Muhimbili University of Health and Allied Sciences
Department of Parasitology and Medical Entomology
School of Public Health and Social Sciences
P O Box 65011 •Dar es Salaam •Tanzania
Telephone: 255 51 153419 Fax: Fax 255-51-150563

1 GENERAL INFORMATION- FULL PROTOCOL	
☐ **Initial** ☒ **Revised; OHRA Protocol #: R01 HD 57941-01A2**	
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Principal Investigator	**Zul Premji, MD, Msc, PhD**
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Introduction

An estimated 1.62 billion people, or more than a quarter of the world's population, are anemic [1]. Anemia is common in pregnancy, and increases the risk of preterm delivery, low birth weight, and perinatal mortality and morbidity [2]. Iron deficiency, the most widespread nutritional problem in the world, is also the leading cause of anemia during pregnancy, and prenatal iron supplementation is standard of care in most countries [3]. However, there is limited evidence from randomized trials in developing regions in support of the safety of this intervention. There are some particular concerns regarding the use of iron supplementation in women who are not iron deficient. Findings from recent randomized trials among children have raised concerns regarding the safety of iron supplementation in malaria-endemic regions [4]. The safety and efficacy of prenatal iron supplementation is similar areas of high malaria burden need to established, particularly among women who are not anemic or iron deficient [4-12].

In order to address this important research gap, we propose a randomized controlled trial among pregnant women in Tanzania. Iron deficiency and malaria are major public health problems in Tanzania. More than 90% of the population resides in malaria-endemic regions, and an estimated 1.7 million women are affected by malaria during pregnancy each year [13]. Together, iron deficiency anemia and malaria represent the leading cause of maternal mortality, accounting for over a quarter of all maternal deaths [14]. We propose to enroll women who not anemic and not iron deficient in a placebo-controlled trial to examine the safety and efficacy of 60 mg daily iron prenatal supplement. In light of documented benefits of iron supplementation among anemic women, those who are severely anemic (defined in Tanzania as Hb < 8.5 g/dL) or iron deficient (serum ferritin < 12 μg/L) will receive iron, as per standard of care, and will not be enrolled in the trial. We will use the cutoff level of < 8.5 g/dL as the threshold for providing iron supplementation to study participants, in accordance with Tanzanian national guidelines. In our study population, the causes of anemia are multifactorial and may include folate deficiency, vitamin B12 deficiency, zinc deficiency, parasitic infections, other chronic infections and inflammations, and hemoglobinopathies such as thalassemia and sickle cell disease [15]. Because we will also be excluding women who are iron deficient at baseline, it is likely that anemia between 8.5 and 11.0g/dL will be largely attributable to these other underlying factors rather than iron deficiency and these participants are unlikely to respond to iron supplementation.

Specific Aims

Primary Aims

1. To determine the **safety** of iron supplementation among pregnant Tanzanian women, as measured by **(a) the incidence of placental malaria**, and **(b) placental malaria parasite density,** compared to placebo.

2. To determine the **efficacy** of iron supplementation among pregnant Tanzanian women, as measured by **maternal hemoglobin** concentrations, compared to placebo.

3. To determine the **efficacy** of iron supplementation among pregnant Tanzanian women, as measured by infant **birth weight**, compared to placebo.

Secondary Aims

1. To determine the **efficacy** of iron supplementation among pregnant Tanzanian women, on the **incidence of maternal anemia,** compared to placebo.

2. To determine the **efficacy** of iron supplementation among pregnant Tanzanian women, on the **incidence of low birth weight,** compared to placebo.

3. To determine the **efficacy** of iron supplementation among pregnant Tanzanian women, on the **incidence of maternal malaria infection,** compared to placebo.

4. To determine the **efficacy** of iron supplementation among pregnant women on **infant hemoglobin concentrations at 6 weeks,** compared to placebo.

5. To determine the **efficacy** of iron supplementation among pregnant Tanzanian women on **infant malaria parasitemia at 6 weeks**, compared to placebo.

6. To determine the **adherence** to iron supplementation intake by pregnant women compared to placebo**.**

Background and Significance

Burden of Iron Deficiency

Iron deficiency is the most common form of anemia worldwide; approximately 50% of anemia is attributable to iron deficiency [16-18]. Iron deficiency has a detrimental impact on human health, nutrition, and socioeconomic development. Globally, iron deficiency accounts for 841,000 deaths and 35,057,000 disability-adjusted life years lost, and ranks ninth globally among the 26 leading risk factors worldwide [19]. Pregnant women are particularly vulnerable to iron deficiency and anemia, and anemia is the most common medical disorder in pregnancy. Approximately 41.8% of pregnant women (56 million) are anemic worldwide [1], with iron deficiency anemia accounting for the majority of cases. Anemia has been related to increased maternal morbidity and mortality and adverse perinatal outcomes [20-27] and reduced cognitive development and functioning [28-30] and work capacity [30, 31]. Iron deficiency anemia is a severe public health problem in malaria-endemic countries of Africa. Approximately 17.2 million pregnant women and 83.5 million children under five are anemic in sub-Saharan Africa [1]. Iron deficiency contributes to 22% of all maternal mortality (30-44 years), and 33% of all perinatal mortality [32].

In Tanzania, iron deficiency is also recognized as a severe public health problem. According to the National Bureau of Statistics Tanzania Demographic and Health Survey 2004–05 and the WHO Global Database on Anemia, 58.2% (54.0-62.3%) of pregnant women and 47.2% (45.8-48.7%) of non-pregnant women (15-49 yrs) are anemic (Hb < 11 g/dL) in Tanzania [1, 33]. Anemia during pregnancy has been found to be a significant public health burden in both rural and urban settings of Tanzania. An estimated 97,000 disability adjusted life years are lost due to iron deficiency anemia annually in the country [19].

Burden of Malaria and its Complications

An estimated 350 to 500 million cases of clinical malaria occur annually worldwide, resulting in more than one million deaths. The majority of the global burden of malaria resides in sub-Saharan Africa, where 10% of the world's population accounts for approximately 60% of clinical cases and 90% of deaths [34-36]. Malaria in pregnancy is a major public health problem in sub-Saharan Africa. An estimated 25 million women become pregnant in malaria-endemic areas in Africa, leading to 200,000 infant deaths each year [37]. In particular, *P. falciparum* is more frequent and of higher parasite density in pregnant than non-pregnant women [38], and is associated with increased risks of maternal anemia prematurity and low birth weight.

In Africa, the prevalence of maternal malaria and placental malaria are high. In a recent review of 20 studies from eight African countries, the median prevalence of maternal malaria infection (defined as peripheral or placental infection) in all gravidae was 27.8% [37]. A review by Guyatt produced a similar estimate of 26% for placental malaria (range 5–52%) [39]. Prevalence of malaria infection has been found to be higher among primigravida [40-43]. In a study from Kenya, among primigravida, 23% had peripheral malaria, 22% had placental malaria based on smears, and 64% had histological evidence of placental malaria. The corresponding numbers for secundigravidae were 18%, 14% and 54% [44]. In the same study, histologic malaria was associated with significantly higher risks of anemia and low birth weight.

The most severe consequences of malaria in pregnancy are low birth weight, maternal anemia and maternal death [45]. The association between maternal malaria and low birth weight has been recognized for at least fifty years [46, 47]. Several observational studies in Africa have confirmed differences between the mean weight of newborns from mothers whose blood and/or placenta appeared infected with malaria and babies from uninfected mothers [38]. The differences in birth weight range from around 50 to 300 grams, according to the setting, season and population under study [48-51]. In areas of high malaria transmission, placental malaria infection is associated with a two-fold increase in the risk of low birth weight [39]. The greatest effect of placental malaria infection is observed among primigravida; the odds ratio of low birth weight associated with malaria is two to seven times higher in primigravida than multigravidae women [52]. In sub-Saharan Africa, nearly 20% of low birth weight deliveries are attributable to malaria in pregnancy, which accounts for 35% of all preventable low birth weight. Malaria-related low birth weight is estimated to account for between 62,000 and 363,000 infant deaths every year in Africa, or three to 17 deaths per 1000 live births [53]. An estimated 11.4% of neonatal deaths and 5·7% of all infant deaths in malaria-endemic areas of Africa are attributable to malaria-related low birth weight [54]. This effect was greatest among infants born to primigravida at 17·6% of neonatal deaths and 9·8% of infant deaths. Malaria in pregnancy in these settings may be responsible for up to 70% of intrauterine growth restriction, whereas its contribution to preterm delivery, although still substantial, is relatively lower at up to 36% % [37]. Low birth weight associated with malaria in pregnancy is estimated to result in 100,000 infant deaths in Africa each year [55].

A recent review of nine mainly hospital-based studies showed that placental malaria was associated with twice the risk (odds ratio 2·19) for stillbirth [57]. Additionally, the risk of all-cause anemia is estimated to be approximately three-fold higher among infants born to mothers with placental malaria infection [57-59]. Further, in a study in Tanzania, infants born to women with placental malaria infection had a 41% increased risk of malaria infection [60]. In a meta-analysis of 20 studies from Africa, *Plasmodium falciparum* malaria in pregnancy contributed to anemia and low birth weight through both preterm-LBW and IUGR-LBW in a relatively consistent fashion across different studies and settings. The prevalence of malaria infection in pregnancy ranged from approximately 10% to 65% across the settings where these associations were observed. Estimates of malaria's contribution to LBW were modest and consistent across studies—accounting for approximately 8–14% of LBW and IUGR-LBW and approximately 8–36% of preterm LBW [37].

In addition to adverse outcomes during the fetal period and in infancy, maternal anemia is another major complication of malaria during pregnancy. An estimated 26% of severe anemia is attributed to malaria, among pregnant women of all gravidities [61]. About 70% of third trimester women in a Kenyan study had hemoglobin levels <11 g/dl [43] while in an urban population in Blantyre, Malawi, 57% of women had hemoglobin <11 g/dl at their first antenatal visit [62]. Malaria in pregnancy is also an established risk factor for maternal mortality. The percentage of direct and indirect malaria-related maternal deaths range from 0·5% to 23·0% in hospital studies and from 2·9% to 17·6% in community-based studies [63].

Studies of intervention with chemoprophylaxis aimed at decreasing malaria infection during pregnancy have shown that such an action is beneficial for birth weight, especially in primigravida. Birth weight differences between treatment and control groups in primigravida range from 85 to 252 grams, and in multigravidae from 28 to 149 [64-66]. In Cot *et al*'s trial of chloroquine to prevent malaria in primigravidae in Cameroon [67], a significant mean difference of 207 grams in birth weight was found between treatment and control groups (230 g after adjustment for imbalances in the distribution of potential confounders among groups). There was an adjusted 80% reduction in the proportion of low birth weight associated with the use of prophylactic chloroquine. In Malawi [68], prophylaxis with mefloquine was significantly more effective in preventing low birth weight than chloroquine (12.5% vs. 15.5%). This protective effect was attributed to the reduction on placental and umbilical cord blood malaria infection. The authors state that malaria prevention programs for pregnant women in hyper-endemic areas may reduce the amount of preventable low birth weight by 30% or more. Meta-analyses of intervention trials suggest that successful prevention of malarial infections reduces the risk of severe maternal anemia by 38%, low birth weight by 43%, and perinatal mortality by 27% among paucigravidae [55].

Despite a comprehensive national malaria control program, malaria is still a major public health problem in the United Republic of Tanzania. Dar es Salaam is characterized as an area with endemic and perennial malaria, with malarial transmission occurring throughout the entire year [14]. *P. falciparum* accounts for more than 95% of all malaria infections in Tanzania, and an estimated 1.7 million women are affected by malaria during pregnancy each year [13]. More than 90% of the population lives in malaria-endemic areas, and malaria is the leading cause of outpatient and inpatient health service attendance and the leading cause of death in both children and adults [14]. There are an estimated 14 to 18 million new malaria cases being reported each year resulting in 100,000 to 125,000 deaths annually. Combined with severe anemia, malaria is the leading cause of maternal mortality, accounting for more than 25% of maternal deaths [14].

Iron Requirements during Pregnancy

During pregnancy, iron requirements are increased in order to support maternal metabolism, iron transfer to the fetus, and fetal growth and development [69, 70]. The total iron cost of pregnancy is estimated at 1040 milligrams, and iron demands during pregnancy are approximately two times greater than the non-pregnant state [71]. Iron deficiency anemia accounts for the majority of anemia cases in pregnancy; more than 90% of the iron deficiency anemia in pregnancy is due to depleted iron stores associated with parasitic infections and inadequate dietary intake [1]. Low bioavailability of iron is also a major etiological factor in the development of iron deficiency. In general, non-heme iron (found in plants and fortified foods) is less bioavailable and more sensitive to other foods ingested in the same meal (e.g., ascorbic acid, phytic acid), compared to heme iron (found in animal food sources)[72]. In resource-limited settings, bioavailable iron may comprise as little as five percent of the diet, compared to 18 to 25% in typical Western diets [73]. Socioeconomic factors such as prohibitive cost, food insecurity, low availability of heme iron sources, and vegetarian dietary patterns contribute to low intake and bioavailability of dietary iron. Additionally, the presence of infectious diseases such as malaria, intestinal helminthes, and chronic inflammation, may further elevate iron requirements and compromise intestinal iron absorption.

Anemia may be due to inherited hemoglobinopathies, of which sickle cell disease and thalassemia are the most common disorders. The sickle-cell gene is distributed widely throughout sub-Saharan Africa, where carrier frequencies range from 5% to 40% or more of the population. In Tanzania, data on carrier frequency is scarce, but it is estimated that the carrier rate for sickle cell disease is 13-15%. The sickle cell mutation in the β-globin gene may present as either heterozygous (sickle cell trait) or homozygous (sickle cell disease). The homozygous state of the sickle-cell gene leads to changes in the shape of erythrocytes that shorten their survival and render them unable to pass through capillaries, leading to anemia and vaso-occlusion. In contrast, the heterozygous state confers a protective effect against malarial disease [97].

