Statistical Analysis Plan

PRENATAL IRON AND MALARIA (PIMAL) STUDY: Safety and efficacy of iron interventions in African pregnant women

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Table of Contents

1 Introduction ................................................................................................................................. 4
  1.1 Definitions .............................................................................................................................. 4
  1.2 Purpose of the analyses ......................................................................................................... 4
2 Participants flow chart .............................................................................................................. 5
3 Analyses populations .............................................................................................................. 5
  3.1 Full Analysis Population .................................................................................................... 6
  3.2 Per Protocol Population ...................................................................................................... 6
  3.3 Safety Population .............................................................................................................. 6
4 Baseline descriptions ............................................................................................................. 6
5 Study endpoints ...................................................................................................................... 7
  5.1 Primary outcome: .................................................................................................................. 7
  5.2 Secondary outcomes .......................................................................................................... 8
  5.3 Justification ......................................................................................................................... 8
6 Primary analysis ..................................................................................................................... 11
  6.1 Efficacy Analyses ................................................................................................................ 12
  6.2 Adjustment for baseline covariates ..................................................................................... 11
7 Subgroup analyses ................................................................................................................ 11
8 Safety Analyses ..................................................................................................................... 13
References..................................................................................................................................... 14
## Abbreviations and Definitions

A list of the abbreviations and acronyms used in the Statistical Analysis Plan (SAP) and their definitions are shown here below in alphabetical order.

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>AE</td>
<td>Adverse Event</td>
</tr>
<tr>
<td>AGP</td>
<td>Alpha-1 acid glycoprotein</td>
</tr>
<tr>
<td>BMI</td>
<td>Body Mass Index</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>Hb</td>
<td>Haemoglobin</td>
</tr>
<tr>
<td>HIV</td>
<td>Human Immunodeficiency virus</td>
</tr>
<tr>
<td>IPT</td>
<td>Intermittent preventive treatment</td>
</tr>
<tr>
<td>IUGR</td>
<td>Intra uterine growth restriction</td>
</tr>
<tr>
<td>NaFeEDTA</td>
<td>sodium ethylenediaminetetraacetate</td>
</tr>
<tr>
<td>NTBI</td>
<td>Non Transferrin Bound Iron</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>SAE</td>
<td>Serious Adverse Event</td>
</tr>
<tr>
<td>SAP</td>
<td>Statistical Analysis Plan</td>
</tr>
<tr>
<td>sTfR</td>
<td>Serum transferrin</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
<tr>
<td>ZPP:Heme ratio</td>
<td>Zinc protoporphyrin to haemoglobin ratio</td>
</tr>
</tbody>
</table>
1 Introduction
A recent trial in children reinforced earlier concerns that supplemental iron can lead to an increased burden of malaria, perhaps through the transient production in plasma of non-transferrin bound iron (NTBI). An expert group convened by the World Health Organization (WHO) recently recommended that iron supplementation in children should be restricted in malaria-endemic areas, but that these restrictions are not to be applied to food fortification with iron. The Kenyan government has recently made mandatory iron fortification of industrially milled flour a legal requirement. Universal iron supplementation continues to be recommended for women during pregnancy and 3 months postpartum. Potential effects of iron on infection are likely to be most pronounced in pregnancy, when iron absorption is very high. This seems to be confirmed by observational studies, which indicate that iron deficiency in parturient women is associated with a marked reduction in the prevalence and density of malarial parasites in the placenta. Such infections have devastating effects on the foetus and neonate, causing low birth weight, intrauterine growth retardation, preterm delivery, spontaneous abortion, stillbirth and neonatal mortality. This study was designed to compare daily high-dose iron (i.e. iron-fortified foods plus iron supplements) versus low-dose iron (i.e. iron-fortified foods only) during pregnancy regarding the presence of \textit{Plasmodium} infection at delivery.

