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STUDY PROTOCOL

REVISED **Safe and effective delivery of supplemental iron to healthy older adults: The double-blind, randomized, placebo-controlled trial protocol of the Safe Iron Study** [version 2; peer review: 3 approved]

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## Abstract

The forms of iron currently available to correct iron deficiency have adverse effects, including infectious diarrhea, increased susceptibility to malaria, inflammation and detrimental changes to the gut microbiome. These adverse effects limit their use such that the growing burden of iron deficiency has not abated in recent decades. Here, we summarize the protocol of the "Safe Iron Study", the first clinical study examining the safety and efficacy of novel forms of iron in healthy, iron-replete adults. The Safe Iron Study is a double-blind, randomized, placebo-controlled trial conducted in Boston, MA, USA. This study compares ferrous sulfate heptahydrate ($FeSO_4 \cdot H_2O$) with two novel forms of iron supplements (iron hydroxide adipate tartrate (IHAT) and organic fungal iron metabolite (Aspiron™ Natural Koji Iron)). In Phase I, we will compare each source of iron administrated at a low dose (60 mg Fe/day). We will also determine the effect of $FeSO_4$ co-administrated with a multiple micronutrient powder and weekly administration of $FeSO_4$. The forms of iron found to produce no adverse effects, or adverse effects no greater than $FeSO_4$ in Phase I, Phase II will evaluate a higher, i.e., a therapeutic dose (120 mg Fe/day). The primary outcomes of this study include *ex vivo* malaria (*Plasmodium falciparum*) infectivity of host erythrocytes, *ex vivo* bacterial proliferation (of selected species) in presence of host plasma and intestinal inflammation assessed by fecal calprotectin. This study will test the hypotheses that the novel forms of iron, administered at equivalent doses to $FeSO_4$, will produce similar increases in iron status in iron-replete subjects, yet lower increases in *ex vivo* malaria infectivity, *ex vivo* bacterial proliferation, gut inflammation. Ultimately,

## Open Peer Review

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this study seeks to contribute to development of safe and effective forms of supplemental iron to address the global burden of iron deficiency and anemia.

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## Introduction

The forms of iron currently available have adverse effects that limit their use in addressing prevalent iron deficiency. Iron-deficiency, and the anemia it can produce, affects some 1.2 billion people worldwide, mostly children and women in resource-poor countries, comprising a global disease burden that has not abated in recent decades. Efforts to address iron deficiency through iron supplementation programs have been frustrated by the serious side effects of inorganic forms of iron, which due to lower enteric absorptive efficiency compared to heme-iron in foods (2–20%), must be given in relatively high levels[1]. Those adverse effects include infectious diarrhea[2–5], detrimental changes in the gut microbiome[5,6] and increased serious morbidity among iron-replete children in malaria-endemic areas[7,8], as well as less serious consequences such as dyspepsia and constipation. One of the underlying causes of these effects are thought to involve stress on the gut due to unabsorbed iron, which can be pro-oxidative and pro-inflammatory. In addition, unabsorbed, soluble iron can be used by the gut microbiome and favor the proliferation of pathogenic enteric bacteria[9], which can contribute to the inflammatory response[5,6,10,11] that leads to down-regulation of iron absorption[12]. Further, the formation of non-transferrin-bound iron (NTBI) from the high supplemental sources of iron is believed to be involved in the increase of malaria infection severity[13,14]. In capillaries of the intestine and brain, NTBI is proposed to increase sequestration of erythrocytes infected with malaria[15].

The serious adverse effects of current iron supplements are problematic because they have deterred addressing prevalent iron deficiency in regions of need. Specifically, the finding that routine iron supplementation led to increased severity of clinical malaria among children in Tanzania[7] effectively halted efforts to address iron deficiency in malaria-endemic areas. Lack of a safe and effective treatment leaves large numbers of children iron deficient, many with associated anemia. Thus, the burden of disease, which includes growth, cognitive and physical performance deficits as well as increased risk of infection, continues to climb in this age group.

## Rationale

The overall goal of this project is to generate evidence needed for the development of a modality of providing bioavailable iron that minimizes or eliminates adverse effects in iron-replete individuals. We will employ the commonly used iron supplement, ferrous sulfate ($FeSO_4$) to compare with two novel forms of iron with features that suggest each may be a useful nutritional source of iron with fewer side effects than $FeSO_4$.

The first novel form of iron is a nanoparticulate formulation of iron hydroxide adipate tartrate (IHAT), which was developed and donated to this study by the Medical Research Council, Cambridge University and, currently, Nemysis Ltd. IHAT is a nanoparticle-based supplement composed of three generally recognized as safe (GRAS) substances, iron hydroxide, tartaric acid, and adipic acid. The particle itself resembles the normal metabolite ferritin, which is a larger polyatomic particle. Like ferritin, IHAT can be absorbed by endocytosis, but dissociates within the enterocyte and is subsequently metabolized as ferrous iron. In pre-menopausal, iron-deficient women, the relative bioavailability of IHAT to $FeSO_4$ was approximately 75%[16]. Once absorbed, IHAT appears to enter the metabolic iron pool at efficiencies comparable to iron from $FeSO_4$, and it is insoluble in the lumen of the gut[16,17]. That appears to prevent its being utilized by enteric bacteria, as evidenced by the finding that it did not affect the recovery of the hindgut microbiome in iron-deficient mice[18]. Thus, we anticipate that it is likely to be less irritating to the gut and, likely, less pro-inflammatory than $FeSO_4$. In addition, IHAT appears to enter the metabolic iron pool more slowly than iron from $FeSO_4$[19], suggesting that its post absorptive surge in pro-oxidative NTBI may be less than that of $FeSO_4$, thus, producing less oxidative stress.

The second novel form of iron is an organic fungal iron metabolite, Aspiron™ Natural Koji Iron, which has been developed and donated to this study by Cura Global Health Inc. Aspiron is a product of the natural fermentation of Koji (*Aspergillus oryzae*) in the presence of $FeSO_4$. The iron-rich Koji biomass is heated, harvested and dried resulting in the inactivation of Koji powder that contains 8–10% iron. Koji (*A. oryzae*) is widely used for making such foods as soy sauce, tempeh, miso, and for producing food-grade α-amylase. Koji is considered safe by Joint FAO/WHO Committee on Food Additives and has been accepted as a GRAS constituent of food. The Codex Alimentarius International standards included Koji as an approved food additive. In non-pregnant, non-anemic women with marginal iron stores (ferritin concentration of <40 μg/L), the absorption of Aspiron was comparable to $FeSO_4$, suggesting similar tolerability as $FeSO_4$[20]. The kinetics of Aspiron absorption shows it to appear in the plasma later, and over a longer period of time, than iron in $FeSO_4$. Therefore, we anticipate that any post absorptive surge in pro-oxidative transferrin-iron and NTBI will be less than that of $FeSO_4$, thus, also producing less oxidative stress. Moreover, Aspiron was found to have significantly lower number of side effects compared to $FeSO_4$[20].

This study is divided to two phases. In Phase I, we will determine effects of both forms of low-dose supplemental iron. For the forms of iron found to produce no adverse effects at the 60 mg Fe/day dose level, Phase II of the study will be conducted in which such forms will be tested at a higher, therapeutic dose of 120 mg Fe/day against the same outcomes.