Due to increased iron requirements during pregnancy, the Recommended Dietary Allowance for iron intake for pregnant women is 27 milligrams per day [73]. However, due to inadequate intake and bioavailability of dietary iron and other essential nutrients, iron requirements may be difficult to achieve *via* diet alone [3, 74, 75]. Current World Health Organization guidelines are 60 milligrams of supplemental elemental iron for all pregnant women, once daily to prevent anemia and twice daily to treat anemia during pregnancy [32]. However, these guidelines were developed before evidence accumulated on the possible risks associated with iron supplementation, particularly among individuals not suffering from iron deficiency and living in malaria-endemic areas, as reviewed below.

Biomarkers of Iron Status

Iron deficiency anemia is generally defined as low serum transferrin saturation (<15%), a low serum ferritin concentration (< 12 μg/L), and an elevated soluble transferrin receptor (> 6 mg/dL) in the context of microcytic anemia [72]. There are a variety of biomarkers available to assess iron status. However, some of the indicators are invasive, expensive, and/or technically demanding, and may fluctuate in the context of dietary iron intake, pregnancy, or inflammation. For example, stainable iron in bone marrow aspirate is the gold standard method for iron status assessment, and hepatic iron stores are definitive markers for iron stores; however, these techniques are invasive and not feasible in resource-poor field settings. Hemoglobin concentration and serum iron are not optimal indicators of anemia, since body iron stores can be depleted in the presence of normal hemoglobin levels, and serum iron can vary with short-term dietary intake of iron as well as with acute infections. Hemoglobin or serum ferritin may also be altered in the context of pregnancy or infectious diseases, due to hemodilution or inflammation.

However, serum ferritin represents a comparatively better indicator of iron stores and iron deficiency anemia, and is less costly and invasive compared to bone marrow aspirate methods.

Hemoglobin: Hemoglobin is the most commonly used diagnostic indicator for anemia, due to its low cost and feasibility. According to the World Health Organization, anemia is defined as hemoglobin levels below 11 g/dl for pregnant women, and less than 12 g/dl for non-pregnant women [3]. In Tanzania and other countries in sub-Saharan Africa, 8.5 g/dl is conventionally used as a cut-off for anemia. However, hemoglobin does not reflect iron stores, and varies due to physiological changes such as hemodilution. For example, low hemoglobin concentrations at the end of the second trimester are considered normal and even beneficial because they signify appropriate plasma volume expansion (hemodilution), unless the mean corpuscular volume is reduced [76]. Both hemoglobin and hematocrit have low sensitivity and are not as informative as other methods of iron status assessment. These methods are, however, simple and inexpensive to perform and feasible in resource-poor settings. Iron stores, hemodilution, and inflammation are important factors to consider when interpreting hemoglobin values in the context of pregnancy.

Serum Ferritin: Serum ferritin is an excellent indicator of iron status of populations [72]. The WHO Consultation considered serum ferritin to be the best indicator of total body iron stores, depleted iron stores, defined as 12 μg/L (approximately 100 mg of storage iron) [71]. Although a higher cut-off of 30 μg/l has been proposed for iron deficiency assessment in the presence of infection for children under five [77] and pregnancy [74,78], few studies have examined this cut-off in pregnant women in developing countries. The cutoff of SF<12 has been used to define iron deficiency in several studies among HIV-uninfected pregnant women in sub-Saharan Africa [79-83]. As an acute phase protein, serum ferritin concentrations increase in response to inflammation, even if iron stores are low [72]. Therefore, in settings with high infectious disease burden, the measurement of an indicator of inflammation can further interpretability of serum ferritin as a marker of iron stores. Utilization of an acute phase protein may improve the interpretability and utility of serum ferritin as an indicator of iron status. Serum ferritin cutoffs have also been proposed for iron overload among men (>200 μg/l,>300 μg/l) [84, 85] and women (>150 μg/l, >200 μg/l) [84-86].

Serum Transferrin Receptor: Serum transferrin receptor is derived from the formation of red blood cells, and reflects the intensity of erythropoiesis and iron demand. Concentrations of serum transferrin receptor are elevated when tissue iron levels are low. The concentration of transferrin receptor rises in iron deficiency anemia; it is a marker of iron deficiency severity when iron stores have been exhausted, and there are no other causes of erythropoiesis. Reference ranges are 2.2 to 5.0 mg/L for men and 1.9 to 4.4 mg/L for women; a cutoff level of >8.5 mg/l has been proposed for serum transferrin receptor to characterize tissue iron deficiency [78]. However, this criterion has not been validated in pregnant women or resource-limited settings. Increased erythrocyte production, such as in malarial infection, may also elevate serum transferrin receptor concentrations. Therefore, it is important to assess inflammatory markers, in order to rule out inflammation when using serum transferrin receptor as an indicator of iron status [62]. Appropriate cutoff levels need to be established for pregnant women, particularly in regions of high malarial burden.

Hepcidin: Recent studies have identified a prominent role for hepcidin in the etiology of anemia of inflammation [87, 88]. Hepcidin is a liver-derived peptide regulator of iron homeostasis [89-91]. Hepcidin inhibits ferroportin, a protein that transports iron from storage cells, as an essential mediator of hypoferremia. Hepcidin acts at several levels of erythropoiesis, and may account for the hypoferremia associated with inflammation and other clinical features of anemia of inflammation [87, 88]. Hepcidin levels are increased in inflammation and act to suppress iron

absorption, ferroportin activity, and release of iron from storage sites. The overall result is a chronic hypoproliferative anemia with classic changes in iron metabolism and reduced survival time of red blood cells [87]. Hepcidin inhibits the efflux of iron from cells such as enterocytes, hepatocytes, and macrophages, which decreases intestinal iron absorption and serum iron levels, and reduces availability of iron stores to pathogens [92]. Reference ranges for hepcidin have been identified as 58.9 to 158.1 ng/mL; however, cut-offs have not been established for pregnant women, particularly in areas of high infectious disease burden.

Zinc Protoporphyrin: Zinc protoporphyrin has been identified as a potential indicator of iron status. Zinc protoporphyrin is a measure of iron deficiency severity, and reflects a shortage in iron supply at the final stages of hemoglobin production. In a study of children from Zanzibar, zinc protoporphyrin was significantly associated with hemoglobin concentration, and less affected by malarial infection, compared to serum ferritin or transferrin receptors [96]. However, malarial infection and recent fever were slightly associated with zinc protoporphyrin, suggesting that this indicator may be somewhat affected by the acute phase response [96]. Zinc protoporphyrin and zinc protoporphyrin/hemoglobin ratios are potential indicators of iron status that need to be further explored in other populations in malarial endemic areas. However, the threshold for zinc protoporphyrin needs to be established in pregnant women and resource-limited settings, and prohibitive cost remains challenging for large-scale implementation in field settings [93].

Inflammatory Markers: Inflammatory cytokines such as TNF and interferon-γ may play a role in iron metabolism, such as inhibition of erythropoiesis and ferroportin expression. Both serum ferritin and serum iron are acute phase reactants to inflammatory cytokines; the presence of inflammation and other infections therefore needs to be considered in the diagnosis of iron deficiency [92]. However, according to the WHO, further research is needed regarding the relationship between serum ferritin, transferrin receptor, and different acute phase proteins, in order to identify optimal biomarkers for iron status in the context of endemic infectious diseases [93]. Several acute phase proteins have been identified to facilitate assessment of iron status in the context of inflammation. For example, C-reactive protein, α-1-antichymotrypsin, α-1 acid glycoprotein, serum amyloid A, fibrinogen and haptoglobin have been used in the interpretation of serum ferritin levels in the context of inflammation. C-reactive protein (CRP) is the most commonly used acute phase protein in iron status assessments. CRP is highly sensitive to inflammation but also subsides rapidly in concentration; α-1-antichymotrypsin has also been identified as a potential acute phase protein in iron assessment; levels of α-1-antichymotrypsin also rise rapidly in response to inflammation, but remain at a high concentration longer than C-reactive protein. In contrast, α-1 acid glycoprotein is slower to respond to inflammation than C-reactive protein or α-1-antichymotrypsin but remains at a high concentration for longer than either indicator [94, 95]; therefore it may be a better indicator of changes in iron status in the context of sub-clinical infections and chronic inflammation. C-reactive protein is, however, the most commonly measured acute phase protein in field settings, and is the only acute phase protein with established international reference standards.

In summary, accurate, sensitive, affordable, and feasible methods are needed to assess iron status in resource-poor field settings characterized by high burden of endemic infectious diseases. In 2001, the World Health Organization recommended hemoglobin, serum transferrin receptor, and serum ferritin or bone-marrow iron as the best combination of indicators to reflect iron status across the entire continuum [84]. In the context of inflammation, acute-phase response proteins, such as C-reactive protein and α-1 acid glycoprotein, may improve assessment of iron deficiency in the context of chronic inflammation and endemic infectious diseases. The recent WHO Consultation proposed that where possible, hemoglobin concentration, serum ferritin and transferrin receptor, and at least one acute phase protein should be measured [93]. More recently,

findings from several research studies have identified hepcidin as an important indicator of iron metabolism and a measure of iron status in the context of anemia of inflammation. Further research is needed to examine the relationship between hemoglobin, serum ferritin, transferrin receptor, and acute phase proteins, and to validate appropriate cutoffs in the context of pregnancy and malaria-endemic regions.

Anemia and Pregnancy Outcomes

During pregnancy, concentrations of hemoglobin, serum iron, and serum ferritin, and percentage saturation of transferrin decrease, largely due to hemodilution [74]. However, transferrin concentrations increase almost two-fold in the third trimester, from mean non-pregnant values of 3 mg/L to 5 mg/L, in order to facilitate iron transfer to the fetus [74]. Iron from maternal blood via transferrin-receptor mediated endocytosis [98], and transfer of iron to the fetus are regulated by the placenta [99], with approximately two-thirds of fetal accretion in the third trimester [99]. Intestinal iron absorption increases two- to three-fold during the second and third trimesters, in order to assist with meeting increased requirements for iron [74]. However, if maternal iron stores become depleted, the mother becomes anemic, and iron transfer to the fetus is compromised [101].

Observational Studies: Several observational studies have been conducted to examine the association between maternal iron status and perinatal health outcomes. Anemia has been identified as an independent risk factor for morbidity and mortality in pregnant women [2, 20-23, 102]. Iron deficiency contributes to an estimated 22% of all maternal mortality in women between 30 and 44 years of age [32]. In an analysis of observational studies, moderate anemia (hemoglobin 40-80 g/L) was associated with a 1.35-fold increased risk in maternal mortality (95% CI: 0.92, 2.00); severe anemia (<47 g/L) was associated with a 3.51 times greater risk of maternal mortality (95% CI: 2.05, 6.00) [52].

Anemia is also a risk factor for adverse perinatal outcomes, including low birth weight and neonatal mortality. An increased incidence of low birth weight in infants born to anemic women has been observed in several studies [19, 103-104]. For example, infants born to women with severe maternal anemia (Hb < 8.0 g/dL) had mean birth weight values that are 200 to 400 grams lower than in women with normal hemoglobin levels [106]. A U-shaped relationship between maternal hemoglobin and birth weight has been established; however, the mechanisms implicated at either end of this curve may be quite different. For example, low hemoglobin levels may be due to iron deficiency, while high hemoglobin levels may be due to factors such as insufficient plasma volume expansion [107]. A substantial body of evidence supports a relationship between severe anemia and perinatal mortality [2, 102, 108]. Anemia increases the risk of perinatal mortality and morbidity [2], and contributes to an estimated 33% of all perinatal mortality [32]. An association between maternal anemia and preterm delivery has also been noted in several studies [2]. However, further research is needed to establish a causal relationship [106].

Randomized Trials: The results and limitations of the observational studies referred to above have prompted the undertaking of several randomized controlled trials to investigate the effects of iron supplementation on health outcomes in pregnant women and their children.

Iron and Anemia: Several studies have evaluated the safety and efficacy of iron supplements in the prevention and treatment of anemia. According to the Institute of Medicine report [109] and a 2001 Cochrane review [110], the evidence to date for either a beneficial or harmful effect of iron supplementation on pregnancy outcomes is inconclusive. The 2001 Cochrane review of five trials provided inconclusive evidence on the effects of treating iron deficiency anemia in pregnancy, due to the lack of good quality trials [110]. Additionally, in many of the previous trials, women

were supplemented with 100 mg Fe/d [110], approximately three times the estimated requirement of pregnancy [74], and greater than current World Health Organization recommended dose of 60 mg. Most trials did not report any tolerance or side effect data associated with high-dose iron supplementation [110]. The updated 2007 Cochrane review evaluated findings from 17 trials of 2578 women, and concluded that daily oral iron treatment improves hematological indices [111]. However, the majority of trials included in this analysis did not assess relevant clinical outcomes or adverse side effects, and had a lack of standardization of dosages of iron supplements. Of the 17 randomized clinical trials included in this review, most focused on laboratory results. Only six trials assessed clinical outcomes; one of these trials was conducted in a malaria-endemic region and did not specifically examine malaria as an outcome measure. Therefore, clear conclusions regarding the safety and efficacy of iron supplementation in malaria endemic regions of the world is difficult.

