death, congenital abnormality. In non-pregnant women at end assessment the proportion infected in the first year was assessed and the same pre-specified outcomes as at ANC1. Adverse events captured incidence of pre-specified gastrointestinal events (definitions and MedRA classification, grading and severity, Supplement 1 and Table S3, supplement 2). Malaria parasitemia prevalence was measured in non-pregnant women during the first rainy season after at least six months supplementation. Adherence to supplementation is reported.

Statistical analysis

A statistical analysis plan was approved before data base lock and release of the data and analyses follow that plan with only minor variation. Analyses followed CONSORT guidelines for non-inferiority trials [14]. Investigators were blinded to allocation (coded as A/B) until completion of the primary outcome analysis. Prevalence analyses based on data at specific time points utilized risk-ratio binomial models unadjusted and adjusted for season of transmission (low or high), LLIN use (proportion of weekly visits with reported use prior to visit date), antimalarial use in 4 weeks prior to visit, and whether menarcheal at baseline. Parasite density and iron biomarkers were analyzed using analogous ordinary regression models following logarithmic transformation. Results are expressed as risk-ratios (or density/biomarker level ratios) for iron treatment with 95%CI. Analyses of outcomes by iron deficiency utilized the same methodology, but with no menarche covariate. Mean hepcidin levels were compared between malaria parasitemia positive and negative women at ANC1 and end assessment using Mann-Whitney U-tests. Differences in proportions with elevated hepcidin were estimated using Fischer's Exact test and based on the 95th percentile value

for a healthy Dutch female population of comparable age [15]. Statistical significance was one-sided at alpha = 0.05.

Malaria incidence was calculated using Poisson regression models for the number of malaria episodes per woman with an offset of the logarithm of the period under observation (i.e. until final assessment, ANC1 or lost to follow up) with adjustment for LLIN use and menarche. Results are presented as incidence ratios for iron treatment with 95%CI. Time to first episode of malaria was analyzed using Cox regression models with participants censored at ANC1, final assessment or loss to follow up with adjustment for season (baseline hazard stratified by enrolment month to allow for timing of rainy season), LLIN use, menarche and iron deficiency at baseline (using adjusted ferritin definition). Results are expressed as hazard ratios for iron treatment with 95%CI. All analyses were performed using R statistical environment version 3.3 [16].

Results

Of 2317 women invited for screening 1959 were randomly assigned weekly supplements (n=980, iron and folic acid; n=979, folic acid); 1954 (99%) comprised the intention to treat population for the primary outcome. 405 pregnancies (21%) occurred during follow-up, with another 73 (4%) early pregnancies identified at or shortly after the end assessment survey (giving 478 pregnancies in total) (Figure 1). Median weekly supplement adherence at ANC1 was 79% (95%CI, 65-90%, iron) and 80% (59-91%, folic acid) and at end assessment for non-pregnant women 83% (72-91%, iron) and 84% (70-92%, folic acid) (Table S1, Supplement 2). Mean plasma ferritin and sTfR/log ferritin ratios were not improved in women with weekly adherence ≥ 80% (Figure 2). Median weekly LLIN use to ANC1 was 48.8%, and to end assessment 46.7%.

Baseline

Characteristics were similar between groups (Table 1). Adolescents (<20 years) comprised 93%. Women who remained non-pregnant were younger with lower body mass index. Women not attending were more illiterate and sexually active (Table S2, Supplement 2). Iron deficiency prevalence at baseline did not differ in women lost to follow-up before ANC1 (9 [2%] of 478), or for non-pregnant women lost to follow-up before end assessment (501, [32%] of 1549) compared to those followed successfully. Iron deficiency prevalence was almost two-fold higher using the sTfR/log ferritin ratio (22%) as biomarker than adjusted ferritin (12%) (Table 1). Elevated hepcidin concentration (>10.5 nmol/l) occurred in a quarter of women.

Prevalence of *P.falciparum* parasitaemia in non-pregnant women during the first rainy season was 36.4% (95% CI, 33.7-39.3, n=1167) with no difference by trial arm.