Here, we summarize the protocol for the Safe Iron Study, the first clinical study examining the safety and efficacy of these novel forms of iron supplements in iron-replete older adults.

## Hypothesis and objectives

We will robustly test our formal hypotheses yielding useful answers to the primary questions about the relative safety and efficacy of these novel forms of iron in iron-replete subjects. This study will address the major side effects of oral iron supplementation of iron-replete subjects. Specifically, we will compare the effects of two novel forms of iron and the widely used ferrous form on malaria (*Plasmodium falciparum*) infectivity of host erythrocytes (assessed *ex vivo*), bacterial proliferation (of selected species) in the presence of host plasma (assessed *ex vivo*), redox stress and inflammatory stress (assessed *in vivo*), gut irritation (assessed *in vivo*), effects on the gut microbiome (assessed *in vivo*). This set of indicators is referred to as the "safety profile". Additionally, iron utilization (assessed *in vivo*) will be compared to evaluate efficacy.

In Phase I, we will test the following research questions underpinning formal hypotheses using the design detailed below:

1. Does IHAT, a novel form of iron, produce beneficial and/or adverse effects comparable to $FeSO_4$ at equivalent iron doses?

IHAT and $FeSO_4$ administered at 60 mg Fe/day will produce comparable responses in malaria infectivity, bacterial proliferation, and iron status parameters. However, IHAT will produce less gut/systemic inflammation, and fewer effects on the microbiome.

2. Does Asprion, a novel form of iron, produce beneficial and/or adverse effects comparable to $FeSO_4$ at equivalent iron doses?

Aspiron and $FeSO_4$ administered at 60 mg Fe/day will produce comparable responses in malaria infectivity, bacterial proliferation, and iron status parameters. However, Aspiron will produce less gut/systemic inflammation, and fewer effects on the microbiome.

3. Are the adverse effects of $FeSO_4$ affected by co-administration of a multi-micronutrient powder (MNP)?

The safety profile of $FeSO_4$ for iron-replete individuals is not affected by its administration with a multiple micronutrient powder.

4. Are the beneficial and/or adverse effects of $FeSO_4$ comparable when administered daily or weekly?

The timing of $FeSO_4$ administration will not affect malaria infectivity, bacterial proliferation, and iron status parameters; however, weekly administration will produce less gut/ systemic inflammation and fewer effects on the microbiome.

In Phase II, we will test the following formal hypotheses using the design detailed below:

1. Does IHAT, a novel form of iron, effect positive improvements in iron status comparable to $FeSO_4$ at equivalent iron doses, while producing fewer adverse effects?

IHAT and $FeSO_4$ administered at 120 mg Fe/day will produce similar increases impacting iron status in Fe-replete subjects. However, IHAT will produce relatively lower increases in *ex vivo* malaria infectivity, *ex vivo* bacterial proliferation, redox stress, gut/systemic inflammation, and fewer effects on the hindgut microbiome.

2. Does Aspiron, a novel form of iron, effect improvements in iron status comparable to $FeSO_4$ at equivalent iron doses while producing fewer adverse effects?

Aspiron and $FeSO_4$ administered at 120 mg Fe/day will produce similar increases on iron status in Fe-replete subjects. However, Aspiron will produce relatively lower increases in the *ex vivo* malaria infectivity, *ex vivo* bacterial proliferation, redox stress, gut/systemic inflammation, and fewer effects on the hindgut microbiome.

## Study design

The Safe Iron Study is a double-blind, randomized, placebo-controlled study with iron supplementation in iron-replete older adults. For Phase I, adults are randomized to one of six intervention groups (Table 1). For Phase II, adults are randomized to one of three intervention groups (Table 2). For both phases, each arm undergoes an intervention period of 4 weeks, after which changes in parameters will be compared between baseline (week 0) and post-intervention (week 4) study visits.

**Table 1. Intervention groups for Phase I of the Safe Iron Study.**

| Subjects | Intervention Group | N | Intervention | Sampling |
|---|---|---|---|---|
| Fe-replete, PMW and men | Intervention 1 | 25 | Placebo | 0 and 4 weeks of intervention |
| | Intervention 2 | 25 | $FeSO_4$ (60 mg Fe/day) | 0 and 4 weeks of intervention |
| | Intervention 3 | 25 | $FeSO_4$ (420 mg Fe/week) | 0 and 4 weeks of intervention |
| | Intervention 4 | 25 | $FeSO_4$ (60 mg Fe/day) + MNP | 0 and 4 weeks of intervention |
| | Intervention 5 | 25 | IHAT (60 mg Fe/day) | 0 and 4 weeks of intervention |
| | Intervention 6 | 25 | Aspiron (60 mg Fe/day) | 0 and 4 weeks of intervention |

PMW, post-menopausal; $FeSO_4$, ferrous sulfate; MNP, multiple micronutrient powder; IHAT, iron hydroxide adipate tartrate

**Table 2. Intervention groups for Phase II of the Safe Iron Study.**

| Subjects | Intervention Group | N | Intervention | Sampling |
|---|---|---|---|---|
| Fe-replete, PMW and men | Intervention 1 | 25 | $FeSO_4$ (120 mg Fe/d) | 0 and 4 weeks of intervention |
| | Intervention 2 | 25 | IHAT (120 mg Fe/d) | 0 and 4 weeks of intervention |
| | Intervention 3 | 25 | Aspiron (120 mg Fe/d) | 0 and 4 weeks of intervention |

PMW, post-menopausal; $FeSO_4$, ferrous sulfate; IHAT, iron hydroxide adipate tartrate

## Methods: Subjects, interventions and outcomes

### Study setting
The majority of the research will be conducted at the Jean Mayer United States Department of Agriculture (USDA) Human Nutrition Research Center on Aging (HNRCA), Boston, MA, USA. The HNRCA has in-house recruiting and human subject management teams, medical oversight, and a wide spectrum of statistical, biochemical and molecular biological analytical expertise. The *ex vivo P. falciparum* infectivity and bacterial proliferation studies will be conducted at the Tufts University Graduate School of Biomedical Sciences, Boston, MA, USA.

### Eligibility criteria
Subjects are healthy older adults from Boston and surrounding area and must meet all of the inclusion criteria and none of the exclusion criteria to be considered eligible for the study.

*Inclusion criteria.* Apparently healthy men and post-menopausal women (defined as no menses for ≥1year); between 50–80 years of age; BMI range between 18–35 kg/m²; typical bowel pattern of at least one stool every other day; willing to comply with study procedures; and will not be undergoing colonoscopy in the 2 months before, or during the course of the study.