The dose of iron supplementation provided has been considered in several studies. Higher dose iron supplements have also been associated with frequent gastrointestinal side effects [110], such as epigastric discomfort, nausea, constipation, and diarrhea [73, 112, 113], and may inhibit the absorption of zinc [114] and adherence. Evidence from prophylactic iron supplementation trials in non-anemic pregnant women in industrialized countries has demonstrated that lower-dose daily iron supplements (18-30 mg/d) are as effective as higher-dose iron supplements in the prevention of iron deficiency and anemia throughout pregnancy [71, 115, 116], with fewer gastrointestinal side effects [71]. In Australia, for instance, women receiving 20 mg iron daily had a reduced incidence of iron deficiency anemia ($P < 0.005$) and iron deficiency ($P < 0.001$) at delivery, and a comparable frequency of gastrointestinal side effects, compared to placebo [71] . A randomized, double-blind trial was conducted in Denmark to evaluate the safety and efficacy of four doses of iron supplements in preventing iron deficiency and anemia. A total of 427 healthy pregnant women were randomly assigned to four dosages of ferrous iron (as fumarate): 20 mg (n = 105), 40 mg (n = 108), 60 mg (n = 106), and 80 mg (n = 108) from 18 weeks of gestation to delivery. The prevalence of postpartum iron deficiency anemia was low and similar in the four groups, and there were no significant differences in the occurrence of gastrointestinal symptoms. There were no significant differences in iron status (ferritin, sTfR, and Hb) between the groups who received 40, 60, and 80 milligrams of iron. However, women who received 20 milligrams of iron had significantly lower median serum ferritin compared to the other three intervention groups ($p < 0.01$). The authors concluded that a supplement of 40 milligrams of ferrous iron daily from 18 weeks gestation to delivery was adequate to prevent iron deficiency in 90% of Danish women and iron deficiency anemia in at least 95% of the women during pregnancy and postpartum [117].

A randomized, double-blind, placebo-controlled iron supplementation trial was recently conducted among 244 pregnant women in Iran who were not anemic or iron deficient (hemoglobin concentration $\geq$ 13.2 g/dL, serum ferritin level > 15 μg/L at 13 to 18 weeks gestation). Women were randomized to take either one 150-mg tablet of ferrous sulfate daily or placebo during their pregnancies. The use of placebo did not cause a considerable decrease in markers of anemia in women with a hemoglobin concentration of 13.2 g/dL or greater in the second trimester of pregnancy, compared to iron supplementation [118]. Further, investigators found that iron supplementation in pregnant women who were not anemic (hemoglobin > 13.2 g/dL) resulted in a reduction in serum levels of copper and zinc [119], suggesting that routine iron supplementation may adversely affect the status of other important micronutrients.

Perinatal outcomes: Prenatal iron supplementation is recommended based on its benefit in preventing and treating maternal anemia. However, there is a lack of well-designed iron supplementation trials in pregnant women that have examined perinatal outcomes, particularly in resource-limited settings and/or those with a high rate of malaria infection. Although

observational studies have found an association between maternal anemia and adverse perinatal outcomes, and iron has been identified as a key growth factor for the developing fetus [62, 120-122], few studies have demonstrated improvements in birth weight or gestational age with prenatal iron supplementation [106]. Randomized controlled trials from the United States [123, 124] and Nepal [125] have suggested that iron supplementation may reduce the risk of low birth weight. For example, two randomized controlled trials in the United States found that iron supplementation led to improved mean birth weight and decreased the risk of low birth weight [123, 125]. In a randomized placebo-controlled trial in Cleveland, Ohio, among 275 low-income non-anemic pregnant women (30 mg iron as ferrous sulfate vs. placebo), iron supplementation did not significantly affect the prevalence of anemia or the incidence of higher preterm births. However, iron supplementation resulted in a significantly higher mean (+/- SD) birth weight (206 +/- 565 g; p=0.010), a significantly lower incidence of low-birth-weight infants (4% compared with 17%; p= 0.003), and a significantly lower incidence of preterm low-birth-weight infants (3% compared with 10%; P = 0.017) [123]. Observed reductions in the prevalence of low birth weight were primarily due to the effects on gestational age and not intrauterine growth retardation.

In a large, cluster-randomized trial in Nepal, no differences were seen in gestational age, but the prevalence of low birth weight was significantly lower among women who received iron-folate supplements compared to placebo (34% vs. 43%) [125]. However, this setting was characterized by high background rates of low birth weight and nutritional deficiencies including iron; all participants received vitamin A; and the intervention included folate in addition to iron, which constrains interpretability of findings with respect to iron supplementation. However, neither of these studies was conducted in areas of high malarial transmission; as a result these birth outcomes could not be assessed with respect to a possible increased risk of malaria during pregnancy. In this randomized trial from Nepal, iron supplementation did not reduce the occurrence of prematurity. However, neither of these studies was conducted in areas of high malarial transmission; as a result these birth outcomes could not be assessed with respect to a possible increased risk of malaria during pregnancy. A recent meta-analysis [126] confirmed the conclusion of earlier reviews [106] that there are insufficient data regarding the efficacy of iron supplementation during pregnancy on birth size or the risk of premature delivery.

Iron and Malaria

Iron is an important nutrient for host requirements and for the metabolism of invading pathogens. Nutritional immunity involves iron-withholding defense systems, such as iron-binding proteins that cause hypoferremia, which effectively reduces the amount of available iron for pathogens. Iron supplementation may exacerbate susceptibility to *P. falciparum* and other pathogens that target juvenile red blood cells, since iron increases erythropoiesis and reticulocyte production. Free iron is essential for multiplication of parasites such as plasmodia [127], bacteria production (*Escherichia coli, Mycobacteria sp, Pasteurella sp, Shigella sp, and staphylococcus*) [128], and viral replication [129]. Limitation of metabolically active iron in pathogen-invaded cells inhibits pathogen growth [127]. Iron may also have indirect nitric oxide-mediated effects. Iron inhibits expression of inducible nitric oxide synthase, blocks the synthesis of nitric oxide by transcriptional inhibition of inducible nitric oxide synthase, and down-regulates the production of nitric oxide in macrophages [130]. Since nitric oxide is essential to macrophage defense against *P. falciparum* [131], iron deficiency may enhance nitric oxide synthase-medicated defenses against malarial parasites. In the absence of iron and after interferon stimulation, nitric oxide synthesis is increased; nitric oxide reacts with enzymes needed for DNA synthesis and electron transfer, and results in death of pathogens [132]. Iron supplementation may also compromise the immune response to infection *via* inhibition of zinc absorption [132].

Iron supplementation increases morbidity and mortality among children in malaria-endemic areas. In particular, findings from the recent randomized trial in Pemba, Tanzania raised concerns regarding the safety and efficacy of iron supplementation in areas of high malarial burden [3, 4]. In this study, children aged one to 35 months were randomized to iron (12.5 mg) and folic acid (50 μg; n=7950), iron, folic acid, and zinc (n=8120), zinc alone (n=8079), or placebo (n=8006); children aged one to 11 months received half of the dose of supplements. The iron and folic acid-supplemented arms of the trial were stopped early, upon recommendation of data and safety monitoring board. Children who received iron and folic acid with or without zinc were 12% (95% CI 2-23, p=0.02) more likely to die or need treatment in hospital for an adverse event and 11% (1-23%, p=0.03) more likely to be admitted to hospital; there were also 15% (-7 to 41, p=0.19) more deaths in these groups. The authors concluded that iron supplementation to children who are not iron deficient may be harmful, and current guidelines for universal supplementation with iron and folic acid should be revised [4]. In a community-based cluster-randomized, double-masked, placebo-controlled, 2x2 factorial trial in children aged 1 to 36 months in southern Nepal, children were randomized to daily oral supplementation to age 36 months with: iron (12.5 mg) and folic acid (50 μg; n=8337), zinc alone (10 mg), iron, folic acid, and zinc (n=9230), or placebo (n=8683); children aged 1 to 11 months received half the dose. Iron supplementation had no effect on the risk of all-cause mortality (IRR = 1.03, 95% CI: 0.78, 1.37); however, iron supplementation resulted in a non-significant reduction in rates for diarrhea, dysentery, and respiratory infections [32]. Overall, routine iron supplementation in children in high malaria endemic regions resulted in increased risk of severe illness and death. However, the effects of iron supplementation among pregnant women in these regions have not been established and warrant further investigation.

Observational Studies in Pregnant Women: Few studies have examined the association between iron status and malaria risk during pregnancy. In an early study from Papua New Guinea, mean (SE) pre-natal hemoglobin was higher in women with postnatal malaria than women without postnatal malaria (8.91 (1.90) vs. 9.78 (1.79)). Intravenous iron injection in the same population was associated with a 5-fold increase in the frequency of malaria after delivery among primiparous women (OR: 5.46, 95% CI: 2.20-13.53) [133]. There was no significant increase in risk observed among multiparous women (OR: 1.12, 95% CI: 0.73, 1.70). Similar findings were reported in an observational study among primigravidae in Thailand [134]. In contrast in Kenya, van Eijk et al compared the occurrence of maternal and placental malaria among pregnant women of > 32 weeks gestation who received hematinic supplementation for the duration of pregnancy to women who received no intervention in an earlier time period [57]. Adjusting for a number of potential confounders, including HIV status, iron supplementation was not associated with an increased risk of maternal or placental malaria (placental malaria OR = 1.07, 95% CI: 0.86, 1.32). The lack of effect, however, may be due to the short duration of supplementation. More recently, Kabyemela et al conducted a longitudinal study among pregnant women in Tanzania [135]. Iron deficiency was associated with a significantly lower risk of placental malaria (OR= 0.19, 95% CI: 0.10-0.34). Stratified analyses suggest that the protection of iron deficiency is limited to primigravidae and secundigravidae. The greatest effect of placental malaria infection on low birth weight has been observed among primigravidae;[52] the odds ratio of low birth weight associated with malaria is two to seven times higher in primigravid than multigravid women [52].

Intervention Studies in Pregnant Women: There is limited evidence from intervention studies on the safety of iron supplementation among pregnant women. An early study by Fleming *et al.* among primiparous women in Nigeria suggested no difference in the number of cases of malaria associated with 60 mg/day of elemental iron supplementation. However, formal tests for the effect of the iron intervention were not provided and the interpretation of these findings is limited by the small sample size (n=228) [136]. A trial among multigravidae pregnant women without

anemia (packed cell volumes ≥ 25%) in the Gambia provided 200 mg ferrous sulphate or placebo daily and assessed the impact of supplementation on the prevalence of maternal malaria (36 weeks and postpartum) and placental malaria [37]. No difference was seen for any outcome between women who received iron or placebo. Iron was, however, associated with an increased risk of malaria among women with the Hgb AS genotype when compared to placebo (76% vs. 58%, p for interaction by genotype = 0.06). In Uganda, Ndyomugyenyi et al. randomized primigravida in their first or second trimester to case management, case management with malaria chemoprophylaxis, or case management with 1220 mg elemental iron and folic acid [137]. No difference was found in the frequency of infant parasitemia at birth or at 1 month, or in infant parasite density. There were marginally significant differences between groups in terms of the frequency of maternal malaria at birth (76% vs. 61% vs. 62% p = 0.06) and placental malaria (74% vs. 54% vs. 64% p = 0.05) [137].

Iron supplementation in pregnancy confers benefits to maternal iron status and hematologic status. There is a consistent finding that anemia is associated with adverse maternal and pregnancy outcomes. However, there is limited evidence that iron supplementation reduces the risks of these adverse outcomes. Further, findings from recent randomized trials raise concerns regarding the safety of iron supplementation in areas of high malarial burden. Overall, there is a paucity of good quality randomized trials assessing the safety and efficacy of iron supplementation in pregnancy, and its effects on perinatal health outcomes. In particular, there is a lack of research on the safety and efficacy of prenatal iron supplementation in developing regions, characterized by extensive burden of iron deficiency, malaria, and other endemic infectious diseases.

Rationale for the Proposed Trial

Iron deficiency anemia and malaria are urgent public health problems in sub-Saharan Africa, including Tanzania. As reviewed above, overall, there is a paucity of good quality randomized trials assessing the safety and efficacy of iron supplementation in pregnancy, and its effects on perinatal health outcomes [111]. Prenatal iron supplementation is recommended based on its demonstrated benefit in preventing and treating maternal anemia. There is limited data on the efficacy of iron supplementation on pregnancy outcomes, including birth weight. There are also concerns regarding the use of iron supplementation, particularly among non-anemic women. In particular, there is a lack of research on the safety and efficacy of prenatal iron supplementation in developing regions, characterized by extensive burden of iron deficiency, malaria, and other endemic infectious diseases. Evidence from randomized controlled trials is urgently needed to examine the safety and efficacy of iron supplements among pregnant women in malaria endemic regions, particularly among women who are not anemic [4-12].

World Health Organization guidelines recommend iron supplementation for pregnant women, women of childbearing age, and children under two years of age in areas with high prevalence of anemia (≥40%), regardless of malarial status [32]. However, WHO recommendations for iron supplementation are controversial in areas of high malaria burden [138, 139]. Iron supplementation may result in increased levels of malaria parasitemia [9, 10], rates of malaria, pneumonia, and diarrhea [10, 139, 140], and malarial morbidity and mortality [4]. In particular, findings from the recent trial in Pemba, Tanzania raised concerns regarding the safety and efficacy of iron supplementation in areas of high malarial burden [4, 141]. Due to these and other findings, the WHO recommends that caution should be exercised in iron supplementation of children, in areas of high malaria burden, and that children with anemia or at high risk of iron deficiency be targeted for possible supplementation [11, 12].

Adverse effects of supplemental iron would be of considerable public health importance in malaria endemic region of sub-Saharan Africa due to widespread use of iron supplements in malaria-endemic areas, especially during the antenatal period. In order to address this important research gap, we therefore propose a randomized controlled trial among pregnant women in Tanzania. Given the benefits of supplementation in treatment of anemia, all anemic women or those are iron deficient will be provided with iron supplements and excluded from the trial.