1.1 Definitions
1. \textit{Plasmodium} infection: see Table 4.
2. Patent infection: an infection that results in detectable parasites or parasite antigens based on HRP2 and/ or pLDH dipstick tests.
3. Sub-patent infection: an infection with a negative result upon blood examination by microscopy or \textit{Plasmodium} antigen dipstick test (HRP2 and/ or pLDH) but with a positive result for a PCR test.
4. Gravidity: the number of times a woman reports to have been pregnant, regardless of the outcome of these pregnancies, with twins and other multiple births counted as 1, and including the current pregnancy.
5. Presence of intrauterine growth restriction (IUGR): a fetus whose weight is below the 10th percentile based on a reference population with similar gestational age, or whose birth weight at term is less than 2500g (Galan, Ferrazzi, & Hobbins, 2002);
6. Fever: axillary temperature $\geq 37.5$ °C;
7. Anaemia: haemoglobin concentration <110 g/L (WHO, 2011a);
8. Iron deficiency: plasma ferritin concentration <15 g/L (WHO, 2011b);
9. Iron deficiency anaemia: presence of both anaemia and iron deficiency;
10. Inflammation: either C-reactive protein (CRP) >10 mg/L(Nielsen, Bek, Rasmussen, Qvist, & Tobiassen, 1990) or alpha-1-acid glycoprotein (AGP) >1 g/L(Filteau et al., 1993);

Parasite density was calculated as the median of four counts by microscopic examination of thick blood films (duplicate counts by each of two laboratory technicians).

Haemoglobin concentration was calculated as the mean of duplicate measurements or median values if more than two recordings were available.

1.2 Purpose of the analyses
This plan is restricted to the statistical analysis of the primary study objective as formulated in the original study proposal, i.e. to compare the presence of \textit{Plasmodium} infection in parturient women who received a combination of iron-fortified foods with iron supplements versus iron-fortified foods only. The plan is based on the original proposal, and has been finalised before the treatment code was known/revealed. No interim analyses were planned or done.
2 Participants flow chart

INVITED FOR SCREENING, n = 2015

- No show: n = 211

SCREENED, n = 1804

- Excluded: n = 674
  - Not pregnant, n = 227
  - Carrying multiples, n = 8
  - Gestation age >23 weeks, n = 137
  - Due to medical reasons, n = 16
  - Not willing to comply with the intervention, n = 286

Received chemotherapy with praziquantel and albendazole

- Excluded: n = 660
  - Declined consent by head of household, n = 210
  - No show for randomisation visit, n = 427
  - Haemoglobin concentration <90 g/L, n = 23

RANDOMISED, n = 470

High-dose iron: (daily supplements, 60mg ferrous fumarate, plus fortification*), n =

- Loss to follow-up:
  - Moved away from study area, n =
  - Refused follow-up by study staff, n =
  - No reason given, n =
  - No maternal Plasmodium infection status determined at birth:
    • Miscarriage, n =
    • Home delivery, n =
    • Referral (to other hospital) delivery, n =

Included in full analysis set: n =
- All maternal samples collected at birth, n =
- Placenta samples missing but venous blood collected, n =
- Venous samples not collected but placental samples collected, n =

- Twin pregnancies, n =
- Consumed ≤90% of scheduled supplements, n =

Included in per protocol analysis set: n =

Low-dose iron: (fortification* only), n =

- Loss to follow-up:
  - Moved away from study area, n =
  - Refused follow-up by study staff, n =
  - No reason given, n =
  - No maternal Plasmodium infection status determined at birth:
    • Miscarriage, n =
    • Home delivery, n =
    • Referral (to other hospital) delivery, n =

Included in full analysis set: n =
- All maternal samples collected at birth, n =
- Placenta samples missing but venous blood collected, n =
- Venous samples not collected but placental samples collected, n =

- Twin pregnancies, n =
- Consumed ≤90% of scheduled supplements, n =

Included in per protocol analysis set: n =

* Target fortification level: participants got approximately 9 mg of fortificant iron (27%RNI) as NaFeEDTA per day
3 Analyses populations

3.1 Full Analysis Population

All subjects who were randomised and who a) received at least one dose of the experimental supplements (iron or placebo); b) Plasmodium infection status could be ascertained from blood or placental samples collected at birth or within 48 h after birth. In case of maternal or (unborn) child death, a study subject contributes only to the full analysis population when meeting both criteria (a) and (b) above.

3.2 Per Protocol Population

The per protocol population comprises the full analysis set but restricted to participants who a) consumed >90% of scheduled supplements; and b) had singleton pregnancies.