*Exclusion criteria.* Any major illness or condition that may interfere with study outcomes at the discretion of the study physician; personal history of glucose-6-phosphate dehydrogenase deficiency; type 1 or type 2 diabetes, or use of any pharmacological treatment for diabetes; endocrine disorders including unstable thyroid disease (dose adjustment of thyroid replacement in the past 6 months), adrenal disease, pheochromocytoma or parathyroid disease; recent history of inflammatory diseases; use of TNF blockade medication, methotrexate, or other immune-modulating drugs; steroid use (except for non-prescription topical and nasal steroids); change in dose regiment in past month or expected change during course of study if using hormone replacement therapy with estrogen, testosterone or growth hormone; history of myocardial infarction, stroke or total ischemic attack, coronary artery bypass graft, stenosis >50% diagnosed within the past year or acute unstable cardiovascular disease; clotting/bleeding disorders or ongoing anticoagulant use; gastrointestinal (GI) diseases, conditions or medications known to influence GI absorption; history of stomach or bowel resection (other than appendectomy), gastric bypass or other bariatric weight loss procedure; regular use (>2 times per week) of acid lowering medication; history of eating disorder anorexia, bulimia or binge-eating in the past 5 years; actively undergoing dialysis; inadequately controlled hypertension, certain psychiatric disorders; immunodeficiency condition, HIV or AIDS; cancer of any type (except for non-melanoma skin) or cancer therapy in past year; actively using cancer chemotherapeutic agents; regular use of ASA, NSAIDs or Cox-2 inhibitors; infection or febrile illness within two weeks prior to study or study blood draws; history, vaccination or treatment for malaria, or antimalarial prophylaxis in past 3 months; vaccinations (excluding malaria vaccine) <72 hours prior to all blood draws; unmanaged seizure disorders; history of splenectomy; chronic liver disease; use of fiber supplements, laxatives or stool softeners; colonoscopy procedure or prep within 2 months prior to or during study; predicted or anticipated antibiotic use within 3 months prior to or during study participation; unwilling to maintain dose regimen during study if using probiotic or prebiotic; inability to deliver stool sample within 18 hours of bowel movement; alcohol use on average >2 servings/day or >14 servings/week or self-reported binge drinking; current use of iron; unwilling to stop all dietary supplement, vitamins (except calcium or vitamin D), minerals, herbal other plant-based preparations, fish oil supplements or homeopathic remedies use at least one month prior to study; inadequate venous access or history of a bilateral mastectomy with nodal dissection; participation in other research study during the same time period; no social security number; iron saturation outside of normal range; hemoglobin <11.7 (females) <13.2 (males); serum creatinine >1.5 mg/dl; fasting blood sugar ≥126 mg/dL; SGOT >1.5 x upper range of normal; SGPT >1.5X upper range of normal mg/dL in absence of benign cause.

### Interventions
This study will be using two novel forms of iron (IHAT and Aspiron) compared to $FeSO_4$. One arm will also include an MNP (MixMe™ Vitamin and Mineral Powder) donated by DSM Nutritional Products (Switzerland). The placebo will be comprised of Melojel® corn starch, donated by Ingredion (Manitoba, Canada). All forms of iron, MNP and corn starch have been approved for use in humans. This trial is only intended to evaluate the different supplement forms of iron on the structure or function of the body, not for a therapeutic purpose. All raw materials will be sent to the Richardson Centre for Functional Foods and Nutraceutical (RCCFN) at the University of Manitoba, Winnipeg, Manitoba, Canada for encapsulation. All interventions will be encapsulated into opaque, double zero capsules and appear identical in size, color and weight. The RCCFN is licensed as a Health Canada Natural

Health Product Site with the ability to produce good manufacturing practices (GMP) and Hazard Analysis and Critical Control Points (HACCP) certified products. For the administration of capsules, each subject will receive a number-coded calendar-style monthly pill planner with a set number of capsules.

*Adherence.* Adherence to supplement use will be assessed by using pill counts, a compliance calendar and iron status. Pill counts will be based on the number of pills remaining in the returned monthly pill planner. Subjects will be sent home with a compliance calendar to record their supplement intake and will be instructed to bring this document with them on the post-intervention study visit. Subjects will be instructed to return the number-coded calendar-style monthly pill planner with any unused sections or pills at the end of the 4-week study visit. The number of unused pills will be recorded at the 4-week study visit. Parameters of iron status will be measured in serum and will serve as additional measures of adherence to supplement protocol.

## Outcomes
The Safe Iron Study has three primary outcomes to test the formal hypotheses in Phases I and II. These outcomes include malaria (*Plasmodium falciparum*) infectivity of host erythrocytes (assessed *in vitro*), bacterial proliferation (of selected species *Staphylococcus aureus*, *Escherichia coli*, *Acinetobacter baumannii*, *Klebsiella pneumoniae* and *Salmonella typhimurium*) in presence of host plasma (assessed *ex vivo*) and intestinal inflammation assessed by fecal calprotectin concentrations. Secondary outcomes include iron status parameters (hemoglobin, transferrin, ferritin, serum iron, total iron-binding capacity (TIBC), percent saturation, NTBI, and fecal iron), assessments of systemic inflammation (CBC/diff, *ex vivo* cytokine production, *in vivo* inflammatory cytokines), additional assessments of intestinal inflammation (fecal neopterin, myeloperoxidase, alpha-1 antitrypsin, cytokines, fecal immunochemical test (FIT) and plasma tryptophan and kynurenine), alterations in gut microbiome (microbiota analysis and short chain fatty acids) and redox status (F2α-isoprostanes and glutathione). A detailed outcome measurement plan is included in Table 3.

## Recruitment and screening
Subjects are recruited from the general public in the greater Boston area using several recruitment strategies including local print and electronic media (such as advertisements in newspapers, craigslist, local newsletters, bulletin boards, different websites and radio) as well as posting flyers in public places such as local YMCAs, supermarkets, libraries, laundromats, local community organizations and health centers. Additionally, a roster of >20,000 names of previous study subjects from which potentially qualifying women and men within the specified age and BMI can be identified and contacted through the use of direct mailings.

Prospective subjects will provide oral consent form prior to completing a telephone pre-screening questionnaire, and if they appear to be qualified, they will be invited to the HNRCA

for screening. This will allow for the recruitment of only those who are confident they are able to complete all study activities. The full screening process will be conducted in a quiet location by an experienced research study nurse at the Metabolic Research Unit (MRU) at the HNRCA, and those interested will be asked to sign the screening consent form (Supplementary File 1, available as *Extended data*[21]) prior to the start of all screening procedures. Subjects will then undergo blood tests and a review of their medical history and general health including gastrointestinal health history, gut irritation questionnaire, use of medications and nutritional supplements including iron. Once an individual has gone through the screening process and is deemed eligible to participate, they will be informed by telephone by a staff at the MRU that they are eligible and if they still agree to participate, they will be scheduled to come to the HNRCA for the enrollment and baseline study visits.

## Study timeline and visits
Illustrated in Figure 1 (Phase I) and Figure 2 (Phase II), eligible subjects will be identified using pre-screening and screening procedures, as described above. Once an eligible subject is identified and wishes to participate, they will complete an enrollment visit for which informed consent (Supplementary File 2, available as *Extended data*[21]) is obtained and instructions for stool sample collection are reviewed. The eligible subject is then randomized to a treatment group. The subject returns for a baseline, pre-intervention visit (week 0) for which a stool sample, fasted blood sample and questionnaire assessing signs and symptoms of gastrointestinal health (Gut Irritation Questionnaire) are collected, followed by standard continental breakfast (English muffin or bagel, spreads, coffee, juice and fruit). Oranges (juice, marmalade, or fresh fruit), and teas will not be offered, as those foods decrease the absorption of iron in the body. Immediately following breakfast, subjects will be administered the assigned intervention agent. After 2 hours, a post-prandial, post-dose, blood sample is collected. Instructions for intervention agents are reviewed with the subjects and they are dispended study supplements sufficient for 4 weeks. During the 4-week intervention period, subjects complete weekly Gut Irritation Questionnaires in addition to three 24-hour dietary recalls via telephone. After the 4-week intervention, subjects return for a post-intervention visit which follows the same procedures as the baseline visit.