Tanzania is an ideal setting for the proposed study as it has a very good scientific infrastructure, great political stability, and a long track record in collaborative research between the Harvard School of Public Health and Muhimbili University of Health and Allied Sciences.

Preliminary Studies

We will briefly present findings from other epidemiologic studies that we have conducted in Dar es Salaam to address the inter-relationships of micronutrients, anemia and iron deficiency status, malaria, and perinatal and child health outcomes.

Micronutrients and Pregnancy Outcomes among HIV-infected Women: This was a randomized double-blind trial to examine the effects of vitamin A and other vitamins on pregnancy outcomes, perinatal transmission and disease progression among pregnant women in Dar es Salaam. To randomize 1,078 consenting HIV-infected subjects over two years, 13,876 eligible pregnant women were tested for HIV. Enrolled women were followed up on monthly basis at a study clinic from enrollment starting in 1995 until the close of follow up at the end of August 2003. Multivitamin supplementation (i.e. vitamins B-complex, C, E) significantly reduced by about 40% the risks of fetal loss, low birth weight, severe preterm birth, and small-for-gestational age birth [142]. Multivitamins excluding A had no effect on the overall risk of transmission of HIV-1, but supplementation of breastfeeding mothers significantly reduced child mortality and transmission of HIV-1 through breastfeeding among immunologically and nutritionally compromised women. Vitamin A alone increased the risk of HIV-1 transmission (RR=1.38, P=0.009) [143]. This finding was unexpected and prompted the DSMB to drop the vitamin A arm of the trial.

Micronutrients and Pregnancy Outcomes HIV-uninfected Women: In the PeriNatal Study, a double-blind trial in Dar es Salaam, Tanzania, we randomly assigned 8,468 HIV-uninfected pregnant women (gestational age of fetus, 12 to 27 weeks) to receive daily multivitamins (including multiples of the recommended dietary allowance) or placebo. All the women received prenatal supplemental iron and folic acid and malaria prophylaxis. Multivitamin supplements significantly reduced the risk of low birth weight by 18%, decreased the risk of small for gestational age by 23%, and reduced the risk of maternal anemia (hemoglobin level, <11 g/dL; relative risk, 0.88; 95% CI, 0.80 to 0.97; P=0.01),but had no significant effects on fetal loss or prematurity [144].

Zinc and Perinatal Outcomes: Poor zinc status has been associated with adverse pregnancy outcomes, but higher levels of zinc intake were significantly associated with higher risks of mortality among US men. We examined the effects of zinc supplementation on pregnancy outcomes and hematologic and T cell counts of HIV-infected women in Dar es Salaam (n=400). Retention rates were high at 95%. Zinc supplements had no effect on pregnancy outcomes or CD4, CD8, or CD3 cell counts during the follow-up period. However, zinc had an adverse effect on hemoglobin, red blood cell count, and packed cell volume. The rise in hemoglobin over this period was significantly lower (P = 0.03) in the zinc group (x +/- SD: 11.5 +/- 17.9 g/L) than in the placebo group (15.2 +/- 18.6 g/L); similarly, the changes in red blood cell count and in packed

cell volume over the same period were significantly lower in the zinc group (P < 0.01 and P = 0.01, respectively) [145]. These adverse effects may be due to adverse effect on iron absorption, as previously observed in other studies.

Anemia and Mortality among HIV-infected Women: Some studies have evaluated the association between iron status and mortality in resource-poor settings. In a prospective observational analysis conducted as part of the randomized clinical trial of multivitamins in Tanzania, investigators examined the relationship between anemia and mortality, among 1,078 HIV-infected pregnant women. All women received 120 milligrams of ferrous iron and 5 milligrams of folate tablets daily, as per standard of care in Tanzania. Anemia was defined as moderate or severe, using the hemoglobin concentrations of 8.5-10.9 g/dl and <8.5 g/dl as cutoffs, respectively. Anemia was an independent predictor of mortality in this population, and associated with increased risk of all-cause mortality (relative hazard [RH]: 2.06, 95% CI: 1.52 to 2.79 for moderate anemia and RH: 3.19, 95% CI: 2.23 to 4.56 for severe anemia) and AIDS-related mortality (RH: 2.21, 95% CI: 1.53 to 3.19 for moderate anemia and RH: 3.47, 95% CI: 2.25 to 5.33 for severe anemia), in adjusted models controlling for CD4 cell count, WHO HIV disease stage, age, pregnancy, vitamin supplementation, and body mass index. Iron deficiency anemia was associated with all-cause and AIDS-related death and a 50% decline in CD4 cell count. In the same study, hypochromasia, either with microcytosis (HR=2.94, 95% CI=2.01-4.29) or without (HR=2.85, 95% CI=2.02-4.03) was associated with AIDS-related mortality, providing evidence that iron deficiency was an independent risk factor for all-cause and AIDS-related death [146].

Iron Status, Anemia, and Disease Progression: We examined the relationship between iron status and anemia as well as HIV-disease progression. Iron status and anemia was assessed at 30 weeks after delivery. Prevalence estimates for iron deficiency and iron deficiency anemia were 39.7% and 23.6%, respectively. Iron deficiency was associated with 48.9% of anemia cases [147]. In cross-sectional analyses hemoglobin and serum ferritin, but not serum transferrin receptor concentrations, were significantly related to C-reactive protein concentrations. Furthermore, serum ferritin was significantly inversely related to CD4 cell counts and positively related to viral load. In longitudinal analyses, serum ferritin was modeled using the cutoffs < 12 μg/L, 12-29.9 μg/L, 30-150 μg/L, and >150 μg/L. Serum ferritin > 150.0 μg/L was related to a non-significantly elevated risk of progression to stage 4 (rate ratio = 1.78; 95% CI = 0.68-4.64; P = 0.24) compared with to serum ferritin <12.0 μg/L, after adjusting for confounding variables such as C-reactive protein concentrations, hemoglobin, CD4 cell count, viral load, body mass index, and mid-upper arm circumference [147].

Malaria in Pregnancy: We examined the associations between malaria infection among pregnant women and the risk of adverse pregnancy outcomes using data from one of our perinatal clinical trials in Dar es Salaam. We recruited 1078 HIV-infected, pregnant women who were 12-27 weeks of gestation at first prenatal visit and intended to deliver in Dar es Salaam. These women were followed monthly during pregnancy at Muhimbili National Hospital and at their homes, and information on the outcome of their pregnancies was recorded. In cross-sectional analyses of the study population at baseline, malaria infection was a strong predictor of severe anemia (<70 g/L) [148]; women with ≥1000 parasites/106L were 2.7 times as likely to be severely anemic (95% CI = 1.58, 4.61; P=0.0003) as those without parasites. There was evidence for a “dose-response” association between parasite load and the risk of severe anemia. In longitudinal analyses, malaria parasitemia at baseline was associated with a significantly higher risk of low birth weight (LBW, <2500 g), after adjustment for various potential confounders (OR=1.81; 95% CI= 1.04, 3.16). This association appeared to be density dependent, with significantly higher risk among women with moderate to heavy parasitemia (≥1000x106/L). The risk of LBW associated with malaria did not differ significantly between primiparous and multiparous women. Malaria was also predictive

of lower birth weight as a continuous variable; mean birth weight decreased monotonically as malaria parasite density increased, with adjusted average differences between women without malaria and those with parasitemias <1000, <10,000, or ≥10,000 (X 106/L) of -26, -140, and -337 grams, respectively. Mothers with malaria parasites in peripheral blood were also 79% more likely to have a small-for-gestational age newborn than mothers without malaria ($P < 0.05$), after adjusting for potential confounding factors. Malaria at baseline was also a significant risk factor for low weight gain during pregnancy, particularly during the second trimester [149]; women with parasite densities ≥1000x106/L had an estimated rate of weight gain at week 18 that was 148 g/wk lower than for women without parasites; the inverse association between malaria parasitemia and the rate of weight gain followed a significant linear trend. In another study among 400 HIV-infected pregnant women between 12-27 weeks of gestation maternal parasitemia (1/μL) at the first antenatal visit was associated with increased risk of low birth weight < 2,500 g (adjusted relative risk [ARR] = 2.66; $P = 0.01$) and preterm delivery < 37 weeks (ARR = 1.87; $P = 0.06$). Maternal parasitemia at delivery was associated with preterm delivery (ARR = 2.27; $P = 0.008$), intrauterine growth retardation (ARR = 1.92; $P = 0.03$), and neonatal death (ARR = 3.22; $P = 0.07$). Cord parasitemia was associated with a large and significant increase in the risk of neonatal death (ARR = 8.75; $P = 0.003$) [150].

Malaria in Children: We examined the effect of oral vitamin A supplements on malaria-related deaths, growth of children with malaria, and incidence of parasitemia in a randomized clinical trial conducted between 1993 and 1995. In this trial, 687 children 6 to 60 months of age who had been hospitalized with pneumonia at Muhimbili National Hospital were randomly allocated to receive 4 oral doses of 200,000 IU vitamin A (100,000 if < 12 months of age) or placebo. The first dose was administered on the day of admission, the second on the following day, and the third and fourth doses at 4 and 8 months after the initial hospitalization, respectively. Children were followed for morbidity and mortality endpoints after discharge from the hospital for an average of 24 months. Among children who died, the cause of death was ascertained through verbal autopsies that were independently reviewed by two senior pediatricians. Vitamin A supplements resulted in a 49% reduction in all-cause mortality ($P=0.02$), and an 86% reduction in malaria-related deaths (95% CI = –7, 100%; $P=0.06$) [144]. Among infants < 12 months who had any malaria parasitemia on the day of hospital admission, vitamin A resulted in significantly greater attained weight after 1 year of follow-up (average effect = 747, 95% CI = 71, 1423) [151]. In contrast, vitamin A had no effect on growth among children without malaria, suggesting that vitamin A could be a limiting factor for growth in children with malaria, independent of anti-malarial treatment. Vitamin A was not significantly related to the incidence of parasitemia (at least one parasite in peripheral blood film) during follow-up [152]. The study was not designed to examine malaria as an endpoint; hence these null results could have been due to low statistical power to examine this particular question and that the surveillance for malaria parasitemia was not intensive.

In summary, throughout the implementation of these and other studies, we have substantial experience conducting large-scale and complex randomized clinical trials in perinatal health and iron status among pregnant women in Tanzania. Of particular importance to the current proposal is the demonstration that we can enroll large numbers of pregnant women into large-scale clinical trials, and achieve high rates of follow-up and adherence after fully informed consent. Our findings highlight the burden of clinical complications associated with iron deficiency and malaria and provide support to the importance of examining the effect of the safety and efficacy of iron supplementation in the context of high malarial burden, and examining immunological and clinical outcomes related to malaria in pregnancy.

Research Design and Methods

Eligibility criteria: Participants will be recruited from pregnant women presenting to a large prenatal care clinic in Dar es Salaam, Tanzania. More than 90% of women present for their first prenatal care visit after the 12th week of gestation. Eligible participants will be pregnant women at or before 27 weeks of gestation, who are primigravida or secundigravidae, not anemic (defined as Hb<8.5 g/dL) or iron deficient (defined as serum ferritin <12 μg/L), HIV-uninfected, and intend to stay in Dar es Salaam until delivery and for at least six weeks thereafter. Women with high iron stores at baseline (i.e., serum ferritin >200 μg/L) will be excluded. HIV-infected women will be referred for standard of care services, including provision of antiretroviral treatment, as needed. The recruitment process will be completed within one week, such that randomization will be completed at the latest by 20 weeks of gestation.

Ascertainment of Gestational Age: In order to more accurately determine gestational age, women may undergo ultrasonography at baseline. Measurement of the fetal biparietal diameter will be used to determine gestational age. The ultrasound will be carried out by an obstetrician trained by an experienced ultrasound specialist at each of the two proposed clinics. When performed in pregnant women up to 18 weeks of gestation, the accuracy of ultrasound in determining gestational age is approximately plus or minus 7 days. Accuracy should have similar variability in pregnant women between 18 and 20 weeks. Verification of ultrasound findings will be performed by an experienced ultrasonographer in a subset of 10% of pregnant women during the first six months of enrolment, as a measure of quality assurance and quality control.

Study Population Characteristics: Recruitment of women for the proposed study will be conducted at the same clinics where we have enrolled cohorts of pregnant women in other randomized trials. In our experience, the median gestation age at enrollment was 21.4 (mean=20.9, SD=3.6)); approximately two percent of women presented at less than 12 weeks gestation, while close to 50% were at 12 to19 weeks gestation. Of all enrolled women, 94.5% of women delivered at a study hospital, while 5.5% delivered at home. More than 50% of women were primigravida or secundigravidae: a quarter of the women were primigravida, another quarter secundigravidae, and the rest were multigravidae. Acceptance of voluntary counseling and testing for HIV has been more than 95%.

A Food Frequency Questionnaire was used to assess dietary intake among 8,265 HIV-uninfected pregnant women in the PeriNatal study in Dar es Salaam, Tanzania in 2005. At baseline assessment, pregnant women consumed an average of 13.4 (s.d. 7) milligrams of iron daily, with a median of 12.0 grams, and an interquartile range of 9.6 to 14.9 grams. An estimated 12.7% of iron intake (1.7 mg) was heme iron, derived from meat, fish, and poultry food sources, with 87.3% of daily iron intake from non-heme sources, on average. Total energy intake was 2,398 calories daily, on average (median: 2,295; IQR: 1843.7). Average consumption of vitamin C was 161.4 milligrams (s.d. 103.5 mg); estimated intake of phytates and zinc were 2263.6 (s.d. 889.2) and 9.6 (s.d. 3.6) milligrams, respectively [145].