ANC1 Outcomes

Mean gestational age was 18.5 (SD 5.5) weeks (iron and folic acid) and 18.0 (6.0) weeks (folic acid). *Plasmodium* parasitemia prevalence was 54.3%. This did not differ by trial arm (adjusted ratio 1.0, 0.97-1.03), nor when associated with fever. Incidence and time to the first symptomatic episode (fever plus parasitemia or RDT positivity), were not different between arms (Table 2).

There was no difference between study arms in anemia or iron deficiency prevalence, or mean concentrations of iron biomarkers (Table 3). Median hepcidin concentration was 3.9 nmol/l (IQR 1.7:9.0) with, and 2.9 nmol/l (0.7:7.0) without, malaria parasitemia (P= 0.005). Elevated hepcidin was more frequent in parasitemic (22%) compared to non-parasitemic women (11%), (P= 0.015).

End Assessment Outcomes

Plasmodium parasitemia prevalence was 49.0% and did not differ by trial arm (adjusted ratio 1.1, 0.95-1.28), or if associated with fever. There was no difference between study arms in prevalence of

anemia or iron deficiency, or mean concentrations of iron biomarkers (Table 3). Median hepcidin concentrations in women with and without malaria parasitemia were respectively 3.4 (1.7:6.5) and 2.9 nmol/l (1.1:6.2; P=0.011). Elevated hepcidin was similar in parasitemic (15%) compared to non-parasitemic women (16%), (P= 0.85).

Adverse events

Supplements were well tolerated but with more frequent gastro-intestinal events with iron supplementation (RR 1.29, 95%CI 0.93-1.79, P=0.12). Adverse events for all categories did not differ by trial arm (Table S3, Supplement 2). We recorded 106 adult and 81 infant SAEs with almost equal frequency between groups (Table 4). 6 adult deaths occurred (3 at delivery), unrelated to the intervention or malaria. All congenital abnormalities (1.4%) occurred in controls. There were 17 infant/perinatal deaths, 12 to mothers who received iron.

Discussion

We found that weekly iron and folic acid supplementation for up to 18 months did not affect significantly prevalence of *Plasmodium* infection, iron deficiency or anemia, compared with folic acid supplements alone in either the pregnant or non-pregnant cohort. Women receiving iron experienced non-significantly increased gastro-intestinal effects. Asymptomatic falciparum malaria infection was highly prevalent despite weekly active surveillance, access to free treatments, and treatment of all RDT positives at the interim safety survey.

Our trial was double blind, tablet consumption was directly observed with balanced adherence covering wet and dry seasons, and active surveillance identified malaria episodes. Inadequate supplementation is unlikely since women absent for a number of weeks were tracked and resumed supplements, allowing them to re-establish their iron levels. Untreated chronic asymptomatic

malaria may lead to tissue pathology within the gut [17] as well as elevated hepcidin concentrations [18], which would be expected to reduce iron absorption. In relation to a safety trial, an effect of iron supplementation on malaria risk becomes difficult to establish if there is limited iron absorption. Baseline prevalence of iron deficiency was comparable to estimates in a recent meta-analysis for low income countries [19]. In the only *in vivo* iron absorption study to date in young women, conducted in Benin, markedly reduced iron absorption with asymptomatic malaria infection was demonstrated, and those participants had lower median parasite densities than we report at ANC1 [20]. Increasing iron dosage may be inadequate to overcome this limitation. Since participant diets also influence iron absorption due to high phytate content or other micronutrient deficiencies, effects of iron supplements may also depend on food habits and bioavailability. Better malaria control for adolescents could potentially both lower infection rates and improve food iron absorption, helping to meet growth requirements.