## Methods: Assignment of interventions
### Randomization and blinding
The allocation sequence constructed using a block randomization with a block size of four. Random ordering of the allocations within each block was performed using for the study was performed using SFMT (SIMD-oriented Fast Mersenne Twister) algorithm (version 1.5.1) based randomization functions in the "StatsBase" Julia package. No stratification was used in the randomization, and the allocation sequence was implemented using the built-in "randomization" module in REDCap. This module references the provided allocation table and assigns participants based on order of enrollment.

For Phase I, subjects will be randomly assigned to one of the six treatment arms outlined in Figure 1 at the time they enroll

**Table 3. Timing and analytical details of questionnaires, biological and chemical analyses conducted at each study visit.**

| Screen Fast | Baseline (0 wks) Fast | Baseline (0 wks) 2 hrs | Post-Intervention (4 wks) Fast | Post-Intervention (4 wks) 2 hrs | Intra Study (0–4 wks) | | Whole Blood | Serum | Plasma | Stool |
|:---:|:---:|:---:|:---:|:---:|:---:|:---|:---:|:---:|:---:|:---:|
| + | + | | + | | | General Health/ Medical History | | | | |
| + | | | | | | Diet History | | | | |
| | | | | | + | 24-h Dietary Recalls | | | | |
| + | + | | + | | + | Gut Irritation Questionnaire | | | | |
| + | | | | | | Standard Health Screening Questionnaire | | | | |
| | + | | + | | | Malarial Infectivity | *In vitro* | | | |
| | + | + | + | + | | Bacterial Proliferation | | Assessed *in vitro* | | |
| + | | | | | | Health Status | | Chem Profile | | |
| + | + | | + | | | Systemic Inflammation | CBC/diff | | | |
| | + | | + | | | Systemic Inflammation | *Ex vivo* cytokine production | | | |
| | + | + | + | + | | Systemic Inflammation | | | *In vivo* inflammatory cytokines | |
| | + | | + | | | GI Inflammation | | | | Fecal immune Chem Test (FIT) |
| | + | | + | | | GI Inflammation | | | Tryptophan, Kyneurenine | Calprotectin, Neopterin, Myeloperoxidase, Cytokines, $\alpha$1-antitrypsin |
| | + | | + | | | Gut Microbiome | | | | Fecal SCFAs |
| | + | | + | | | Gut Microbiome | | | | Microbiota DNA Analysis |
| | + | + | + | + | | Redox Stress | | | F2$\alpha$-isoprostanes, GSH/GSSG | |
| + | + | + | + | + | | Fe Status | Hb | Fe, Transferrin, Transferrin saturation | | |
| | + | + | + | + | | Fe Status | | Ferritin, Transferrin receptor, Hepcidin | | |
| | + | + | + | + | | Fe Status | | Non-Transferrin-bound-Fe (NTBI) | | |
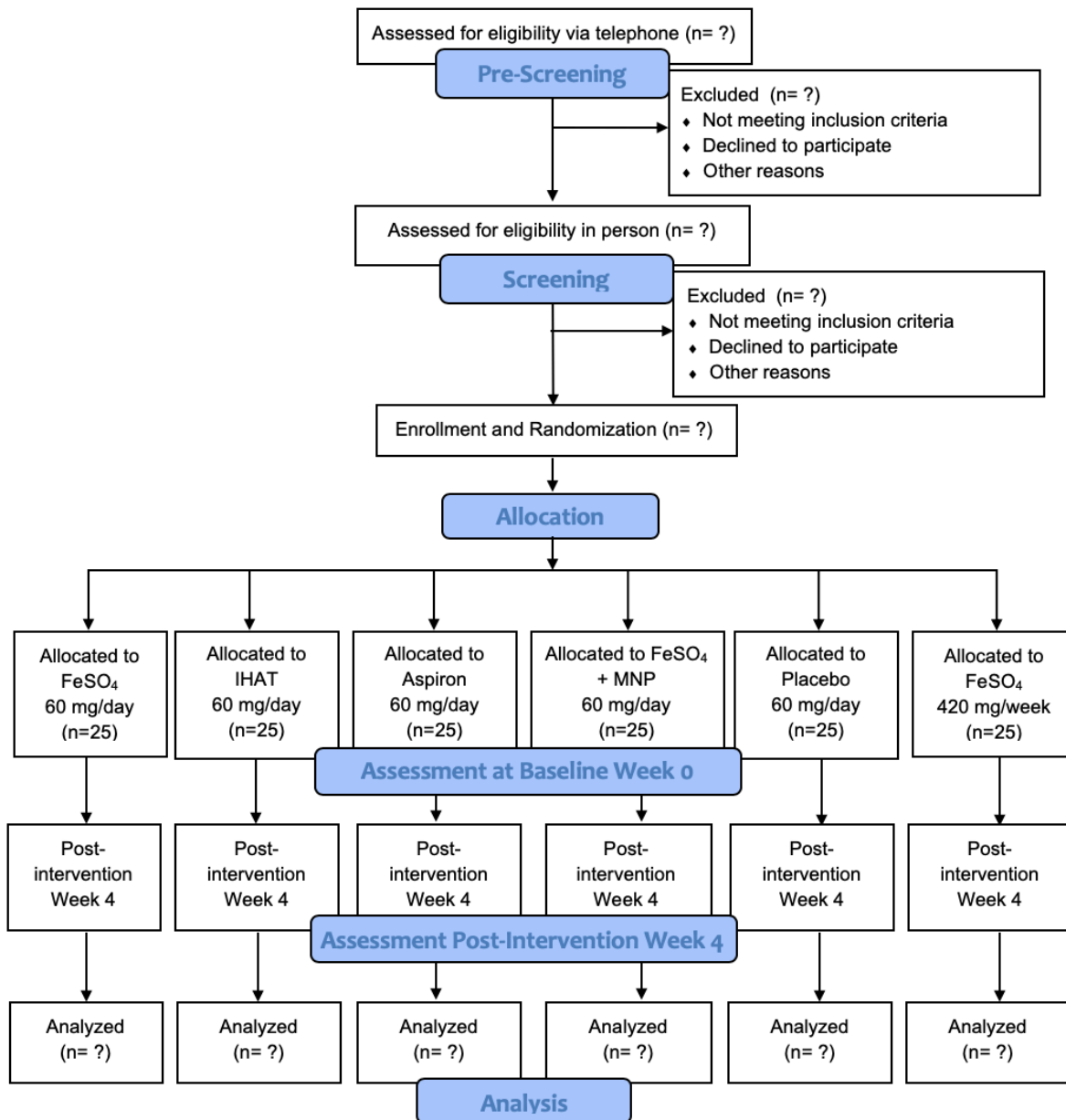
**Figure 1. Phase I CONSORT flow chart.**

in the study. A randomization scheme for all entering subjects will be generated and stored by the Data Manager. The randomization sequence is known only to the Data Manager, and REDCap user roles were used to prohibit access to the allocation sequence until the participant was randomized to a treatment group. The unblinded key is maintained by the data manager and stored in a locked filing cabinet until the data collection for the study is complete. An active control group, using a corn starch placebo, will be used to provide a control for prospective changes over time and mimic any effects of ingesting a daily capsule in case it may be relevant. We will

also provide placebo capsule to all other groups to blind against the $FeSO_4$ plus MNP group. All daily interventions groups will receive three physically similar capsules to ensure blinding of the treatments. The weekly intervention group will receive four physically similar capsules once per week.