Recruitment of Study Participants: Recruitment will take place at Amtulbai clinic in Dar es Salaam in order to expedite enrollment of the desired sample size, 1500. This center was selected as it provides antenatal services to a large number of pregnant women in Dar es Salaam, and reflects a high malarial burden in its patient population. We maintain adequate clinic space and have an excellent working relationship with physicians and nurses there**.** *(a) First visit:* A brief baseline questionnaire and clinical assessment will be administered to every woman presenting for prenatal care and who satisfies the eligibility criteria above. A specimen will be collected at baseline to assess iron status, including serum ferritin and hemoglobin levels, and sent to the lab for testing. They will have the study objectives and design explained to them and pre-HIV-test

counseling will be provided to all women as per standard of prenatal care in Tanzania. Issues of confidentiality of the result will be underscored. Blood will be tested using two rapid HIV assays, with confirmation of discrepant results using ELISA as per national guidelines. Results will be available on the same day. Women will be informed of the result of HIV testing and post-test counseling will be provided. Eligible women will be asked to come back 3 days later for confirmation of gestational age, and for results on their iron status, including hemoglobin levels and serum ferritin. Women with HIV infection, severe anemia (Hb < 8.5 g/dL), and/or low iron status (serum ferritin < 12 μg/L) as well as women with evidence of iron overload (ferritin > 200 μg/L) will be excluded from participation, and will receive standard prenatal care services including antiretroviral therapy and iron supplementation, as needed. *(b) Second visit:* The period between the first and second visit will allow mothers to consider the matter of participation in the study further, thereby reducing the number of defaulters once randomization of the subject has taken place. At this visit, eligibility will be assessed. Written informed consent for participation is the trial will be sought. Consented mothers will have a detailed background questionnaire administered and a full clinical examination will be carried out. Women will be randomly assigned to one of the experimental regimen groups below. Each mother will receive a one-month supply of capsules, and will be given an appointment to come back a month later. They will be escorted home by one of the project staff to record the exact location of the house that will be visited, if necessary.

Experimental Regimens and Randomization Procedures: Women will be randomly assigned to receive a daily oral dose of one of the following two regimens from enrollment and until delivery: (a) 60 mg iron (as ferrous sulphate) or (b) placebo. Tablets will be packaged in identical coded bottles that contain supply for 45 days (although the research visits are scheduled at monthly intervals, the extra tablets are necessary in case a woman is delayed in coming in for her scheduled visit). A list from 1 to 1500 will be prepared according to a randomization sequence in blocks of 20. Each of the two study clinics where randomization will occur will have a subset of the list of numbers. At enrollment, each eligible woman will be assigned to the next numbered bottle of regimen at that site. Experience has shown that unblinding, whether real or based on an impression or perception that develops among subjects or staff, is more likely to occur if regimens are color-coded or bear simple numeric codes. To minimize this risk, we will provide the regimen in bottles labeled with the participant's identification numbers, and will make active tablets and placebo indistinguishable, so that neither the subjects nor the investigators can identify which group of subjects is randomized to the same regimen.

The proposed dose of 60 mg elemental iron (200 μg ferrous sulphate) is the WHO-recommended dose for universal supplementation during pregnancy. It also corresponds to the WHO-recommended dose for daily supplementation over a 3-month period among lactating women and women of reproductive age who live in areas where the prevalence of anemia exceeds 40% [84]. Although the prevalence of anemia among women of reproductive age exceeds 40% in Tanzania [33], the WHO-guidelines during lactation and reproductive age have not been adopted by the Tanzanian Ministry of Health and this practice is thus currently not in use [84].

High-dose iron supplements can cause gastrointestinal side effects, such as upper abdominal discomfort, nausea, and constipation [112], and can inhibit the absorption of zinc [114]. These side effects can be associated with non-adherence, as well as poor dietary intake of other important nutrients. The Institute of Medicine determined the Lowest-Observed-Adverse-Effect Level as 70 milligrams of iron per day, based on studies that identified minor gastrointestinal symptoms occurring at daily intake of 11 milligrams of iron from diet and 60 milligrams of iron from supplementation [73]. The Tolerable Upper Intake Level with no expected adverse effects was identified at 45 milligrams of iron per day among adults, including pregnant women [73]. At

the 60 milligram dosage recommended by the World Health Organization, minor side effects include epigastric discomfort, nausea, diarrhea, and constipation [73]. However, any minor side should be mild in the vast majority of cases, and will be monitored closely throughout follow up. In addition, the dosage is commonly used worldwide among pregnant women in many developing countries.

Follow up Schedule: Each pregnant woman will be seen at one of the two study clinics once a month until delivery. At each visit, trained attendants will administer a questionnaire on the health of the woman in the preceding month, and a medical officer will carry out an obstetric examination. At the end of her monthly visit, every woman will be given a month's supply of capsules. Adherence in the previous month will be assessed through questioning of the mother, capsule count, and measurement of plasma serum ferritin levels in a random sub-sample of women (see section on Adherence below).

Women who do not come for their monthly appointments will be visited at home when possible and asked to come to the study clinic if their health status allows. One of the eligibility criteria for the trial is a woman's intention to stay in Dar es Salaam until delivery and for six weeks thereafter. However, for women who do travel outside of Dar es Salaam, we will attempt to maintain contact with neighbors and relatives in the area to collect information on the outcome of pregnancy, and women will be asked to deliver at the same hospital where they are being followed up (**Amtullabai** clinic). Study midwives will be available 24 hours per day to attend to study women, document details of delivery, and schedule post-natal appointments. We will keep a record of the Expected Date of Delivery, and if the participant does not come to the study clinic or present in labor up to three days after her due date, she will be visited at home; if she was found to have given birth, the child will be examined by the visiting nurse, and the mother will be encouraged to come to the study clinic for a complete examination by a study pediatrician. If the participant has complications during pregnancy or delivery and needs to be referred to either Temeke Hospital or Muhimbili Hospital, one of our study nurses will accompany the participant to the respective hospital.

After delivery, mothers will be given a clinic appointment at 6 weeks post-partum, in order to ascertain survival status, and to collect limited morbidity data and anthropometric measurements on mother and child. Clinical history (including the signs of malaria infection) and medications used by mother and child in the prior 6 weeks will be ascertained. General physical examination of mother and child, including the respiratory, lymphatic systems, skin, and central nervous system will be undertaken. Child measurements of length, weight, head circumference, chest circumference, and mid-upper arm circumference will be performed.

Precise determination of the cause of death is difficult given that postmortem examinations are rarely done. If a woman or child die in a health facility, events before and at the time of death will be transcribed from hospital records. If death occurs out of the hospital, a standard verbal autopsy form will be administered to assess the cause of death. The latter includes open- and closed-ended questions on the date of death; the clinical picture, and treatments received in the period before death. Health facilities accessed by the deceased will be visited and data available at these sites will be transcribed. Three physicians will review the information collected in the verbal autopsy form and from the health facility and a final determination of the cause of death will be assigned.

Each woman will be seen with her infant for an additional visit at 6 weeks postpartum, or until her death, the occurrence of miscarriage/abortion, or loss to follow-up. Loss to follow-up will be kept to a minimum. At each clinic visit during the entire study, women will be asked if they moved in the preceding month; if they have moved, they will be escorted home and the new

address will be recorded. As part of an incentive package to continue participating in the study, mothers will have access to the study clinic throughout the study period. Women and children will be managed at the study clinic during pregnancy, delivery, and early childhood in keeping with standard practice at medical practice of the Ministry of Health in Tanzania. Children will receive appropriate monitoring and vaccination services at the hospital's maternal and child health (MCH) clinic. Women will be encouraged to use the study clinic at any time if they require medical attention. Study physicians will attend to the study subjects who request services. Mothers will be reimbursed for transportation costs and for the costs of medications that have to be purchased (if the physician assesses that the woman is in financial need). Similar procedures in our current perinatal studies have resulted in >95% follow-up by 6 weeks postpartum.

Adherence: Any trial requires a high degree of adherence in taking the experimental regimens since non-adherence can bias the results of the trial and decrease the statistical power of a study to detect any true effects. We will encourage each woman to take her regimen at the same time every day, to put the bottle in a visible place, and to ask a relative to remind her to take the regimen. During each monthly visit, adherence with supplement use will be assessed in three ways: (a) Direct questioning: asking the woman about her ingestion of the capsules in the previous 24 hours, previous week and previous month. (b) Capsule count: the number of capsules remaining in each monthly bottle will be counted. (c) Biochemical assessment: assessment of adherence biochemically is more accurate as a method of assessment, provided there is a reasonable biochemical marker. We intend to assess the subjects' adherence with use of the experimental regimen by measuring the concentration of serum ferritin. A blood specimen will be collected at randomization and at delivery for this purpose from a sample of 300 randomly selected women. Research assistants will define lack of adherence as any deviation from complete adherence defined using pill count. At all visits, they will assess the patient's adherence with treatment, identify potential barriers to adherence, and identify solutions to these obstacles.

Standard of Care: All pregnant women will receive standard prenatal care at all times. Malaria prophylaxis is provided as a dose of sulphadoxine pyremethamine at 20 weeks of gestation and one at 30 weeks of gestation. All study patients with symptomatic malaria will be managed according to the standard national policy for the treatment of malaria. Uncomplicated malaria in will be managed with oral quinine. At randomization and delivery, we will assess Hb levels. If the patient's Hb level falls below the 8.5g/dL cutoff for severe anemia, she will be treated as per standard of care in Tanzania. We will investigate the primary cause of the severe anemia and treat appropriately. For instance, if sf<12mcg/L, we will treat with iron supplementation as per standard of care. Appropriate treatment for any condition (e.g., severe malaria, pre-eclampsia) will be provided. Daily folate (400 mcg) supplementation is established as standard prenatal care practice in many developing countries including Tanzania, based on their demonstrated benefit in prevention of birth defects, particularly neural tube defects [153], and will be provided. All women who are identified to be HIV-infected during the recruitment process will be referred to an HIV/AIDS Care and Treatment clinic and provided with care and treatment, including antiretroviral therapy as needed and in accordance with national and WHO guidelines. HIV/AIDS care and treatment services are provided as part of the Harvard School of Public Health PEPFAR program that is being implemented in Dar es Salaam in collaboration with Muhimbili University and the City of Dar es Salaam Regional office of Health. In light of our recently published findings of protective effects of these multivitamins on pregnancy outcomes among HIV-uninfected women, we will seek to provide such supplements to study participants after consultation with officials at the Ministry of Health.

Pharmacy Procedures and Regimen Disposal: The MAL1 study regimen will be prepared and distributed to clinic sites each week, based upon upcoming Weekly Appointment Lists. Each

Tuesday, the clinic staff will submit a Weekly Appointment List for the following week. The data unit staff will be responsible for creating and submitting Weekly Appointment Lists. The clinic research supervisor will weekly appointment lists before sent to Central Pharmacy.

Preparation of regimen will always be conducted by two pharmacy staff members at Central Pharmacy. Regimen is prepared at the rear of the pharmacy during the less-congested hours of pharmacy operation, so that there is minimal disturbance to pharmacy activities. Pharmacy staff will prepare the study regimen by labeling coded bottles with the appropriate client ID number and name based on the master randomization list. The regimen will then be transported to the clinic staff by Monday morning for delivery to the sites *via* project transportation.

Clinic staff will also return empty and used regimen bottles to Central Pharmacy by Friday afternoon each week.

Central Pharmacy staff will prepare two bottles of study regimen for all new participants. Labels for these study regimen bottles will include a printed ID recruitment and randomization number, and a blank placeholder for the participant's name. The RA will be responsible for requesting the study regimen for new participants, obtaining it from the clinic pharmacy, and writing the participant's name in the appropriate space on the regimen bottles.

Subsequent regimen bottles will be prepared for participants at a rate of one per month, and include preprinted participant ID numbers and names.

Clinic pharmacy and study staff will be responsible for maintaining a supply of two regimen bottles for each participant at the clinic at all times. Regimen bottles should be stored so that the client names and ID numbers are clearly visible. When a new regimen bottle is prepared and received for a participant, the pharmacy staff should store the newest regimen behind the other bottles, such that regimen bottles are administered to participants in the order they are received from Central Pharmacy and client names and ID numbers are clearly visible. This procedure will ensure that an additional bottle of study regimen is always available for participants, in case a client arrives early for a clinic appointment, or if there are any issues with transportation of study regimen.

The clinic Research Supervisor should submit requests for study regimen to Central Pharmacy by the Tuesday of each week, in the form of Weekly Appointment Lists. In the event that additional regimen is needed for a particular participant after this date (e.g., a client is planning to undergo an extended safari), requests for additional regimen should be made no later than the morning of the day before the regimen is needed. Requests for study regimen placed on the same day that the regimen is needed cannot be guaranteed to be filled in time for morning transportation to sites.

At Central Pharmacy, study regimen should be stored in a locked room, accessible only by official pharmacy personnel. At study clinics, medications must be stored in a locked cabinet accessible only to research pharmacy staff.

The iron regimens will be stored in a temperature-controlled pharmacy, between 15 to 30° C. Daily temperatures will be recorded in a temperature log forms, and monitored by the Head Pharmacist.

Study product expiration dates vary according to specific protocol and this is routinely checked. In a situation where the protocol needs a container to be opened and a portion of product be

dispensed, the manufacturer's instructions are followed in order to determine new expiration dates.

The head pharmacist will be responsible for monitoring the master randomization list, managing the regimen supply and distribution, supervising transportation of regimens to the respective clinic sites, maintaining and adequate regimen supply, and notifying the Study Coordinator and or PI when additional regimen should be re-ordered.