In 2016, WHO revised their guidelines on iron supplementation in menstruating women and adolescents girls, distinguishing settings where anemia prevalence is ≥40% from >20%. It recommended daily use (30-60 mg elemental iron) provided for three consecutive months per year where prevalence is ≥40% [11], and weekly intermittent supplementation in three monthly annual cycles where anemia prevalence is 20-40% [3]. Amongst non-pregnant controls in our study anemia prevalence was 45% at end assessment but ≤20% were iron deficient. As we saw no improved iron status at the higher dosage of 60 mg with up to18 months supplementation (maximum 78 doses), it is questionable whether daily dosage, given over three months, would be more beneficial (maximum 90 doses). This population has low iron deficiency prevalence despite high anemia prevalence. Without evidence that iron supplementation is effective in young nulliparous women living in malaria endemic areas, the basis for the WHO recommendation is weak and possibly unsafe in this group.

Adverse events were evenly balanced across trial arms, including miscarriages, which could result from early pregnancy malaria. Gastro-intestinal adverse events were non-significantly increased in iron supplemented women. We have showed a two-fold increased use of antibiotics for treatment of gastrointestinal infections in these women, with increased use of antifungals for lower genital infection in the non-pregnant iron supplemented cohort [21]. Iron supplementation is reported to increase diarrhea frequency in menstruating women from non-malaria endemic areas [2].

Our study has some limitations. Non-attendance was greater than expected with 25% of non-pregnant women not attending end assessment and 30% of pregnant women at ANC1, although contact with their families was retained to monitor AE up to birth. Attrition was equivalent between trial arms. Domestic labor, early sexual activity, pregnancy before marriage and migration outside the study area at marriage were contributory factors in failure to attend [22], and would be higher than in a non-adolescent population. Six percent were identified as non-menarcheal at end assessment, possibly related to intentional misreporting to gain free treatment [23]. Bias due to different premenarcheal iron requirements would be unlikely as there were equal numbers across study arms, but premenarcheal status reduced the conception rate.

Conclusions

In a high malaria transmission area weekly iron supplementation did not increase malaria risk, improve iron status or reduce anemia in young, mostly adolescent menstruating women, nor in early pregnancy. Iron absorption studies are required to clarify whether chronic malaria influences iron uptake. Baseline characteristics were typical for adolescents in low income, rural sub-Saharan Africa, thus our results should apply to similar malaria-endemic areas with high rates of asymptomatic adolescent malaria. Studies are warranted to improve malaria prevention and control in adolescent populations and to shape relevant interfacing iron deficiency reduction strategies. Iron

supplementation, as routinely given to populations such as this, is not helpful and potentially harmful. WHO Guidelines for universal supplementation in young nulliparous women may need to be re-assessed.

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Author Contributions

Drs S.A.Roberts, B.J.Brabin and S.Gies had full access to all study data and take responsibility for the integrity of the data and accuracy of the data analysis.

Study concept and design: B.J.Brabin, S.Gies, L.Brabin, U.D'Alessandro conceived the study and wrote the protocol.

Acquisition, analysis, or interpretation of data: S.Gies, S.Diallo, A.Kazienga, S.Ouedraogo, D.W.Swinkels, A.J.Geurts-Moespot, Y.Claeys, H.Tinto, B.Faragher, B.J.Brabin.

Drafting of the manuscript: B.J.Brabin, S.A.Roberts, L.Brabin

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Study supervision: S.Gies, S.Diallo, B.J.Brabin.

Conflict of Interest Disclosures

All authors have completed and submitted the ICMJE Form for disclosure of potential conflicts of interest. We declare that we have no conflict of interest.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

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Figure 1 Trial profile

Legend to Figure 1. Stippled arrow indicates the number of women identified in early pregnancy at or within two months of the end assessment survey. Secondary outcome in non-pregnant women is malaria parasitemia.

Figure 2 Mean ferritin concentration and sTfR/log ferritin ratio in non-adherent and adherent women by trial arm

Legend to Figure 2. Boxplots (median, interquartile and 90% ranges) for iron biomarkers in controls and iron-supplemented women at the three assessment points, subdivided by adherence to treatment. Adherence defined as receiving ≥ 80% weekly supplement intake up to ANC1, or end assessment in non-pregnant women. Broken reference lines are median baseline levels.