3.3 Safety Population

All subjects who received the study treatment (including control) and are confirmed as providing complete follow-up data (including information on adverse events).

3.4 General information

In the univariate analysis, variables will be checked for distribution (normal, binomial etc.), central tendency (mean, median, mode), dispersion of the values (standard deviation, variance, range, and quartiles), and the presence of outliers/ extreme values. Similar variables will be compared by use of bar graphs, box plots and by comparing their means and distributions.

In case the intervention was stopped before its prescheduled end, whether by decision of the trial participant or the field team, we will report reasons for this withdrawal. In case data or samples were collected after stopping the intervention, we will include these data or information derived from these samples in the full analysis set. Missing data for haemoglobin concentration at the end of the intervention will be imputed blindly, before starting primary analysis, as the mean, geometric mean or median values obtained from participants without missing values in the same intervention group.

Reporting conventions: We will report confidence intervals when possible. P-values \( \geq 0.01 \) will be reported to 2 decimal places; \( p \)-values less than 0.001 will be reported as “\(<0.001\)”. Means, standard deviations and any other statistics other than quartiles will be reported to one decimal place greater than the original data. Quartiles, such as median, or minimum and maximum will use the same number of decimal places as the original data. Estimated parameters, not on the same scale as raw observations (e.g. regression coefficients) will be reported to 3 significant figures.

4 Baseline descriptions

Data will be summarised by intervention group as n, mean, SD (symmetrically distributed continuous variables); n, median and IQR (skewed continuous variables); or numbers and percentage (categorical variables). Means for non-normally distributed variables will be reported as geometric mean.

The following tables will show the study participants’ characteristics per intervention group.
Table 1: Demographic and biochemical characteristics at baseline

<table>
<thead>
<tr>
<th>Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Height, cm</td>
</tr>
<tr>
<td>Weight, kg</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
</tr>
<tr>
<td>Marital status</td>
</tr>
<tr>
<td>Married or living together</td>
</tr>
<tr>
<td>Divorced or separated</td>
</tr>
<tr>
<td>Never married</td>
</tr>
<tr>
<td>Age, y¹</td>
</tr>
<tr>
<td>Gestational age, weeks ¹</td>
</tr>
<tr>
<td>Gestational age</td>
</tr>
<tr>
<td>1st trimester</td>
</tr>
<tr>
<td>2nd trimester</td>
</tr>
<tr>
<td>Gravidity</td>
</tr>
<tr>
<td>Primigravidae</td>
</tr>
<tr>
<td>Secundigravidae</td>
</tr>
<tr>
<td>Multigravidae</td>
</tr>
<tr>
<td>Current fever (axillary temperature ≥ 37.5 °C)</td>
</tr>
<tr>
<td><em>Plasmodium</em> infection, Table 4</td>
</tr>
<tr>
<td>Current or recent <em>P. falciparum</em> infection, by either HRP2- or LDH-based dipstick</td>
</tr>
<tr>
<td><em>P. falciparum</em>, by PCR</td>
</tr>
<tr>
<td><em>P. falciparum</em>, by HRP2- or LDH-based dipstick or PCR</td>
</tr>
<tr>
<td><em>Plasmodium</em> spp. other than <em>P. falciparum</em>, by LDH-based dipstick or PCR</td>
</tr>
<tr>
<td>Any <em>Plasmodium</em> spp., by dipstick or PCR</td>
</tr>
<tr>
<td>HIV infection</td>
</tr>
<tr>
<td>Haemoglobin concentration, g/L</td>
</tr>
<tr>
<td>Anaemia, haemoglobin concentration &lt;110g/L</td>
</tr>
<tr>
<td>Plasma transferrin concentration, g/L</td>
</tr>
<tr>
<td>Plasma sTfR concentration, µg/L</td>
</tr>
<tr>
<td>Plasma ferritin concentration, µg/L</td>
</tr>
<tr>
<td>Iron deficiency, plasma ferritin concentration &lt;15µg/L</td>
</tr>
<tr>
<td>All</td>
</tr>
<tr>
<td>Restricted to those with CRP&lt;10mg/L</td>
</tr>
<tr>
<td>Restricted to those with alpha-1- acid glycoprotein&lt;1.0g/L</td>
</tr>
<tr>
<td>Restricted to those with CRP&lt;10mg/L or alpha-1- acid glycoprotein&lt;1.0g/L</td>
</tr>
<tr>
<td>Whole blood ZPP:haem ratio, μmol/mol</td>
</tr>
<tr>
<td>Erythrocyte ZPP:haem ratio, μmol/mol</td>
</tr>
<tr>
<td>Erythrocyte FEP concentration , µg/L</td>
</tr>
</tbody>
</table>