After the study regimen is transported to the clinic sites, every effort should be made to keep the regimen in a cool well-ventilated area. Two bottles of study regimen should be stored for each participant at the clinic site, with clearly labeled bottles organized by participant ID and name. This supply should be regularly replenished by Central Pharmacy.

At the study clinics, handling of study regimen outside of the prepared bottles should be avoided. If a study site Pharmacist must repackage tablets for a participant, s/he should thoroughly wash his/her hands and prepare medications on a clean washed surface.

There are two study arms for MAL1: iron folate and placebo. The regimens arrive at the pharmacy with two different codes. The pharmacy staff is blinded similar to the rest of the research team. The pharmacy is responsible for assigning the codes to each participant using a randomization list created by the Boston data team. This procedure maintains study blinding, assuring the pharmacists are not aware of which code corresponds to the high and low multivitamin doses.

Participants in MAL1 will be randomized to receive either iron or placebo in a double-blinded manner. All clinic research and pharmacy staff must remain blinded to study regimen composition and intervention group membership throughout the duration of the study. The head pharmacist will be responsible for maintaining the master randomization list and preserving blinding of all study regimen procedures and intervention group membership.

Two pharmacy staff members are involved in assigning codes to the study participants and labeling, with a third person overseeing the process to ensure that it is done properly.

When used regimens are returned to the pharmacy, assigned non-pharmacy staff will take samples and use the randomization list to check retrospectively if any errors were made throughout the process. Findings will be recorded and reported on the appropriate forms on a weekly basis.

Accountability of the study product is held by pharmacy staff. Shipping invoices are stored at the main pharmacy. Actual compliance to study regimen are recorded by both pharmacy and clinical staff. The site pharmacy will store the Patient Study Drug Flow Chart Records.

Study regimen inventory will be conducted once every three months by two pharmacy staff members. The pharmacists will conduct this inventory independently, with tallied results compared afterwards. In the event of any discrepancies in inventory, pharmacy staff should recount the study regimen together, in order to obtain a final total inventory for the study regimen. Results of the study regimen inventory will be recorded in an updated log book stored at Central Pharmacy.

Pharmacy personnel will also conduct clinic site visits every Friday on a rotating basis, so that MAL1 sites will be visited at least once every two months. Pharmacy personnel will evaluate the

storage conditions of the study regimen and supportive medicines, and assess the organization of study regimen bottles and medications. Findings of site visits will be reported and any issues for follow-up will be identified by the Head Pharmacist.

Unused/used drugs are returned to the pharmacy either by a study participant or a research assistant. The returned drugs together with expired ones, are recorded, labeled 'for disposal' and markedly separated from other supplies still in use. At each quarterly visit, Study Monitors review study product ready for disposal and sign off on appropriate accountability form. Destruction request form is filled and sent to the SPONSOR/MANUFACTURER for approval. Once the destruction approval granted, study product will be incinerated under the witness of Head Pharmacist, Study monitor, Municipal and TFDA officials. Before incineration is effected, application letter for study product destruction is submitted to Tanzania Food and Drugs Authority for approval.

Clinical Parameters: A background questionnaire will be administered to all subjects at baseline including questions on age, marital status, socioeconomic and educational status, parity, previous obstetric history, malaria symptoms and other illnesses and treatment during the current pregnancy including use of antimalarial drugs. A complete medical history and clinical examination will also be collected at baseline and at monthly visits to the study clinic. Parameters assessed at these monthly visits will include occurrence of symptoms such as fever and other malaria symptoms since the last visit, antimalarial medication use and any reactions to the medications, cough, diarrhea, body rash, and change in appetite in the preceding month. History of hospitalizations will be recorded. A clinical examination will include: measurement of weight, mid-upper arm circumference, and height; measurement of blood pressure; axillary temperature; general physical examination; obstetric examination (fetal movement, heart rate, presentation, lie, position); reproductive and neurological examinations.

Mothers will be encouraged to deliver at the hospital. At delivery, women will be asked about malaria symptoms, treatment for malaria, and any pregnancy complications since the last study visit. The duration of each stage of labor and complications of labor will be recorded. Thick blood smears will be made from the mother by finger stick, maternal side of the placenta, and umbilical cord. Placentas will be cleaned from blood clots and weighed. The placenta will be put in formalin saline and transferred to the Histopathology Department at Muhimbili as soon as possible. Immediately after delivery, trained research midwives will determine Apgar scores at one and five minutes, and measurement of birth weight, length, mid-upper arm circumference, and head circumference will be undertaken. A physician will carry out a complete physical examination of the newborn to check for physical abnormalities. Stillbirths will also be examined and will be classified as fresh (no sign of decomposition at the time of delivery), or macerated (decomposition had begun before delivery).

At the visit at six weeks postpartum, we will measure maternal anthropometric status including weight and mid-upper arm circumference, and infant's length, weight, head circumference, and mid-upper arm circumference. Infant factors will be assessed at the same visit. Symptoms of diarrheal and respiratory infection in the past 6 weeks will be recorded. Hospitalizations or visits to an outpatient clinic will be also recorded, including the symptoms at that time, physician's diagnosis, laboratory investigations, and medicines prescribed.

Dietary Assessment As part of the ongoing trials on nutrition and infection in Dar es Salaam, Tanzania we have developed experience in the use of dietary assessment methods, including food frequency questionnaires (FFQ) and 24 hour recalls, in this population. The FFQ and food

composition database will be used to assess intake of heme and non-heme iron sources, as well as other nutrients that affect iron absorption, such as ascorbic acid, phytic acid, tannins, and zinc, and assess overall dietary intake and nutritional status. We developed a food frequency questionnaire (FFQ) to assess food intake over the previous 4 months among adults. We will administer a food frequency questionnaire to all women at enrollment in order to assess dietary intake in the preceding four months. Women will be enrolled, on average, at about 20 weeks of gestation, hence the FFQ will ascertain dietary intake in the first half of pregnancy. We have spent considerable time in developing the current version of the adult FFQ. The questionnaire consists of 85 foods and is administered in person by a trained assistant. For each food a subject is asked a series of questions to assess the frequency of consumption of a particular food over the relevant time period. The portion size of each food is also ascertained. This information on frequency of food consumption and portion sizes, in conjunction with food-specific information on the gram weight for each portion size and information on the nutrient content per 100 grams of each food, is then used to calculate a nutrient score (units nutrient per day) for each individual for a given administration of the questionnaire.

The food frequency questionnaire was developed to rank individuals by relative intake of a wide variety of nutrients; the number of foods considered for inclusion was smaller than the number of foods to consider in our studies in the US where a wider variety of foods is available. The food list was compiled by examining published dietary studies and Food Composition Tables for the region, including experience of Tanzanian dieticians, results of 24 hour recalls carried out in the same population; finally, a market survey was carried out to examine if any foods were missed. We piloted various versions of the Frequency Response Questions before deciding on the current format. The dietary data collected affords us the opportunity to quantify intake of various nutrients. The food frequency questionnaire includes a comprehensive list of foods that are consumed in the study setting. We have also developed a food composition table for use in this setting. The nutrient content of all macronutrients, vitamins, and minerals are available.

Laboratory Investigations: A peripheral venous blood sample of 10 ml will be taken from all women at screening, 20 weeks, 30 weeks, delivery, and 6 weeks post-partum. At screening, blood will be tested for a complete blood count and serum ferritin. Following randomization, the following additional assessments will be performed: ABO blood typing as part of standard prenatal care, a sickling test, a blood smear for malaria parasitemia, and c-reactive protein. Malaria parasitemia during pregnancy will be further assessed before ingestion of SP intermittent preventive therapy at 20 and 30 weeks of gestation. At delivery, the following laboratory assessments will be performed in mothers: serum ferritin, c-reactive protein, a complete blood count, and malaria parasitemia. A cord blood specimen and a placental blood specimen from drained retroplacental blood will also be collected at delivery. At the 6 week post-partum visit, malaria parsitemia will be assessed in mothers and a 1 ml specimen will be drawn from the infants through venipuncture for a peripheral blood smear for malaria and a complete blood count. If venipuncture is unsuccessful, however, the heel stick method will be used as an alternative.

Peripheral thick blood smears will be stained with Giemsa and then examined for parasites. Parasite species and density will be recorded. The presence or absence of asexual forms of parasites will be recorded, and the density of such stages will be recorded. Slides will be examined without knowledge of clinical malaria status. In addition, qualitative and quantitative PCR for malaria will be performed at delivery for mothers and at 6 weeks post partum for infants.

After delivery, the placenta will be brought to the laboratory in a sealed plastic bucket containing 500 ml normal saline. A 1 cm3 biopsy will be taken from the maternal side of the placenta

approximately one fourth of the distance from the center to the edge of the placenta [154]. The specimens will be fixed in 10% neutral buffered formalin and processed for routine paraffin embedding. Paraffin sections (□5 μm thick) will be stained with haematoxylin and eosin (H&E), Giemsa's stain, and the periodic acid-Schiff technique. Sections will be examined by light microscopy and under polarized light for the presence of malaria pigment and parasitized erythrocytes. The presence of parasites in maternal erythrocytes in intervillous spaces and fetal erythrocytes in fetal stem vessels will be assessed, and, when present, the percentage of parasitized erythrocytes will be estimated. Malaria pigment will be assessed semi-quantitatively (0 = absent; 1 = mild; 2 = abundant) in maternal and fetal parts of the placenta.

Routine examination of urine and stool (i.e. macroscopic and microscopic assessment including presence and burden of parasites) will be done at baseline. On a subset of 300 individuals, iron markers will be measured at randomization and delivery to assess patients' adherence with the use of the regimen and to ascertain the impact of iron supplementation on these indicators. Retinol binding protein and zinc will be measured in order to examine potential interactions between iron and vitamin A and zinc. An extra 5 ml of blood will be drawn from participants in the sub-sampe for these tests. **The above-mentioned laboratory tests will be performed at the Department of Pathology at MUHAS, Muhimbili-Harvard Laboratory in Dar es Salaam**. All laboratory staff, including the pathologist who reads the placental histopathology slides, will be blinded to the mother's randomization group and to any treatments for malaria.

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Hemoglobin: Hemoglobin will be measured as part of a complete blood count, measured using a CBC5 Coulter Counter (Coulter Corp. Miami, FL). Sickle cell anemia will be diagnosed using a hemoglobin electrophoresis test.

Serum Ferritin: Ferritin will be measured by a particle-enhanced immunoturbidimetric assay using the Hitachi 917 analyzer (Roche Diagnostics, Indianapolis, IN) and Kamiya Biomedical reagents (Seattle, WA). The reference range for serum ferritin is 15 to 200 mcg/L for women.

C-Reactive Protein (CRP): The concentration of CRP will be determined using an immunoturbidimetric assay on the Hitachi 917 analyzer (Roche Diagnostics - Indianapolis, IN), using reagents and calibrators from DiaSorin (Stillwater, MN). In this assay, an antigen-antibody reaction occurs between CRP in the sample and an anti-CRP antibody that has been sensitized to latex particles, and agglutination results. This antigen-antibody complex causes a decrease in transmitted light, which is detected spectrophotometrically, with the magnitude of the change being proportional to the concentration of CRP in the sample. This assay has a sensitivity of 0.03 mg/L. The day-to-day variabilities of the assay at concentrations of 0.91, 3.07 and 13.38 mg/L are 2.81, 1.61 and 1.1%, respectively. The reference range for C-reactive protein is < 5.0 mg/L.

Forms	Screening	Enroll/Rando	Monthly	30 Weeks	Delivery	6WKPP
Screening	**X**					
Rando		**X**				
Background		**X**				
Eligibility		**X**				
Consent		**X**				
Nurse		**X**	**X**	**X**		
Phys		**X**	**X**	**X**		
FFQ		**X**				
24HR		**X**				

Malaria Symptom Diary		**X**	**X**	**X**	**X**	**X**
Delivery					**X**	
Newborn					**X**	
Neonatal					**X**	
6WK-Infant						**X**
6WK-Adult						**X**
Lab Tests	**Screening**	**Enroll/Rando**	**20 Weeks**	**30 Weeks**	**Delivery**	**6WKPP**
Serum Ferritin		**X**			**X**	
C-Reactive Protein		**X**			**X**	
Hepcidin		**X***			**X***	
Retinol Binding Protein		**X***			**X***	
Zinc		**X***			**X***	
Transferrin Receptor		**X***			**X***	
Maternal Hemogram		**X**			**X**	
Infant Hemogram						**X**
ABO Blood Typing		**X**				
Sickling		**X**				
Maternal Blood Smear for Parasites		**X**	**X**	**X**	**X**	**X**
Infant Blood Smear for Parasites						**X**
Placental and Cord Blood Smears					**X**	
Stool General		**X**				

*These tests will be done on subset of 300 participants

Soluble Transferrin Receptor (sTfR): sTfR is measured by a particle-enhanced immunoturbidimetric assay using the Hitachi 917 analyzer and Roche Diagnostics reagents (Indianapolis, IN). In brief, latex-bound anti-sTfR antibodies in the reagents react with the sTfR present in the samples and form an antigen-antibody complex. The turbidity of the agglutination is measured, which is directly proportional to the amount of sTfR present in the sample. sTfR at the concentrations of 2.25 and 7.00 mg/L have a day-to-day variability of 2.2 and 1.4%, respectively. The reference ranges for serum transferrin receptor is 1.9 to 4.4 mg/L for women (2.2 to 5.0 mg/L for men).

These laboratory techniques will be performed in Dar es Salaam where we have established a research laboratory that has a strict quality control system.. In order to maintain uniform reporting of blood smear results, a senior lab technologist will counter check at least 5% of all the slides (randomly selected) once a week. Retraining of the primary parasitology technician will be done if there is a discordance of ≥10% or a negative slide is found to be positive or the converse. External quality control will be achieved by sending a 5% subsample of slides to an external reader at the National Malaria Control Program for blinded reading. External quality control will also be done on 50 blood smears taken at baseline and from 25 of the first 50 patients with parasitemia.