Table 1: Baseline characteristics of the intention to treat dataset

Characteristic		All Iron n=978	All Control n=976	Non-pregnant Iron n=766	Non-pregnant Control n=783	Pregnant Iron n=258	Pregnant Control n=220
Socio-demographic							
Mean age, years (SD)		16.8(1.7)	16.8(1.8)	16.7(1.7)	16.7(1.8)	17.1(1.7)	17.1(1.7)
Age <20 years, n (%)		78 (93.5)	910/976 (93.2)	719/766 (93.9)	737/783 (94.1)	239/258 (92.6)	199/220 (90.5)
Mossi ethnicity, n (%)		936/978(95.7)	946/975 (97.0)	730/766 (95.3)	757/782 (96.8)	250/258 (96.9)	214/220 (97.3)
	Missing (%)	0/978 (0)	1/976 (0.1)	0/766 (0)	1/783 (0.1)	0/258 (0)	0/220 (0)
Religion, n (%)	Catholic	414/978 (42.3)	405/975 (41.5)	317/766 (41.4)	327/782 (41.8)	118/258 (45.7)	86/219 (39.3)
	Protestant	126/978 (12.9)	120/975 (12.3)	113/766 (14.8)	106/782 (13.6)	18/258 (7.0)	19/219 (8.7)
	Muslim	251/978 (25.7)	250/975 (25.6)	187/766 (24.4)	203/782 (26)	75/258 (29.1)	52/219 (23.7)
	Traditional	187/978 (19.1)	200/975 (20.5)	149/766 (19.4)	146/782 (18.7)	47/258 (18.2)	62/219 (28.3)
	Missing (%)	0/978 (0)	1/976 (0.1)	0/766 (0)	1/783 (0.1)	0/258 (0)	1/220 (0.5)
Education, n (%)	None	595/975 (61.0)	578/974 (59.3)	447/763 (58.6)	450/781 (57.6)	176/257 (68.5)	142/220(64.5)
	Primary	212/975 (21.7)	201/974 (20.6)	168/763 (22.0)	169/781 (21.6)	54/257 (21)	38/220 (17.3)
	Secondary	168/975 (17.2)	195/974 (20.0)	148/763 (19.4)	162/781 (20.7)	27/257 (10.5)	40/220 (18.2)
	Missing (%)	3/978 (0.3)	2/976 (0.2)	3/766 (0.4)	2/783 (0.3)	1/258 (0.4)	0/220 (0)
Literate, n (%)		329/966 (34.1)	347/966 (35.9)	278/756 (36.8)	297/774 (38.4)	68/255 (26.7)	62/218 (28.4)
	Missing (%)	12/978 (1.2)	10/976 (1.0)	10/766 (1.3)	9/783 (1.1)	3/258 (1.2)	2/220 (0.9)

Occupation, n (%) [a]	Student	300/978 (30.7)	317/976 (32.5)	255/766 (33.3)	274/783 (35)	60/258 (23.3)	51/220 (23.2)
	Trading	32/978 (3.3)	31/976 (3.2)	24/766 (3.1)	21/783 (2.7)	10/258 (3.9)	10/220 (4.5)
	Domestic	534/978 (54.6)	515/976 (52.8)	401/766 (52.3)	407/783 (52)	154/258 (59.7)	120/220(54.5)
	Farmer	375/978 (38.3)	380/976 (38.9)	269/766 (35.1)	290/783 (37)	130/258 (50.4)	101/220(45.9)
	Other	5/978 (0.5)	4/976 (0.4)	3/766 (0.4)	2/783 (0.3)	3/258 (1.2)	3/220 (1.4)