5 Study endpoints

5.1 Primary outcome:
The primary outcome is defined as the presence of maternal *Plasmodium* infection in samples collected at parturition, regardless of species, as indicated by one or more positive results for the presence of parasite-derived LDH or HRP2 in plasma (dipstick tests), past infections (as indicated by *Plasmodium* pigment upon histological examination of placental biopsies), or
Plasmodium DNA (any species, by PCR test) (Table 2, case 1). We excluded the results of microscopy from this case definition because these results would be considered incorrect if discordant with results from PCR tests.

5.2 Secondary outcomes
As secondary outcomes, we will consider the following indicators:
- Patent and sub-patent Plasmodium infection (Table 2, cases 2 and 3 respectively);
- Current or recent Plasmodium infection (Table 2, case 4);
- Birth weight (g);
- Gestational age at delivery (weeks);
- The presence of intrauterine growth restriction (IUGR);
- Maternal and neonatal iron status at 1 month after delivery, assessed by haemoglobin concentrations, iron deficiency anaemia and iron stores (ratio of ferritin:transferrin receptor concentrations); with and without restriction to those with inflammation.

5.3 Justification
When formulating our outcome definitions, we considered Plasmodium infections in pregnant women to be important in so far as they are associated with adverse maternal or pregnancy outcomes. However, the pathological consequences of maternal infection likely depends on population-specific factors such as immunity (and thus transmission intensity, parity and maternal age), access to treatment, coverage and quality of antenatal services and drug resistance. Discordant results between studies in the association between maternal Plasmodium infection and adverse maternal or pregnancy outcomes are also likely due to differences in the sensitivity and interpretation of diagnostic tests (Uneke, 2007).

Histological examination of placental biopsies is often considered the ‘gold standard’ of malaria diagnosis in pregnancy, and results in infection status being classified into four categories depending on the presence and distribution of Plasmodium parasites and haemozoin (Bulmer, Rasheed, Francis, Morrison, & Greenwoods, 1993):
  a) Current infection: parasites are present in the intervillous spaces with or without pigment in intervillous monocytes;
  b) Chronic infection: parasites are present in the intervillous spaces along with pigment as deposits or in macrophages within fibrin;
  c) Past infection: pigment is present in the absence of parasites; and
  d) Not infected.

Pigment deposition has generally been found to be associated with low birth weight, particularly in the presence of parasites in the intervillous spaces (Menendez et al., 2000). There is uncertainty, however, about the effect of past infections (i.e. with pigment deposition but without parasitaemia), on neonatal outcomes: such infections have been found to be associated with reduced birth weight in several studies Walter, Garin, Blot, & Philippe, 1981, Watkinson & Rushton, 1983, Rogerson et al., 2003, but not in others (Matteelli et al., 1996, Menendez et al., 2000, Muehlenbachs, Mutabingwa, Fried, & Duffy, 2007, Muehlenbachs et al., 2010).

PCR is considerably more sensitive in detecting maternal infection than either microscopy or rapid dipstick tests that assay the presence of Plasmodium-specific proteins (e.g. HRP2 or LDH). Several studies have found that sub-patent infections (i.e. low parasitaemias that are detected by PCR but not by microscopy or dipstick tests) are associated with reduced birth weight ((Malhotra, Dent, Mungai, Muchiri, & King, 2005), (Adegnika et al., 2006), (Arango,
Maestre, & Carmona-Fonseca, 2010), (Mohammed et al., 2013)) but others have not ((Mankhambo, Kanjala, Rudman, Lema, & Rogerson, 2002), (Mockenhaupt et al., 2006), (Rantala et al., 2010)). With regards to Plasmodium species other than *P. falciparum*, we are not aware of studies on the maternal or pregnancy outcome of *P. malariae* or *P. ovale* infection during pregnancy. *P. vivax* is also associated with low birth weight but most of these infections cause symptoms ((Luxemburger et al., 2001), (Nosten, ter Kuile, Maelankirri, Decludt, & White, 1991), (Nosten et al., 1999)) and this species rarely occurs in Africa.
Table 2. Case definitions of *Plasmodium* infection