For Phase II, subjects will be randomly assigned to one of the three treatment arms outlined in Figure 2 at the time they enroll in the study. The randomization scheme will be the same as Phase I. We will not employ a control group in Phase II because any prospective changes over time or effects of ingesting a
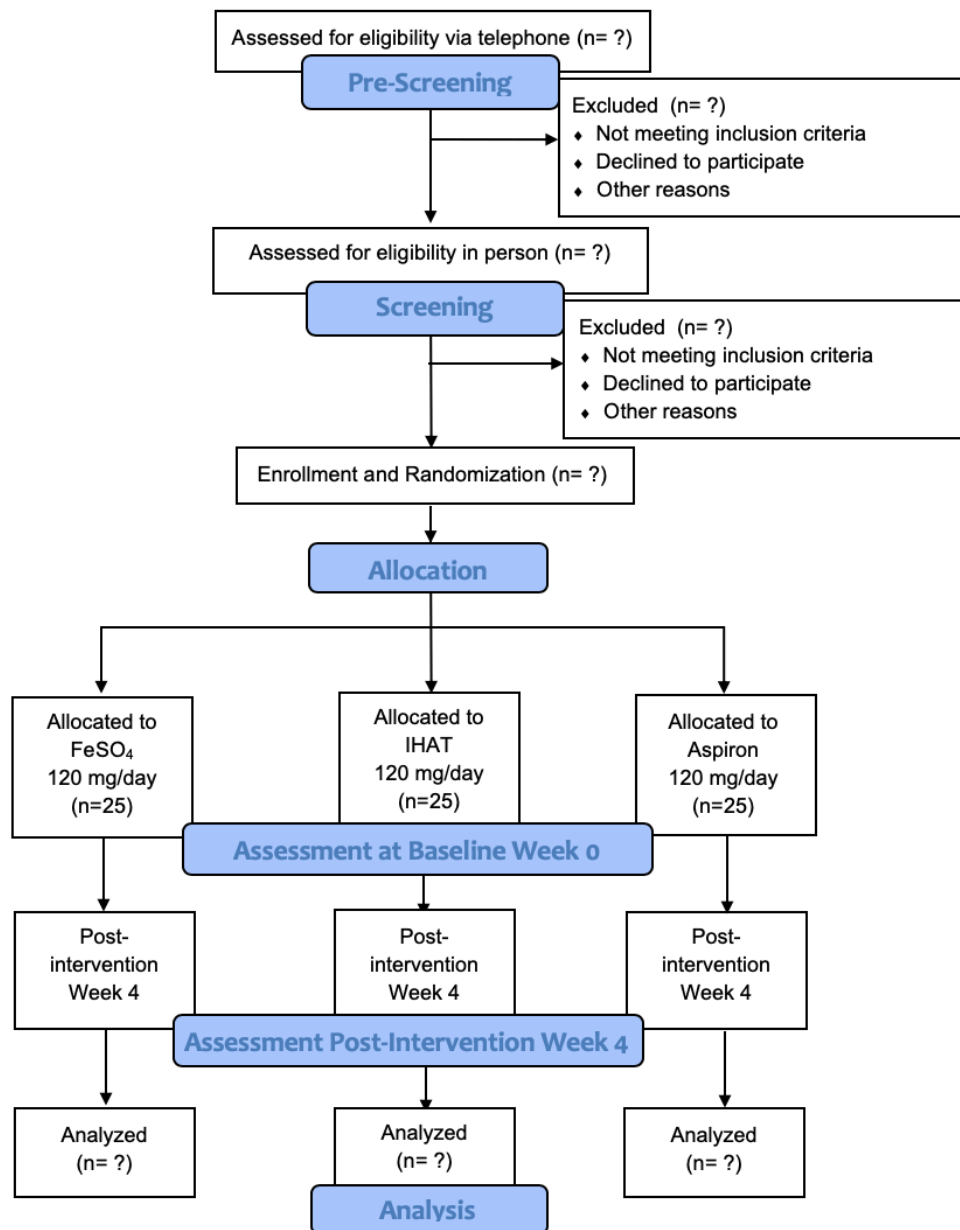
**Figure 2. Phase II CONSORT flow chart.**

daily capsule will be determined from Phase I. All groups will receive four physically similar capsules to ensure blinding of the treatments.

## Withdrawal of subjects

Subject participation may be terminated should they no longer meet any of the study criteria cited above, or if they fail to comply with study requirements or if a reported adverse reaction occurs that is considered severe. Subjects are free to withdraw from participation at any time during the study by writing, calling or emailing the study PI. The decision to terminate a participant from the study will be based on the judgment of the study physician and the study PI. If a participant does not complete the entire study, their stipend will be prorated accordingly depending on where the participant is in the study prior to withdrawal. If a subject withdraws or is withdrawn from the study, any data or specimens collected before withdrawal will still be used in the study.

## Methods: Data collection, management and analysis
### Data collection methods
*Laboratory analyses.* At baseline (week 0) and post-intervention (week 4), subjects will provide blood and stool samples as

well as complete several questionnaires. Blood pressure and pulse rates as well as anthropometric measurements will also be taken. Table 3 outlines the questionnaires, biological and chemical analyses conducted at each study visit. Details of the analyses are described below.

***Blood samples.*** Blood samples will be collected at baseline and post-intervention. Depending on the analyses, 12-hour fasted and 2-hour postprandial, post-supplementation blood samples will be collected (Table 3) and the following parameters will be assessed: CBC/differential; *P. falciparum* infectivity of erythrocytes will be determined *ex vivo*[22]; bacterial proliferation potential will be conducted *ex vivo* on plasma[23]; plasma cytokines and pro-inflammatory markers (Meso Scale Discovery, MSD); *ex vivo* mitogen or antigen-induced cytokine production) commercial ELISAs, R&D Systems, Minneapolis, MN[24,25]); iron and unsaturated iron-binding capacity (AU400 clinical chemistry analyzer, Beckman Coulter, Inc., Brea, CA); serum ferritin (commercial human ferritin ELISA, Thermo Scientific, Frederick, MD); transferrin (immunoturbidimetric procedure using the AU400 clinical chemistry analyzer, Beckman Coulter); transferrin saturation (determined from the relationship of the iron to total iron-binding capacity); transferrin receptor (commercial ELISA, R&D Systems); serum NTBI (serum treated with *tris*-carbonatocobalate(III) trihydrate to saturate non-liganded transferrin binding sites followed by precipitation of proteins and ultrafiltration. Iron in the ultrafiltrate will be determined by electrothermal atomic absorption spectrophotometry or mass spectrometry (MS); hepcidin (commercial competitive ELISA, Bachem Group, Torrance, CA[26]); tryptophan and kynurenine (liquid chromatography (LC)/MS/MS[27]; F2α-isoprostanes (in-line solid phase extraction prior to quantitative analysis by ultra-performance LC/MS/MS[28]); reduced glutathione/oxidized glutathione (Hypercarb HPLC column and quantitative detected by LC/MS/MS[29]).