Clinical

Menarcheal, n (%)	844/978(86.3)	829/976 (84.9)	639/766 (83.4)	645/783 (82.4)	241/258 (93.4)	203/220 (92.3)
Sexually active, n (%)	249/978(25.5)	241/975 (24.7)	159/766 (20.8)	164/782 (21.0)	98/258 (38)	82/220 (37.3)
Height, cm (SD)	159.0 (6.0)	159.2 (6.0)	158.6 (6.0)	159.1 (6.1)	160.0 (5.7)	159.4 (5.6)
Weight, kg (SD)	50.2 (6.8)	50.2 (7.2)	49.6 (6.9)	49.7 (7.2)	51.6 (6.0)	51.6 (6.7)
BMI, kg/m ^{2} (SD)	19.8 (2.1)	19.7 (2.2)	19.7 (2.1)	19.6 (2.2)	20.1 (1.8)	20.3 (2.1)
BMI <18.5 kg/m ^{2}, n (%)	257/978 (26.3)	277/976 (28.4)	222/766 (29.0)	246/783 (31.4)	46/258 (17.8)	39/220 (17.7)
MUAC [cm] (SD)	23.7 (2.1)	23.7 (2.2)	23.6 (2.1)	23.6 (2.2)	24.1 (1.8)	24.3 (2.2)

Serum Iron Biomarkers

Median CRP, mg/l [IQR]	0.59 [0.23-1.47]	0.51 [0.20-1.35]	0.58 [0.23-1.43]	0.50 [0.18-1.29]	0.72 [0.27-1.64]	0.70 [0.22-1.66]
Missing (%)	13/978 (1.3)	13/976 (1.3)	6/766 (0.8)	12/783 (1.5)	7/258 (2.7)	2/220 (0.9)
CRP ≥10 mg/l, n (%)	38/965 (3.9)	41/963 (4.3)	30/760 (3.9)	31/771 (4.0)	9/251 (3.6)	10/218 (4.6)
Median ferritin µg/l [IQR]	49.0 [28.0-78.0]	49.0 [26.0-82.0]	49.0 [28.0-79.8]	50.0 [27.0-81.0]	48.0 [28.0-74.5]	44.0 [26.0-81.0]

	Missing (%)	12/978 (1.2)	14/976 (1.4)	8/766 (1.0)	12/783 (1.5)	4/258 (1.6)	3/220 (1.4)
Median sTfR , mg/l [IQR]		6.32	6.28	6.32	6.26	6.20	6.34
		[5.08-7.87]	[5.14-7.90]	[5.10-7.95]	[5.09-7.92]	[5.02-7.67]	[5.31-7.80]
	Missing (%)	11/978 (1.1)	18/976 (1.8)	7/766 (0.9)	16/783 (2.0)	4/258 (1.6)	3/220 (1.4)
Median sTfR/log_{10} ferritin		3.80	3.74	3.80	3.69	3.77	3.87
ratio [IQR]		[2.88-5.13]	[2.88-5.36]	[2.88-5.13]	[2.86-5.25]	[2.88-5.13]	[2.94-5.61]
	Missing (%)	14/978 (1.4)	18/976 (1.8)	9/766 (1.2)	16/783 (2.0)	5/258 (1.9)	3/220 (1.4)
Median hepcidin, nmol/l		4.80	4.30	4 .80	4.40	4.90	4.10
[IQR]		[2.00-10.75]	[1.90-10.10]	[1.90-10.75]	[1.90-10.00]	[2.32-10.67]	[1.80-10.70]
	Missing (%)	11/978 (1.1)	16/976 (1.6)	7/766 (0.9)	14/783 (1.8)	4/258 (1.6)	3/220 (1.4)
Elevated hepcidin >10.5nM/l		251/967 (26.0)	222/960 (23.1)	196/759 (25.8)	172/769 (22.4)	65/254 (25.6)	55/217 (25.3)
n, (%) [b]							
	Missing (%)	11/978 (1.1)	16/976 (1.6)	7/766 (0.9)	14/783 (1.8)	4/258 (1.6)	3/220 (1.4)
Iron deficiency, n (%) [c]		105/962 (10.9)	125/961 (13)	84/758 (11.1)	100/770 (13)	25/250 (10)	26/217 (12)
(adjusted ferritin)							
	Missing (%)	16/978 (1.6)	15/976 (1.5)	8/766 (1.0)	13/783 (1.7)	8/258 (3.1)	3/220 (1.4)
Iron deficiency, n (%) [d]		205/964 (21.3)	218/958 (22.8)	162/757 (21.4)	168/767 (21.9)	52/253 (20.6)	55/217 (25.3)
(sTfR/log/ferritin ratio >5.6)							
	Missing (%)	14/978 (1.4)	18/976 (1.8)	9/766 (1.2)	16/783 (2.0)	5/258 (1.9)	3/220 (1.4)