<table>
<thead>
<tr>
<th>Case</th>
<th>HRP2-based dipstick test</th>
<th>LDH-based dipstick test</th>
<th>Microscopy of peripheral blood&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Placental biopsy</th>
<th>PCR, peripheral blood</th>
<th>PCR, maternal placental blood</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>P. falciparum</em></td>
<td>Other spp.</td>
<td><em>P. falciparum</em></td>
<td>Pigment</td>
<td>Parasites&lt;sup&gt;2&lt;/sup&gt;</td>
<td><em>P. falciparum</em></td>
</tr>
<tr>
<td>Case 1 (primary outcome)&lt;sup&gt;3&lt;/sup&gt;</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>○</td>
<td>○</td>
<td>●</td>
</tr>
<tr>
<td>Case 2 (patent)</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>○</td>
<td>○</td>
<td>●</td>
</tr>
<tr>
<td>Case 3 (sub-patent)</td>
<td>○</td>
<td>○</td>
<td>○</td>
<td>○</td>
<td>○</td>
<td>○</td>
</tr>
<tr>
<td>Case 4 (current or recent)</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>○</td>
<td>○</td>
<td>○</td>
</tr>
</tbody>
</table>

Test with positive (●) or negative (○) results are linked through 'OR' Boolean operators.

<sup>1</sup>Regardless of parasite stage (i.e. asexual or sexual stages).
6 Primary analysis
The primary measure of intervention efficacy will be the difference in prevalence between intervention groups, calculated using Newcombe’s method (Altman, Machin, Bryant, & Gardner, 2000) in the per protocol population (see section 3.2.). We will also report the prevalence difference as a percentage relative to the placebo group, as well as the prevalence ratio.

7 Secondary analyses

7.1 Adjustment for baseline covariates
We will assess possible confounding by comparing crude and adjust prevalence ratios, the latter being obtained by including baseline covariates that may be prognostic for outcome in a Cox regression analysis with constant time at risk (Lee & Chia, 1993), (Lee, 1994; Osborn & Cattaruzza, 1995), (Barros & Hirakata, 2003). These covariates are divided into two groups:

a) Variables that are likely to be prognostic for the primary outcome, as predefined on the basis of the literature review:
- Gravidity (Desai et al., 2007)): entered as a dummy-coded categorical variable with 3 classes: primigravidae, secundigravidae, multigravida;
- Maternal age (Desai et al., 2007) (Tako et al., 2005): dummy-coded categorical variable with 2 classes: < 20 years and ≥ 20 years;
- HIV infection status (ter Kuile et al., 2004) : binary variable.

b) Variables that are possibly prognostic for the primary outcome:
- Plasmodium infection status (any Plasmodium spp., by dipstick or PCR): binary variable;
- Iron status: haemoglobin concentration as continuous variable centred around the mean as well as plasma ferritin concentration as a binary variable (<12 μg/L or ≥12 μg/L);
- Gestational age at delivery (continuous variable, centred around the mean).

We will examine the influence of these factors using a backward elimination procedure from a full model that includes all of the above variables. When judging the degree of confounding, a diversion of >15% of the crude prevalence ratio will be considered to be of public health importance. When the potential value of this adjustment is in doubt, we will consider the unadjusted analysis as the one of primary attention, and the adjusted analysis being supportive.

7.2 Full analysis set
An exploratory analysis will be conducted on the full analysis set.

7.3 Subgroup analyses
As a first step, we will use stratified analysis to assess to what extent the effect of iron on Plasmodium infection is influenced by gravidity, age, HIV infection and iron status, indicated by anaemia and iron deficiency (Table 3). In this analysis, we will consider variables with the following categories: gravidity: primigravidae, secundigravidae and multigravidae; age: ≤20 years, >20 years; HIV infection: infected, non-infected.
In a second step, we will assess such effect modification directly using multivariate logistic regression analysis.