Additional Laboratory Investigations: We collect and store -70° C an aliquot of plasma from each screened person at baseline and for every enrolled person at all time points for future ancillary analyses of genetic, nutritional, and immunological questions that were not possible to include in the current application due to funding limits. These will include examining novel indicators of iron status and biomarkers of iron metabolism, including hepcidin, zinc, and Hb/Zinc protoporphyrin ratio, and other indicators at baseline; and genetic polymorphisms of haptoglobin.

Data Management and Biostatistical Component: We have developed a stringent data management system for use in our ongoing trials. All questionnaires and laboratory forms that accompany specimens for analysis are tracked using a 'batch' system. All forms of a particular type filled over a certain period of time are processed together as a batch. The processing steps include several stages of review and editing for checking completeness and consistency, double data entry, supervisory checks of the inconsistencies discovered at second data entry, and finally "retirement" and archiving of the form. The batch system ensures that all omissions and most inconsistencies are detected and resolved in a timely manner. Thus, the number of errors in the resulting data files is minimized. A system is in place to ensure that every specimen that was collected at the clinic and sent to the laboratory is accounted for in a returning form. Data entry and management is done using MS-Access software. Data updates will be sent regularly to Harvard, via secure links, where the data is converted to SAS data sets. A software package called Dataweb, developed at the Channing Laboratory in Boston, will be used to display graphically the values for all variables in the data sets. Additional logical and longitudinal data checks will be examined. These outputs will be regularly sent to Tanzania to aid in data cleaning; any data updates will be made in the primary database in Tanzania after review of source documentation. Quality assurance and randomization checking will be undertaken in Boston and results will be communicated to the investigators in Dar es Salaam. A full-time data manager will be hired in Dare es Salaam; s/he will be assisted by a research fellow who is trained at the doctoral level in epidemiology; the fellow will contribute to an overall system of quality assurance with respect to study implementation on a day-to-day basis.

An internal medical monitor will be hired in Dar es Salaam to ensure that the study design is implemented according to protocol. S/he will be a physician with research training and will undertake regular meetings with the site coordinator and with members of the study staff; review of data collection forms and related records to assess their completeness; observation of the study personnel carrying out specific procedures; review of other documents and files at the site to assess whether they are up-to-date; physical or verbal walk-through of certain procedures; conversations with key support personnel to assess their practices with regard to data collection; and inspection of storage facilities (laboratory and data).

Definition of Main Endpoints

Primary endpoints: These will be the incidence and parasite density of placental malaria. Placental infection status will be categorized as infected if there are asexual parasites in the placenta blood; not infected if the placental blood smear is negative; or status unknown if no placental smear is available. Placental malaria parasite density will be defined as number of parasites per μL of blood or 200 white blood cells; the latter will be converted to a count per μL

of blood assuming a count of 8000 WBC/μL [155]. The continuous measurements of maternal hemoglobin and birth weight will be two other primary endpoints.

Secondary endpoints: Low birth weight will be defined as birth weight less than 2500 grams. Maternal malaria will be defined in 2 ways: fever within the last 72 hours with any parasitemia on a peripheral blood smear. High density malaria will be defined as fever with parasitemia >5000. Anemia will be defined as hemoglobin less than 11 g/dl. Severe anemia is less than 8.5 g/dl.

Data Analysis

Analysis of primary endpoints: Intent-to-treat analysis of treatment effects will be used as the primary analysis strategy for all endpoints.

Placental Malaria Infection: We will compare the proportions of randomized women who have placental malaria infection in the iron group, compared with women on placebo**.** To test the statistical significance of any difference observed we will use the $\chi 2$ test. There are numerous risk factors for placental malaria which will be measured in this study at baseline (defined as the time of randomization), including maternal body mass index, gravidity, maternal mid upper arm circumference, hemoglobin, gestational age, maternal age, parity, and indicators of socioeconomic status as measured by education, occupation, possessions, and marital status. Although randomization ensures that, on average, treatment groups will be balanced with respect to all of these risk factors, this may not be true in the particular instance of the current study. Thus, we wish to assess the success of randomization by first comparing the baseline characteristics of women in the treatment groups using $\chi 2$ and t-tests and then using multivariate modeling techniques such as logistic regression to identify the strongest independent imbalances. Then, we will re-assess treatment effects as observed in the original intent to treat analysis after adjusting for the risk factors associated with the strongest imbalances. A logistic regression model will be used for this analysis. We will compare treatment effects estimated from the original analysis to those obtained from the adjusted analysis to decide whether adjustment is necessary. Because placental malaria is not likely to be a rare event in this study population, the odds ratio may not be a good approximation to the parameter of interest, the risk ratio. Multivariate regression models for the risk ratio are now available in commercial software, and we will apply binomial regression models with the log and identity link functions to the data as well. Linearity of multiple risk factors on one scale, e.g. the logistic, generally implies that there will be interactions of these risk factors on another scale, e.g. with the identity link. Goodness-of-fit of the data to various models forms will be explored, and interactions reported.

Modification of any treatment effects observed by a third variable, such as gravidity or maternal mid-upper arm circumference, will be explored by stratification and testing for significance of the difference between the treatment effects observed in two strata. For effect modifiers which are continuous or have more than two levels, the logistic regression model will be used to quantify effect modification and test for statistical significance, through the creation of model interaction terms and use of the likelihood ratio test to assess significance. Since there are no *a priori* effect modifiers hypothesized, our study is not designed to detect any which may occur, and we acknowledge that unless there is strong modification of an observed treatment effect, the power of our study to detect these will be quite low.

Placental malaria, defined as a continuous variable of parasite density will be compared between the two treatment arms. The difference in these measurements for the two treatment groups, and the statistical significance of any difference observed will be assessed by a two-sample t-test and non-parametrically by the Wilcoxon rank-sum test. Linear regression models will be used to evaluate effect modification, such as by baseline iron status, or dietary intake by creating appropriate cross-product terms and evaluating their significance by F-tests. To evaluate

treatment-related differences in change after adjusting for risk factors which remained imbalanced after randomization, linear regression models of the change variables on treatment status and other risk factors will be used.

Hemoglobin: This parameter will be assessed three times (at baseline, 30 weeks of pregnancy and at delivery). In the primary analyses we will compare the mean change between baseline and delivery. This change will be calculated as the average within-women difference in these measurements for the two treatment groups, and the statistical significance of any difference observed will be assessed by a two-sample t-test and non-parametrically by the Wilcoxon rank-sum test. Linear regression models will be used to evaluate hemoglobin levels between the treatment arms, adjusting for factors that were imbalanced after randomization, and effect modification.

Birth Weight: The difference between mean birth weights in the iron and placebo arms will be calculated, and the statistical significance of any observed difference will be assessed by a two-sample t-test and non-parametrically by a Wilcoxon rank-sum test. A linear regression model will be used to adjust for residual treatment imbalances by risk factors, and to evaluate effect modification by these risk factors, using the methods for multivariate modeling described above.

Analysis of Secondary Endpoints

Low Birth Weight (<2500 g): Analyses of the effects of iron supplements on low birth weight will parallel those outlined above for placental malaria infection, also a binary endpoint.

Maternal anemia (Hb < 11.0 g/dL) and Maternal malaria infection: These binary secondary endpoints, as noted in the Definition of Endpoints section, will be analyzed in a similar fashion as the primary endpoints as described above, although only women without these conditions at baseline will contribute to these analyses.

Analysis of Adherence and Iron Status Biomarkers: Pill counts will be undertaken at monthly clinic visits. Serum concentrations of ferritin will be assessed on all subjects at baseline and among a subset of 300 women at delivery. Increased intake of iron is usually paralleled by increases in serum ferritin values, and concentrations of serum ferritin and pill counts will be compared between the active treatment and placebo arms as proxy indicators for adherence with the supplements. The analysis will be based on a regression model for the change from baseline to delivery and supplemented by Wilcoxon rank-sum tests. Median changes (and associated order-statistic-based 95% CI's) will be summarized by treatment arm. The effect of iron supplementation on serum transferrin receptor, hepcidin, and C-reactive protein will be also examined, following a similar approach.

Statistical Power Calculations

Power for the primary aims, placental malaria infection and density, maternal hemoglobin levels, and birth weight, have been calculated based on a nominal Type I error rate (alpha) of 0.05, and a 10% sample size adjustment for loss-to-follow-up and fetal loss. Power is based on a 2-sided test of proportions, based on a Z-statistic using the asymptotic variances of the observed proportions. The table below displays the power of the study to detect treatment effects on placental malaria infection. The assumed placebo arm rate of 20-30% is supported by studies reviewed above [37, 39]. Displayed is the power (for treatment effects expressed as % reductions) based on a 2-sided test for comparison of proportions, total enrolled n=1500, with a 10% sample size adjustment to account for loss to follow-up and fetal loss.

Statistical Power for Incidence of Placental Malaria				
Incidence in Placebo Arm	**Effect of Intervention on the Incidence**			
	HR=0.75	**HR=0.70**	**HR=0.65**	**HR=0.60**
20%	48%	65%	80%	90%
25%	57%	74%	87%	95%
30%	64%	80%	92%	98%

The study will have good power to detect a 35% effect size, for placental malaria rates as low as 20% in the placebo arm. If the placental malaria rates are larger (25% or 30%), the study will have good power to detect a 30% effect size and excellent power to detect a 40% effect size.

Continuous Variables: The table below displays the power of the study to detect treatment effects on continuous variables, including placental malaria density, maternal hemoglobin concentration, and birth weight. Displayed is the power (for treatment effects expressed as percent changes in effect size) based on a 2-sided test for comparison of proportions, total enrolled n=1500, with a 10% sample size adjustment to account for loss to follow-up.

Placental malaria density: The table below displays the power of the study to detect treatment effects on placental malaria density. The assumed mean placental malaria density in the placebo arm of 9,000 parasites/μL is supported by studies reviewed above [156]. The study will have good power to detect an effect size of 8.5%, and excellent power to detect a 10-20% change, based on a 2-sided test for comparison of proportions, total enrolled n=1500, with a 10% sample size adjustment to account for loss to follow-up and fetal loss. The study will have good power to detect an 8.5% effect size, and excellent power to detect an effect size of 10% or greater.

Maternal hemoglobin concentrations: The table below displays the power of the study to detect treatment effects on maternal hemoglobin concentrations. The assumed mean hemoglobin levels in the placebo arm of 11.4 g/dL is supported by studies reviewed above [157]. The study will have good power to detect a 5% effect size, and excellent power to detect a 10% change or greater.

Statistical Power for Continuous Variables							
Variable (Units)	**Mean**	**SD**	**Minimal effect size for 80% power**	**Power for 5% change**	**Power for 10% change**	**Power for 15% change**	**Power for 20% change**
Placental malaria density (parasites/μL)	9000	5000	8.5%	38%	91%	>99%	>99%
Maternal hemoglobin concentration (g/dL)	11.4	3.8	5.0%	79%	>99%	>99%	>99%
Mean birth	3083	400	2.0%	>99%	>99%	>99%	>99%

weight (grams)							

Birth weight: The table above displays the power of the study to detect treatment effects on birth weight. The assumed mean birth weight in the placebo arm of 3,083 grams is supported by studies reviewed above [144]. The study will have good power to detect a 2% effect size, and excellent power to detect a 5% change or greater.

Data Safety Monitoring Board (DSMB)

We have constituted a DSMB to oversee another maternal health study, and we will ask this board to oversee the proposed study as well. Members of the DSMB provide varied and complementary expertise and include: Dr. Mohamed Bakari (clinician, infectious diseases, Tanzania), Dr. Henrik Friis (nutrition, epidemiology, clinical trials, Denmark), Dr. Hassan Mshinda (epidemiology, malaria, Tanzania), and Dr. David Wypij (Biostatistics, clinical trials, USA). Dr. Jeff Griffiths (nutrition, epidemiology, USA) will serve as the chair of the board. DSMB members will not be paid.

It is anticipated that the first review will occur before the initiation of the study, with subsequent reviews biannually thereafter. Each meeting will include an administrative review to assess accrual, retention, and the progress of the study. In addition, the DSMB will monitor the occurrence of any adverse effects; these will include clinical signs and results of laboratory investigations done within the context of the trial. The DSMB will define stopping and unblinding rules at its first meeting. Additional reviews or an altered schedule may be instituted at the discretion of the DSMB.

Open and closed reports will be available to DSMB members at least five working days prior to the scheduled meeting. Open reports will be used to provide findings relating to study progress (across intervention group status), including: accrual rate, baseline characteristics, safety outcomes, and other relevant information. These reports will be prepared by trial investigators. Closed reports will be confidential, and may contain grouped data pertaining to safety and efficacy outcomes. The DSMB chair will designate a person to prepare summary minutes for all meetings. He will compile and distribute draft summaries to DSMB members within one week of the board meeting for review and approval. Finalized DSMB meeting summaries will be distributed to the board members, Principal Investigator, study statistician, and others as per the discretion of the DSMB. Meeting summaries may contain general feedback on study progress, follow-up items, timeline for future meetings, recommendations regarding study protocol and continuation, and justification for these recommendations. After each DSMB meeting, the committee will prepare and submit a report to the Principal Investigator, which will then be forwarded to the Institutional Review Boards at the Harvard School of Public Health and the Muhimbili University of Health and Allied Sciences. This report will document the date and content of the data review, and summarize the board's recommendation regarding any modifications to the study protocol and/or continuation of the trial.