a Multiple answers possible for subsistence activities, ie trading, farming and domestic work

b Hepcidin 95% range for women aged 18-24 years from reference Dutch population was: median 2.6nM; 2.5th percentile 0.7nM; and 97.5th percentile 10.5 nM [15]

c Ferritin < 15 µg/l if C-reactive protein (CRP) < 10 mg/l or, ferritin < 70 µg/l if CRP ≥ 10 mg/l

d Ratio: sTfR (µg/ml) to log_{10} ferritin (µg/l) >5.

Table 2 Malaria at first antenatal visit (ANC1) and at end assessment in non-pregnant nulliparae

Endpoint	Iron	Control	Ratio	P value	Adjusted ratio	P_{adj} [a]
Prevalence - ANC1						
N	163	152				
RDT positive [b], n (%)	95/161 (59.0) [51.3:66.3]	89/152 (58.6) [50.6:66.1]	1.01 [0.84:1.21]	1.000	1.00 [0.98:1.03]	0.718
RDT positive & fever, n (%)	15/163 (9.2) [5.7:14.6]	6/152 (3.9) [1.8:8.3]	2.33 [0.93:5.85]	0.072	2.30 [0.91:5.79]	0.075
Microscopy positive [c], n (%)	86/161 (53.4) [45.7:61.0]	84/152 (55.3) [47.3:62.9]	0.97 [0.79:1.18]	0.820	1.00 [0.97:1.03]	0.975
Clinical malaria [d], n (%)	9/161 (5.6) [3.0:10.3]	4/152 (2.6) [1.0:6.6]	2.12 [0.67:6.75]	0.259	2.14 [0.68:6.76]	0.194
Parasite density, parasites/cmm [e]	2000 [1483:2696]	2244 [1674:3008]	0.89 [0.59:1.35]	0.585	0.89 [0.58:1.36]	0.584
Prevalence - end assessment [a]						
N	441	475				
RDT positive & fever, n (%)	19/432 (4.4) [2.8:6.8]	30/464 (6.5) [4.6:9.1]	0.68 [0.39:1.19]	0.188	0.69 [0.39:1.21]	0.193
Microscopy positive [c], n (%)	189/441 (42.9) [38.3:47.5]	186/474 (39.2) [34.9:43.7]	1.09 [0.93:1.28]	0.282	1.10 [0.95:1.28]	0.180
Clinical malaria [d], n (%)	13/440 (3.0) [1.7:5.0]	22/474 (4.6) [3.1:6.9]	0.64 [0.32:1.25]	0.228	0.67 [0.34:1.31]	0.237
Parasite density, parasites/cmm [e]	302 [248:368]	316 [259:386]	0.96 [0.72:1.27]	0.754	0.96 [0.73:1.27]	0.793
Incidence to end assessment in non-pregnant [f]						
Person-years follow-up, (n)	1114.9 (936)	1121.1 (942)				
RDT positive, (n/N)	0.40 (445/1115)	0.40 (444/1121)	1.01 [0.88-1.15]	0.908	1.01 [0.89-1.15]	0.867
RDT positive & fever, (n/N)	0.16 (174/1115)	0.17 (188/1121)	0.93 [0.76-1.14]	0.494	0.94 [0.76-1.15]	0.525
Microscopy positive, (n/N)	0.25 (282/1115)	0.26 (286/1121)	0.99 [0.84-1.17]	0.919	1.00 [0.85-1.18]	0.971

Clinical malaria [d], (n/N)	0.07 (79/1115)	0.08 (87/1121)	0.91 [0.67-1.24]	0.558	0.92 [0.68-1.25]	0.582
Proportion infected in first year [g]						
Number at risk	978	976				
Number censored at one year, (%)	294 (30.1)	294 (30.1)				
RDT positive & fever, %	13 [11:15]	14 [12:16]	0.89 [0.72:1.11]	0.295	0.91 [0.73:1.13]	0.386
Clinical malaria [d], %	6 [5:8]	7 [5:8]	0.92 [0.67:1.25]	0.588	0.92 [0.68:1.26]	0.617