We will interpret the results of subgroup analysis cautiously (Assmann, Pocock, Enos, & Kasten, 2000), (Wang, Lagakos, Ware, Hunter, & Drazen, 2007),(Fletcher, 2007; Oxman, 1992; Sun, Briel, Walter, & Guyatt, 2010; Yusuf, 1991).

In the analysis of haemoglobin concentrations in maternal blood at 1 month post-partum, we expect little or no treatment effect in individuals who are iron replete, because iron absorption is known to decrease with the magnitude of the iron stores (Hallberg, 2001).

<table>
<thead>
<tr>
<th>Effect modifier</th>
<th>Justification</th>
</tr>
</thead>
<tbody>
<tr>
<td>a. Gravidity</td>
<td>In highly endemic areas, primigravidae are at greater risk of malarial infection (Nosten et al., 1991) (Greenwood, Armstrong, Byass, Snow, &amp; Greenwood, 1992), probably because they have a reduced immunity. Thus we expect that their ability to suppress a possible increase in parasitaemia resulting from iron to be reduced.</td>
</tr>
<tr>
<td>b. Age</td>
<td>Plasmodium infection is expected to be higher in women ≤20 years old. Young primigravidae and multigravidae are at greater risk of malaria and its adverse effects than older primigravidae or multigravidae, respectively(Espinoza, Hidalgo, &amp; Chedraui, 2005; Leenstra et al., 2003; Marques et al., 2005)(S J Rogerson et al., 2000)(Walker-abbey et al., 2005). This is probably because age-associated immunity plays a role in controlling malaria infection during pregnancy in highly endemic areas. We expect that women ≤20 years old have a reduced ability to suppress a possible increase in parasitaemia resulting from iron.</td>
</tr>
<tr>
<td>c. HIV infection</td>
<td>HIV infection compromises malarial immunity such that HIV infected multigravidae have at least as high a risk of placental infection as non-HIV-infected primigravidae (Desai et al., 2007). HIV exacerbates the burden of malaria and increases the degree to which malaria is associated with maternal severe anaemia. We expect that due to compromised malarial immunity, women that are HIV infected will be more susceptible to iron-induced <em>Plasmodium</em> infection.</td>
</tr>
<tr>
<td>d. Iron status at baseline</td>
<td>Low iron status at baseline may be protective against malaria infection but will result in increased absorption of supplemented iron. The vice versa is also true thus we expect the effect of iron supplementation on malaria parasitaemia to be influenced by iron status at baseline.</td>
</tr>
</tbody>
</table>

### 7.4 Intermittent preventive treatment (IPT)

Intermittent preventive treatment data was obtained retrospectively from the participants ante-natal clinic attendance booklets. The information in these booklets is filled in by nurses during the antenatal clinic visits. Because IPT data was recorded after randomisation, theoretically, it could be influenced by the intervention and thus it is considered separately in the analysis. We will examine whether intermittent preventive treatment has an effect on malaria parasitaemia at birth.
Intermittent preventive treatment will be a categorical variable with 5 classes: no dose (participants whose booklets were available but had no data on IPT thus no IPT dose received), single dose, two doses, three doses, unknown (participants whose booklets were not available).

7.5 **Efficacy Analyses**

The most important indicator for evaluating intervention benefits will be haemoglobin concentration at 1 month post-delivery. Group differences in haemoglobin concentration will be measured by linear regression analysis, adjusting for possible group differences in time between delivery and blood collection (centred around 30 days post-partum). We will assess possible confounding of the intervention effect by comparing the difference in mean haemoglobin concentration with and without adjustment for baseline haemoglobin concentration (continuous variable). We will also assess the influence of haemoglobin concentration at baseline (continuous variable) on the haemoglobin response to the iron intervention, using multivariate linear regression analysis.

We will use similar procedures to assess intervention effects on plasma ferritin concentration at 1 month post-delivery.

All analyses of the continuous efficacy variables will be performed as analysis of variance with treatment group adjusting for inflammation etc. All assumptions for regression models will be assessed by viewing plots of the residual values. Dependent variables will be log-transformed as appropriate.

8 **Safety Analyses**

Summary statistics per intervention group will be produced for deaths and serious adverse events that are judged to be related to the treatment. Each subject will only be counted once and any repetitions of adverse events will be ignored; the denominator will be the total population size.
References