***Stool samples.*** Subjects will be asked to provide a sample at baseline and post-intervention visits Subjects will receive stool sample collection kit at the enrollment visit to bring the samples to study visits. The stool sample in the collection container will be placed in a plastic bag, surrounded by frozen gel packs to keep it cool for up to 18 hours. Aliquots of homogenized stool will be created and the following parameters will be assessed: fecal immunochemical tests (FIT) (OC-Auto Micro 80 Analyzer); calprotectin (commercial ELISA, R&D Systems,[30]); myeloperoxidase (commercial ELISA, R&D Systems); neopterin (competitive ELISA, ALPCO, Salem, NH); inflammatory cytokines (Meso Scale Discovery, MSD); alpha-1 antitrypsin (commercial ELISA, R&D Systems); short chain fatty acids (derivatized and analyzed by LC-MS[31]); microbiome DNA sequencing (stool DNA extracted using the QIAamp DNA Stool Mini kit, Qiagen, Valencia, CA, and amplicons of the V4 region of the bacterial 16S ribosomal DNA will be generated using primers[32]. Amplicons will be purified on Angencourt AMpure XP beads (Beckman Coulter, Brea, CA) before submission to the Tufts University Core Facility for 250 bp paired-end sequencing on an Illumina MiSeq. Marker-gene sequencing data

will be processed using the Quantitative Insights Into Microbial Ecology (QIIME) package[33].

## Questionnaires
***Gut irritation questionnaire.*** This questionnaire[34] is designed to be self-administered but subjects will be given the opportunity to ask questions about how to fill out the questionnaire. This questionnaire is slightly modified from a validated questionnaire designed to address symptoms of gastrointestinal (GI) irritation after FeSO$_4$ supplementation[34]. It will be completed at screening, baseline (week 0) and post-intervention study visits. Subjects will be given a copy of the questionnaire to take home. Subjects will be administered this questionnaire during weekly phone calls in which they will use to copy of the questionnaire to follow along with questions as they are asked over the telephone.

***Dietary assessment.*** Study subjects will receive a Food Amount Booklet and information about the 24-hour recalls at the baseline visit (Day 0). Three separate 24-hour recalls will be collected by phone during the 4-week study. Dietary intake data will be analyzed using Nutrition Data System for Research (NDSR) software version 2016, developed by the Nutrition Coordinating Center (NCC), University of Minnesota, Minneapolis, MN. Recalls will be collected using the multiple-pass 24-h recall method, assisted by the NDSR software and the goal for each participant will be to collect two weekdays and one weekend day of intake. Outcome data from NDSR will include daily estimated energy and nutrient intake, as well as food, food group, and dietary supplement data.

## Data capture and management
The research data for this clinical trial will mainly consist of questionnaires and clinical measures in whole blood, serum, plasma and stool related to the proposed interventions. These data will be captured and managed using a REDCap database designed specifically for this study using the HNRCA's deployment of REDCap which are both HIPAA and 21CFR11 compliant. Any data imported into the REDCap database will undergo external validation checks to ensure that the data have been imported correctly. Additionally, metadata files and data dictionaries/codebooks will be used to provide all information necessary to properly use and understand the data files. The HNRCA data management program records and stores subject identifiers separately from clinical information and results of study analyses. A study code number will identify each laboratory specimen and the master list which links the identity of the subject with the laboratory specimens will be kept in a locked file cabinet in the office of the PI and on a password-protected database on a secure Tufts server. The study team will design a tracking and data management system that will include features to improve data quality such as double data entry and edit resolution to ensure that the study variables accurately represent data on the study data collection forms. There will be a tracking and data-management system, trouble shooting for any data entry problems, an audit trail of database editing and range checks for cleaning, daily backup, cleaning of transitional

databases, and transfer of master databases to SAS or SPSS for analysis by the study statistician.

## Data access

Access to the study database will be restricted to only the forms relevant for each investigator until completion of the study and the unblinding of the investigators. Additionally, protected health information will automatically be de-identified for all viewing and data exports. The data analyst will be responsible for managing the user access groups and masking all non-relevant information from the investigators.

## Audit trail and backups

A complete audit history for all data and data collection forms will be maintained using the REDCap audit feature, which records every action performed by each user while in REDCap. The study database will be subject to daily backups to tape array and daily, weekly and monthly backup copies will be maintained by Scientific Computing at the HNRCA. After key points in data acquisition, clean up and analysis, additional an additional off-site copy of the data will be created and stored within an electronic laboratory notebook using the LabArchives electronic laboratory notebook software.

## Statistical methods

*Summary statistics on subject characteristics.* We will summarize subject characteristics across the intervention groups with mean and standard deviation for continuous measures and frequency and proportions for categorical variable using demographic information collected during screening, health status information, food habits data, and compliance to treatment, with special attention to covariates that may influence iron utilization, namely total energy, total protein, and ascorbic acid intakes.

*Planned comparisons.* Statistical testing is structured to align with the study hypotheses and objectives in a series of planned pairwise comparisons. Comparisons will be made between each of the novel forms of iron and $FeSO_4$, administration with multiple micronutrient powder and $FeSO_4$, and weekly administration and $FeSO_4$. The familywise error rate is set at alpha of 0.05 and is controlled separately for indicators of the safety profile within each research objective.

*Statistical procedures for hypothesis tests.* Treatment groups will be analyzed following an intention-to-treat approach. Subjects will not be included in their assigned randomization group for analysis if determined to be nonadherent to the intervention based on compliance and iron status as described under *Interventions*. Responses in malaria infectivity, bacterial proliferation potential, and fecal calprotectin to iron supplementation, quantified as the changes in the respective outcome for each subject over 4 weeks, will be used for comparisons of $FeSO_4$ to the other treatment groups. Pairwise comparisons will be made with two-sample t-tests, or linear models allowing adjustment for covariates. Data will be evaluated graphically to assess modeling assumptions including outlier detection, normality, and linearity with covariates. Missing cases in our study will be evaluated separately to assess bias in our sample due to drop outs. Adherence to the intervention will be summarized across all

treatment groups based on returned supplement capsules and iron status. Any changes in the primary treatment comparisons after exclusion of nonadherent participants will be reported from secondary per-protocol analysis. All statistical analysis will be performed using SAS v. 9.4 (SAS Institute Inc., Cary, NC) unless otherwise specified.

Specific analysis techniques will be applied to each primary and secondary outcome as follows:

1) <u>Malaria infectivity:</u> Data will be evaluated using the 3D7 sialic acid independent strains of *P. falciparum.* Mean infectivity of erythrocytes will be calculated from experiments performed in duplicate. Four-week change will be quantified for each subject using the logarithm of the ratio of percent parasitized erythrocytes at baseline and post-intervention. Planned comparisons will be tested using two-sample t-tests. Random effects regression will be used to incorporate day-to-day variation and test for batch effects.

2) <u>Bacterial proliferation potential:</u> Bacterial proliferation potential will be evaluated using polynomial growth curve models[23]; 18-hour growth curves will be fit from mean optical density over three replicates for each subject's baseline and post-intervention sample. Positive and negative control values on each plate will be used to standardize values to account for day-to-day variation and utilization of different plate readers. Statistical testing will be based on planned comparisons for doubling time during the exponential phase of growth and the maximum level of growth as measured by optical density, calculated from the fitted growth curves. Two-sample t-tests will be used for pairwise group comparisons. We will adjust for multiple tests across the five selected species, using 0.05/5=0.01 type I significance level.