Square brackets: 95% confidence interval

a Prevalence adjusted for: season at visit date; bed net use up to visit date; antimalarial use in month prior to visit and menarcheal status at baseline. Ratio is risk ratio for iron treatment.

b RDT: Rapid diagnostic malaria test

c Parasite positive on blood smear

d *P.falciparum* blood smear positive and fever ≥ 37.5^{o}C

e Geometric mean [95%CI]

f Incidence per person-year adjusted for: weekly bed net adherence and menarcheal status at baseline; ratio is incidence ratio

g Cox regression model of time to first malaria episode, adjusted for baseline iron deficiency (ferritin), bed net use over observation time, visit and menarcheal status at baseline and stratified by enrolment month; ratio is hazard ratio

Table 3 Anemia and iron biomarkers at ANC1 and at end assessment in non-pregnant nulliparae

Endpoint	n	Iron	Control [a]	Effect [b]	P	Adjusted effect [c]	P_{adj}
ANC1							
Mean hemoglobin, g/dl	314	10.17 [9.96:10.38] (1 missing)	10.22 [9.99:10.46]	-0.06 [-0.36:0.25]	0.722	-0.01 [-0.32:0.3]	0.944
Anemia [d]	314	112/162 (69.1) [61.6:75.7]	107/152 (70.4) [62.7:77.1]	0.98 [0.85:1.14]	0.902	0.96 [0.83:1.10]	0.524
Severe Anemia [e]	314	2/162 (1.2) [0.3:4.4]	4/152 (2.6) [1.0:6.6]	0.469 [0.087:2.524]	0.435	0.443 [0.082:2.401]	0.343
Iron deficiency, (%) (adjusted ferritin) [f]	310	11/160 (6.9) [3.9:11.9]	19/150 (12.7) [8.3:18.9]	0.54 (0.27:1.10)	0.123	0.53 (0.26:1.09)	0.083
Iron deficiency, (%) (sTfR/log ferritin ratio) [g]	312	18/162 (11.1%) [7.1:16.9]	19/150 (12.7%) [8.3:18.9]	0.88 (0.48:1.61)	0.728	0.84 (0.46:1.54)	0.578
Mean ferritin, µg/l	313	91 [78:107] (1 missing)	87 [74:103] (1 missing)	1.05 [0.84:1.31]	0.694	1.07 [0.86:1.33]	0.563
Mean sTfR, mg/l	313	6.0 [5.6:6.4] (1 missing)	5.9 [5.6:6.3] (1 missing)	1 [0.91:1.1]	0.949	1 [0.91:1.1]	0.962
Mean ZPP, µmol/mol Heme	285	105 [100:111] (14 missing)	106 [101:112] (16 missing)	0.99 [0.92:1.07]	0.841	0.99 [0.92:1.07]	0.857
Mean hepcidin, nmol/l	311	3.01 [2.5:3.7] (3 missing)	2.86 [2.3:3.5] (1 missing)	1.05 [0.79:1.41]	0.729	1.07 [0.80:1.43]	0.664
Elevated hepcidin, >10.5nM/l [h]	311	29/160 (18.1) [12.9:24.8]	25/151 (16.6) [11.5:23.3]	1.09 (0.67:1.78)	0.766	1.12 (0.69:1.83)	0.649
Non-pregnant	**n**	**Iron**	**Control**	**Effect [b]**	**P**	**Adjusted effect**	**P_{adj}**
Mean hemoglobin, g/dl	913	12.02 [11.91:12.12] (1 missing)	11.93 [11.82:12.04] (2 missing)	0.09 [-0.07:0.24]	0.261	0.06 [-0.09:0.22]	0.420
Anemia [i]	913	179/440 (40.7) [36.2:45.3]	217/473 (45.9) [41.4:50.4]	0.89 [0.76:1.03]	0.124	0.90 [0.78:1.05]	0.168
Severe Anemia [j]	913	0/440 (0.0) [0.0:0.9]	3/473 (0.6) [0.2:1.8]	NA	0.250	NA	0.996
Iron deficiency	910	38/439 (8.7)	50/471 (10.6)	0.82 [0.55:1.22]	0.369	0.82 [0.55:1.23]	0.336