Innovation

We appreciate that the design and statistical methods do not represent innovation as these are the "gold standard" methods. We feel that the study represents innovation in that few randomized studies have been conducted among pregnant women in developing countries, and none on this specific issue, i.e. the safety and efficacy of iron supplementation among pregnant women in areas of high malaria burden. The majority of research to date has focused on the efficacy of micronutrients in malaria among children. We will also develop a repository of specimens that

will allow us to further examine the potential mechanisms of action of iron status in future ancillary studies.

Time Schedule

Total duration of the study is 48 months. The first **3 months** will be used for preparing instruments, hiring project staff and training. The following **3 months** will be utilized for pretesting and standardizing all research procedures and forms in a pilot study, and upgrading training of staff as needed. Recruitment will be done in **21 months.** In order to randomize 1500 pregnant women, we anticipate that we will need to screen approximately 7500 women. Of these, we anticipate that 90% will be HIV-uninfected, that 50% will be either primigravida or secundigravidae, that 50% will have Hb > 8.5 g/dL, and that of these a further 90% will have normal measures of iron status and be otherwise eligible for enrollment. We believe that this estimate of time is conservative, however if we find that the recruitment rate is lower than expected, we plan to request referrals of eligible patients from other prenatal centers that are part of our network in Dar es Salaam. The minimum follow-up time for each participant is **9 months** (7 months during pregnancy and 6 weeks postpartum). The first **6 months** of year 4 will be used to focus on lab tests and data management and analyses. Completion of analysis and write-up will be undertaken in the last **6 months** of the study.

Limitations of the Proposed Study

• Ethics: Ethical concerns are of course always paramount when undertaking clinical research in humans, especially when the research subjects are pregnant women. We adhere to all institutional and federal regulatory and ethical requirements in performing our trials in maternal and child health, and the proposed study is not different in this regard. Mention should be made of the design of our proposed study in that a placebo arm is included. As noted in the Background section, while data generally support the recommendation for universal iron supplementation during pregnancy in industrialized countries, these data are less compelling for women living in malaria-endemic regions of the world. Moreover, as concern has re-emerged from recent studies concerning the safety of universal iron supplementation, we feel that a placebo-based trial is ethically justifiable and scientifically appropriate. By excluding women who are either anemic or iron deficient (or have evidence of iron overload) from the proposed trial, we feel that we are safeguarding the welfare of women enrolled in this trial, regardless of to which arm they are randomized.

• HIV Infection: The burden of malaria in pregnancy is exacerbated by co-infection with HIV. In sub-Saharan Africa, approximately 25 million pregnant women are at risk of malaria infection every year,[36, 60, 61] where concurrently two-thirds of HIV infections, 72% of AIDS-related deaths[157], and 80% of deaths due to malaria occur [3]. A review of 11 studies from Africa highlights the deleterious effect of HIV on malaria, reporting higher risks of placental malaria (RR: 1·66, 95% CI 1·48–1·87), high-density parasitemia, and febrile illness in HIV-infected women [61]. Further, iron supplementation among HIV-infected individuals deserves further study given the potential adverse effects of such supplements on clinical and virological HIV disease stage [158]. Due to the financial restrictions of this proposed trial, we unfortunately will not be able to enroll a large enough sample of both HIV-infected and HIV-uninfected pregnant women in order to evaluate the potential modification by HIV status of iron supplementation. Further research is clearly warranted to explore the interaction between iron, malaria and HIV co-infections, and their impact maternal and infant health outcomes.

• Dose of Iron Supplementation: Some studies have suggested that lower dose iron supplementation (e.g., 30 mg, 40 mg) may be as efficacious in prevention of iron deficiency [70, 114, 115] compared to the standard WHO dose of 60 milligrams, with a less frequent occurrence

of gastrointestinal side effects [71]. However, these studies have been conducted in developed countries; there has been a lack of standardization of iron supplement dosage and inadequate assessment of side-effects in these studies. However, the safety and efficacy of iron supplementation in general and different doses of administration specifically, and effects on malaria-related outcomes have not been examined in developing countries and areas of high malaria burden. We therefore propose to examine the safety and efficacy of a standard 60 milligram dose of daily iron supplementation in pregnant non-anemic women residing in malaria-endemic regions. Due to the financial restrictions of this proposed trial, we unfortunately will not be able to enroll a large enough sample to evaluate the effect of different doses of iron supplementation. Further research and funding is warranted to explore the safety and efficacy of various doses of iron supplementation in pregnancy in areas of high malarial burden.

• Accrual: Recruitment will take place at a large prenatal clinic. This will require a good organizational and supervision system. We have used a system of similar complexity to screen approximately 40,000 women every year as part of previous and ongoing perinatal/child health trials.

• Retention: A major limitation in large trials is the operational difficulty in following up subjects for long periods of time. We have experience with studies of similar design in Tanzania in which we instituted a package of non-coercive incentives for patients to continue coming for follow-up. As a result of these procedures we are able to achieve follow-up rates of 85-95%.

• Bias: The potential for observation bias exists in intervention studies such as this one if the researchers are aware of the experimental regimen to which a subject has been assigned. To avoid the researchers' assessment from being influenced by the regimen received, this will be a double-blind study such that during the study neither the investigators nor the study subjects will know what each regimen contains.

• **Adherence**: We recognize that non-adherence decreases the statistical power of a study to detect any true effect of the regimens. Our power calculations show adequate power even after realistic amount of non-adherence. In addition, we intend to perform statistical analyses to adjust for non-adherence. During the implementation of the study, we will monitor adherence by questioning the subjects, by pill count, and by measurement of plasma serum ferritin levels in a sub-sample of subjects. We will attempt to maximize adherence of the study subjects by providing counseling and allowing the patients adequate time to think through the issues during the recruitment phase, thus eventually selecting a study population that is interested and reliable. Furthermore, we believe that the set of incentives we will provide, as discussed above, will reduce non-adherence.

• Capacity: Research in many developing countries is limited by the lack of well-equipped laboratories and well-trained research staff. Tanzania is one of the exceptions since a number of research projects with substantial laboratory and field research components have been implemented. Several of these studies have been carried out as collaborative efforts between Harvard School of Public Health and Muhimbili University of Health and Allied Sciences.

Summary

Iron supplementation is generally recommended to control iron deficiency and iron deficiency anemia in pregnancy in the developing world. However, there is limited evidence regarding the safety and efficacy of iron supplementation among pregnant women in developing country settings, particularly among non-anemic women. Findings from recent randomized trials among children have raised concerns regarding the safety of iron supplementation in malaria-endemic

regions. However, few studies have examined the impact of iron supplementation on malarial risk in pregnancy, and the safety and efficacy of prenatal iron supplementation have not been established in areas of high malaria burden. To date, studies have not resolved whether antenatal iron supplementation increases the risk of malaria, and few studies have examined the impact of iron status on malaria risk during pregnancy. Adverse effects of supplemental iron would be of considerable public health importance in malaria endemic region of sub-Saharan Africa due to the high prevalence of iron deficiency, malaria and other infectious diseases, and the large number of iron supplements that are routinely distributed, particularly during the antenatal period. Evidence from randomized controlled trials is urgently needed to examine the safety and efficacy of iron supplements among pregnant women in areas of high malaria burden.

Human Subjects
The proposal will be reviewed by the Institutional Review Board at Harvard School of Public Health, and by the Ethical Committee of Muhimbili University of Health and Allied Sciences.

(1) Eligible participants will be HIV-uninfected pregnant women who are primigravida or secundigravidae at or before 27 weeks of gestation, not anemic (defined as Hb<8.5 g/dL) or iron deficient (defined as serum ferritin <12 μg/L), and intend to stay in Dar es Salaam until delivery and for at least six weeks thereafter. Women with high iron stores at baseline (i.e., >200 μg/L serum ferritin) will be excluded. HIV-infected women will be referred for standard of care services including provision of antiretroviral treatment, as needed. Women who have given informed consent to participate in the study will be enrolled.

(2) At each visit to the study clinic, patients will have a complete medical history and physical examination carried out. Background characteristics and dietary data will also be collected. A peripheral venous blood sample will be taken from all women at the time of randomization, at 20 and 30 weeks of gestation, at delivery, and at 6 weeks post partum. If a patient's Hb level falls below the 8.5g/dL cutoff for severe anemia, she will be treated as per standard of care in Tanzania. We will investigate the primary cause of the severe anemia and treat appropriately. For instance, if sf<12mcg/L, we will treat with iron supplementation as per standard of care. At delivery, a cord blood specimen and a placental blood specimen from drained retroplacental blood will be collected. No blood specimens will be collected from the newborn. Malaria parasitemia during pregnancy will be assessed at randomization, before ingestion of SP intermittent preventive therapy, at delivery, and in cord blood. Peripheral thick blood smears during pregnancy and at delivery will be stained with Giemsa and then examined for parasites. Parasite species and density will be recorded. The presence or absence of asexual forms of parasites will be recorded, and the density of such stages will be recorded. Slides will be examined without knowledge of clinical malaria status. At delivery, a cord blood specimen and a placental blood specimen from drained retroplacental blood will be collected. At the 6 week post-partum visit, a specimen will be drawn from the infants through venipuncture. These clinical and laboratory data will be used for research purposes, as well as for provision of appropriate clinical management to the patients.

(3) Informed consent will be obtained from each subject by our full-time research assistants and will be in 2 stages as follows: (a) At the first encounter with a potentially eligible patient, she will be given an overview of the rationale and methods of the study. (b) At the subsequent visit, when the patient will have the details of the study explained to her. In the process of seeking informed consent, the research assistant will make it clear to every patient that participation in the research is voluntary and that she could stop participating at any point after consent is granted without any penalty. **Participants will be given the contact information for the Site Principal Investigator as well as the Chairman of the MUHAS Senate Research and Publications Committee, and**

will be encouraged to contact them with any questions or concerns regarding the trial. In addition to the study investigators, a person Muhimbili who is not part of the trial will be designated as someone to whom the patients may go with any concerns regarding the study, or if they would like to terminate their participation in the trial. The consent process will be conducted in Kiswahili, the local language.

(4) Confidentiality will be strictly observed. The information collected will never be revealed to or discussed with third parties. To further protect confidentiality, study subjects will attend a study clinic that is located within the larger routine clinics; we will use the same clinic flow pattern, same clinic cards, and health education sessions. Our research assistants will dress in the same way as the clinic nurses. A study research assistant and a study physician will available at the study clinic to see clients who prefer to have their routine research visits at ordinary clinic hours and even on Saturdays. Examination and counseling of clients will also be carried out in private cubicles. Laboratory specimens will be taken to the laboratory by one of our research assistants and given only to authorized personnel working in the laboratory. The registers and forms are kept in a locked office space that is not accessible to any other staff except one of the supervisors or the project coordinator.

(5) The doses of iron given to subjects are considered to be safe for use by general population, and for use by pregnant women. The safety of iron supplementation is reviewed in detail above.

Vertebrate animals: None

Contractual arrangements: If an award is made as a result of this application, Harvard University will be prepared to enter into subcontracts with appropriate terms and conditions with Muhimbili University of Health and Allied Sciences, Dar es Salaam, Tanzania.
Consultants: None

Data Sharing Plan: Sharing of data generated by this project is an essential part of our proposed activities, and we plan to do so as per NICHD requirements.

Research seminars at Harvard School of Public Health and Muhimbili University: Regular research seminars are held at both sites, and the investigators are regular participants and lecturers at these symposia. The results of the trial will be presented regularly at these seminars. In addition, investigators regularly teach courses at their respective universities on topics relevant to the research (nutrition, child health, clinical trial methodology) where the results of the study will be discussed in lectures to medical and other graduate student audiences.

Community Advisory Board (CAB) for Harvard-Muhimbili research: Several community members, including current and past participants in trials based in Tanzania, make up our CAB in Dar es Salaam. This group meets regularly with the research team and helps to inform the research team about the social norms and values of the community from which the study participants are recruited. The CAB also engages in discussions with the research team about results of ongoing trials. We propose to update the CAB with the results of any related trial as they become available. The CAB will also help to share the results of the trial with the larger community in culturally appropriate ways.

Symposia with policy-makers in Tanzania**:** We regularly meet with personnel of the Ministry of Health of Tanzania to update them of our plans for future research as well as review results from prior and ongoing trials as they become available. As an example, the MOH has been made aware of our findings concerning the beneficial effect of vitamin supplementation of HIV-infected

pregnant women, and has begun changing policy to encourage widespread use of vitamins for these women. We will to continue this process for the current proposal.

Sharing data with other investigators**:** We realize that the data collected from the proposed trial may provide other investigators with the opportunity to answer scientific questions about a number of ancillary issues, whether examined alone or in combination with other data sets in a meta-analysis. On the other hand, we strive to respect the autonomy of the study participants in determining how their identifiable data and/or biological specimens will be shared. We will draft informed consent materials that set forth the feasible options for each study participant to consider with respect to the possible future uses we may seek to apply their identifiable data and/or specimens that would involve sharing them with other investigators. Each study participant will choose to consent or decline to consent to proposed future sharing of their data with other investigators. The consent process will also make it clear that publications (journals, seminars, etc., as described above) will not identify them, and will use only aggregated data. Further, we will make the study data set and associated documentation available to users under a data-sharing agreement that provides for: (1) a commitment to using the data only for research purposes and not to identify any individual participant; (2) a commitment to securing the data using appropriate computer technology; and (3) a commitment to destroying or returning the data after analyses are completed. In our previous studies, we have supported and encouraged the development of ancillary studies to take place within the larger common protocol. Such ancillary studies can contribute to the acquisition of new data, cement collaborative relationships, and lead to new questions for future studies. Future research with archived data and laboratory samples will only be conducted after ethical approval is granted from both HSPH and MUHAS IRBs. Source data will not be moved away from Dar es Salaam.

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