3) <u>Fecal calprotectin:</u> Four-week change in fecal calprotectin will be performed for planned comparisons using two-sample t-tests with log transformation to satisfy normality assumptions.

4) <u>Iron status parameters, redox Stress, gut and systemic inflammation, gut irritation:</u> Changes in continuous-valued clinical and biochemical outcomes will be evaluated using two-sample t-tests. Variable transformations will be used for skewed outcomes, such as number of GI symptoms.

5) <u>Gut microbiome:</u> Diversity measures will be used to compare overall microbiome changes between treatment groups and testing using PERMANOVA. Functional and taxonomic profiling will be used to describe differences between treatment groups. Two-sample tests will be used to test differences in OTU relative abundances and functional predictions, with false discovery rate adjustments for testing across multiple taxa and functions. We will use clustering methods for microbiota data from gut microbiome samples to characterize the association of iron agent and presence of OTU types.

***Bias, confounding, effect modulators.*** Due to the completely randomized design, there will be protection of bias in treatment effects from known and unknown confounding variables. We will also compare subject characteristics across treatment groups and adjust for confounding effects for any variables not balanced through randomization. We are unaware of effect modulators of iron supplementation on *P. falciparum* infectivity and bacterial proliferation. However, outcomes will be evaluated separately at different covariate levels to look for possible modulating effects to be included as interaction terms in our models. Potential effect modulators for fecal calprotectin include age and sex[35], and we plan to include them in the models for this outcome.

***Power calculations and sample size.*** The sample size of Phase I and Phase II was determined based on power calculations for each primary outcome as follows:

1) <u>Malaria infectivity:</u> The sample size is based on standard deviation estimates from the *in vitro* assay of malaria infectivity by Clark *et al.* (2014)[36]. We pooled the estimated variability of percent increase of *P. falciparum* growth analyzed in Clark *et al.* (2014). Using an independent two-sample t-test, an estimated standard deviation 0.202, with a type I error of 0.05, we have at least 90% power to detect a 20% reduction in *P. falciparum* growth using a minimum of 25 subjects per group.

2) <u>Bacterial proliferation potential:</u> Bacterial proliferation potential due to oral iron supplementation was previously studied from a similar *ex vivo* assay[23]. Between subject estimates of standard deviation were extracted for doubling time during the exponential phase of growth available from three species, *S. aureus*, *E. coli*, and *S. Typhimurium* as 0.2470, (0.530 and 0.212), and (0.813 and 0.071), respectively, with unequal variance estimates used for *E. coli* and *S. Typhimurium* from pre- and post-iron supplementation. Based on two-sample t-tests with type I error at 0.01 (adjusted for planned analysis of five species), and assuming 25 subjects per group, with a minimal detectable difference of 1 hour doubling time, the power is >0.99 for all three species, *S. aureus*, *E. coli*, and *S. Typhimurium*.

3) <u>Fecal calprotectin:</u> Estimates of between subject variation are from subjects with various forms of mild gastrointestinal disease[30]. Mean fecal calprotectin was reported at 27.6 μg/g in subjects with collagenous colitis, representing mild inflammation, and at 1.9 μg/g in subjects with irritable bowel syndrome, reflecting minimal inflammation. Standard deviation in log concentration of fecal calprotectin used in the sample size calculation is 1.3 μg/g. For our study, in order to detect a 3.5 fold change in fecal calprotectin with 90% power and alpha level of 0.05, we require a minimum of 25 subjects per group. Sample size is based on two-sample t-tests with log-transformed values.

With rolling recruiting and screening, we expect enroll 10–12 subjects/month, randomly assigning them to the intervention treatment groups in blinded fashion. The relatively short length and personalized nature of the intervention will reduce the likelihood of drop outs and allow us to recruit continuously until the recruitment goals are met.

## Methods: Monitoring
### Data and safety monitoring
Ongoing health oversight for subjects in the study will be overseen by study physician. Specific participant complaints will be addressed on an individual basis and appropriate referrals to primary care providers or other health care providers will be made. If problems are reported the subjects will be assessed to determine if the adverse event is potentially study-related and then counseled to seek appropriate assistance. To monitor tolerance of the iron supplements, GI irritation will be assessed by subject interview using a validated GI symptom questionnaire[34].

This study presents a minimal risk for subjects; therefore, safety monitoring may be conducted continually by the PI. Despite not requiring an external safety board, this study has an external advisory board who will be involved in the study. The advisory board will review the de-identified data provided to them on a quarterly basis to evaluate the data and monitor subject safety, assessing both harms and benefits. Any reports generated from this advisory board will be submitted to the Tufts Medical Center and Tufts University Health Sciences Campus Institutional Review Board (IRB).

### Trial monitoring
Trial oversight is provided by the study sponsor through the Tufts Medical Center and Tufts University Health Sciences Campus IRB.

## Ethics and dissemination
### Ethics statement
This study has been approved by the IRB at Tufts Medical Center and Tufts University Health Sciences Campus (Phase I IRB #: 12455 and Phase II IRB#: 13341). This study is being conducted in accordance with ICH GCP requirements. Protocol modifications are communicated to the Institutional Review Board at Tufts Medical Center and Tufts University Health Sciences Campus (currently Phase I: Amendment 16). This paper is written following the SPIRIT 2013 guidelines[37]; a completed SPIRIT checklist is available on Open Science Framework[21]. This study is registered on ClinicalTrials.gov with the identifier: NCT03212677.

### Dissemination
Key study findings will be presented at relevant international conferences including American Society for Nutrition annual meeting and Micronutrient Forum. The results of the study will be disseminated by submitting manuscript for publication in relevant, peer-reviewed journals. All publications resulting from this research will be published under CC BY 4.0 and made accessible and open immediately in accordance with the Bill and Melinda Gates Foundation open access policy. The data underlying each publication will be combined with the appropriate metadata necessary for reuse and then deposited

in the Harvard Dataverse under the Bill & Melinda Gates Foundation Dataverse to ensure that the data is immediately accessible to the greater research community and located in a well-known and easy to locate data repository. Request for use of study data must be approved by the study sponsor and IRB.

## Confidentiality

Participant identifiers will be kept in a separate file from data. Data collection forms will not have participant identifiers. The participant's identity and records relating to the telephone prescreening and screening information will be kept confidential in locked cabinets at all times at the Volunteer Recruitment Services, and at the MRU, respectively, whether the participant qualifies for the study. All study records for subjects will be kept confidential and the study co-PI, Co-Investigators, Study Physician, Study Coordinators, and Study Statistician will have access to participant identifying information, if needed; otherwise only de-identified data will be transferred to co-investigators. All data will be kept in secure files and locked data cabinets. Identifiers will not be destroyed at any point.

## Study status

For Phase I, screening for enrollment began in June 2017. As of May 2019, 941 subjects have been pre-screened, and 335 subjects have been screened. The first subject baseline (week 0) visit occurred on August 1, 2017 and as of May 2019, 159 subjects have been enrolled in the study. It is anticipated that data collection will be completed by June 2019, with primary outcome analyses and submission for publication completed by August 2019. Current protocol version is April 1, 2019. It is anticipated that for Phase II, screening for enrollment will begin June 2019 and data collection will be completed in March 2020. Current protocol version is April 26, 2019.