(adjusted ferritin) [f]		[6.4:11.7]	[8.1:13.7]				
Iron deficiency (sTfR/log ferritin ratio) [g]	909	89/437 (20.4) [16.9:24.4]	97/472 (20.6) [17.2:24.4]	0.99 [0.77:1.28]	1.000	0.99 [0.77:1.28]	0.960
Mean ferritin, µg/l	912	49 [46:53] (2 missing)	51 [47:55] (2 missing)	0.97 [0.87:1.09]	0.632	0.97 [0.86:1.08]	0.564
Mean sTfR, mg/l	909	6.4 [6.1:6.6] (4 missing)	6.5 [6.2:6.7] (3 missing)	0.98 [0.94:1.04]	0.558	0.98 [0.93:1.04]	0.534
Mean ZPP, µmol/mol Heme	914	101 [97:104] (1 missing)	101 [98:105] (1 missing)	0.99 [0.94:1.04]	0.783	0.99 [0.94:1.04]	0.784
Mean hepcidin, nmol/l	909	2.85 [2.5:3.2] (3 missing)	2.83 [2.5:3.2] (4 missing)	1.01 [0.86:1.17]	0.939	1.0 [0.86:1.17]	0.960
Elevated hepcidin, >10.5nM/l [h]	909	70/438 (16.0) [12.8:19.7]	72/471 (15.3) [12.3:18.8]	1.05 [0.77:1.41]	0.785	1.05 [0.78:1.43]	0.736

Square brackets: 95% confidence interval

a N (%) for binary variables, mean [95%CI] for hemoglobin; geometric mean [95%CI] for iron biomarkers
b Risk ratio for binary outcomes; difference between arms for hemoglobin; ratio between arms for iron biomarker levels
c Anemia measures adjusted for: baseline menarche, season at assessment and use of antimalarials in the previous month. Iron measures adjusted for baseline menarche.
d Hemoglobin < 11g/dl
e Hemoglobin <8 g/dl
f Ferritin < 15 µg/l if C-reactive protein (CRP) < 10 mg/l or, ferritin < 70 µg/l if CRP ≥ 10 mg/l
g Ratio: sTfR µg/ml to $\log_{10}$ ferritin >5.6
h Hepcidin 95% reference range for women 18-24 yrs age from reference Dutch population was: median 2.6nM; 2.5th percentile 0.7nm; and 97.5th percentile 10.5 nM, [15]
i Hemoglobin < 12g/dl
j Hemoglobin <7 g/d

Table 4 Serious Adverse Events

	Iron	Control
Adult SAE	**N=978**	**N=976**
Accidental death [1]	0	1
Obstetric death	2	1
Other obstetric SAE [2]	12	7
Severe malaria	27	20
Other death [3]	1	1
Other [4]	12	22
Total SAE	54	52
Infant SAE	**N=231**	**N=206**
Miscarriage	16	11
Stillbirth	13	17
Perinatal death [1]	6	0
Neonatal death	4	3
Infant death	1	1
Death from congenital abnormality [5]	0	1
Other Congenital abnormalities [6] [2]	0	5
Other obstetric SAE [2]	2	0
Other Death [7]	1	0
Total SAE	43	38

[1] P= 0.021 (Fishers exact test)

[2] P=0.011 for all abnormalities

[1] One death from drowning; [2] SOC= Pregnancy, puerperium and perinatal conditions, excluding deaths; [3] Neoplasms: benign, malignant and unspecified, (liposarcoma and thoracic pain); [4] see supplementary file for summary of adverse events; [5] Spina bifida; [6] Club foot; congenital anomaly; polydactyly (all classified as unlikely or definitely not treatment related). Excludes abnormalities associated with stillbirth/miscarriage (one microcephaly); [7] Paralytic ileus