## Discussion
### Rationale for enrolling healthy, iron-replete adults as the population of study

There is strong evidence to support the efficacy of iron supplementation in reducing the risk of iron-deficiency and/or anemia in pre-menopausal women. A recent Cochrane review of ten iron supplementation trials in non-pregnant, pre-menopausal women (12–50 years of age) demonstrated that daily oral iron supplementation reduced the risk of anemia (relative risk: 0.39, 95% CI: 0.25-0.60)[38]. Despite the efficacy of iron supplementation in reduction of deficiency risk, there are growing concerns of iron supplementation in iron-replete populations. The aforementioned systemic review did not assess potential risks of iron supplementation, including iron overload, oxidative stress, infection and inflammation, as the trials included only physical signs and symptoms associated with supplementation such as GI discomfort[38]. Based on the available literature, it has been hypothesized that iron-replete individuals may have increased risk of potential adverse effects associated with iron supplementation, particularly in malaria-endemic regions with high levels of inflammation. Most notably, the Pemba sub study, which included 2,413 children undergoing routine iron supplementation, observed an increase in clinical malaria and mortality in iron-replete children[7]. Although this finding was not

statistically significant, it led to the WHO and UNICEF recommending no iron supplementation for young children in malaria-endemic areas and effectively halted programs to address iron deficiency in these areas. The overall goal of the present study is to examine the safety and efficacy of novel forms of iron. Therefore, the rationale for enrolling iron-replete individuals in this study was based on the fact that if adverse effects from iron supplementation were to occur, it would be in this population, rather than an iron-deficient/anemic population.

Regarding the age of our study population, post-menopausal women and age-comparable men in the greater Boston area are being enrolled. It is well recognized that the target population for safe and effective iron supplementation programs is iron-deficient, anemic pre-menopausal women, infants and young children in resource-poor regions. Therefore, the rationale for establishing safety and efficacy in a healthy, older adult population instead of young children was based primarily on ethical considerations. The health of each subject enrolled in this study is monitored weekly, and our study physician, a practicing gastroenterologist, is available for support. While we anticipate the rate of adverse health effects to be low in each of the intervention groups, ethically we wish to first establish safety and efficacy of novel forms of iron supplements in older adults prior to examining the effects in a more vulnerable population such as young children.

### Rationale for daily intervention doses in Phases I and II
Our justification for providing 60 mg iron/day in Phase I is based on the WHO recommendation for daily supplementation for non-anemic, pregnant women with 30–60 mg iron/day[39]. If the forms of iron are found to produce no adverse effects, or not to worsen adverse effects, at the 60 mg Fe/day dose level, we will carry out the Phase II study in which such forms will be tested at a daily therapeutic dose of 120 mg iron/day against the same outcomes. While it is possible that risks (constipation, diarrhea, black stools and GI irritation) may be greater in Phase II due to the use of this therapeutic dose, the published literature suggests that this may not be the case. The Food and Nutrition Board[40] found evidence supporting low adverse effects levels (LOAELs) of $FeSO_4$ at doses of 120 mg Fe/day. A more recent meta-analysis of more than 40 clinical trials[41] found that the prevalence of gastrointestinal complaints associated with $FeSO_4$ administration appear to be idiosyncratic and not related to dose. We anticipate few, if any, complaints associated with either IHAT or Aspiron, as the insolubility of each in the post-duodenal gut would suggest that neither form interacts with the epithelial surfaces.

### Rationale for inclusion of weekly intervention group in Phase I
This study will examine the effects on the "safety profile" of $FeSO_4$ administered daily (60 mg iron/day) compared to weekly (420 mg iron administered in one dose/week). In the treatment of iron deficiency, weekly supplementation has been recommended as it presents feasibility and economic advantages to daily supplementation. In various populations, including iron-deficient, anemic lactating women[42], non-anemic pregnant

women[43], non-anemic pregnant women[44,45] and iron-deficient, anemic school-aged children[46], it has been demonstrated that a weekly dose of $FeSO_4$ can be as effective in improving iron status parameters, but produce lower GI complaints, compared to daily supplementation. Although these studies support the hypothesis that weekly $FeSO_4$ supplementation is advantageous over daily supplementation, few studies have directly assessed adverse effects of weekly iron supplementation administration. Inclusion of a weekly $FeSO_4$ intervention group in Phase I presents great practical value to support the existing literature in pregnant and early-postpartum women that weekly supplementation of $FeSO_4$ is advantageous over daily supplementation.

### Rationale for inclusion of MNP group in Phase I

The primary rationale for use of MNP has been to prevent iron-deficiency and treat iron-deficiency anemia in infants and children[47]. However, MNP are also administered to other target populations, including pregnant women and may be administered as part of national programs. To address the concerns with iron supplementation in malaria-endemic regions, the World Food Program (WFP) and UNICEF formulated MNP with lower iron content for use in these regions. According to a recent Cochrane review, the use of MNP supplementation reduces both anemia and iron deficiency in young children by 31% and 51%, respectively[48]. This assessment agrees with the WHO recommendations of utilization of home fortification of foods with MNP[49] for improvements in iron status and reduction in iron-deficiency anemia. Although efficacious in the treatment of iron-deficiency anemia, the safety and efficacy of these MNP with and without additional iron has yet to be investigated. In a systemic review examining intervention with MNP in pregnant women, there have been no published studies that have examined the potential benefits or risk of MNP on health outcomes[50]. Thus, the inclusion of an MNP and $FeSO_4$ intervention group in Phase I aims to examine the safety of this recommended, common practice.

### Rationale for the placebo control in Phase I

According to the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) guidelines "Choice of Control Group and Related Issues in Clinical Trials" (ICH E10), the placebo-controlled design acts to control for all potential influences[51]. As most of our treatment effects focus on the change in our primary outcomes (malaria infectivity, bacterial proliferation and fecal calprotectin), the primary rationale for including a placebo in Phase I is to control for the potential influence in change over time and seasonal effects in these outcomes. The placebo control group will receive the same number of capsules of other treatment groups and contain cornstarch, sourced from Ingredion. Additionally, the ICH guidelines state that "placebo-controlled trials seeks to show a difference between treatments when they are studying effectiveness, but may also seek to show lack of difference in evaluating a safety measurement"[51]. As this study aims to estimate the "safety profile" between iron supplemented and non-iron supplemented adults, inclusion of a placebo is essential to the study objectives of Phase I. Conclusions on the estimated placebo effect of study participants will be determined from data collected in Phase I.

### Rationale for bacterial species selected for assessing proliferation potential

Five pathogenic bacterial strains were selected for these studies, all serious human pathogens capable of causing bacteremia and spreading through the bloodstream[52–56]: two gram-positive (*A. baumannii* and *S. aureus*), and three gram-negative (*K. pneumoniae*, *S. typhimurium*, and ExPEC (Extraintestinal Pathogenic *E. coli*)). *A. baumannii* is predominantly an opportunistic pathogen[52]. *K. pneumonia* causes fatal bacteremia in infants[53]. *S. typhimurium* is the predominant cause of food poisoning in western countries[54]. *S. aureus* and ExPEC infections result in high global morbidity and mortality rates[55,56].

### Conclusion

Overall, this double blind, placebo-controlled, randomized controlled trial will robustly test our formal hypotheses and yield useful answers to the primary questions regarding the safety and efficacy of these novel forms of iron supplements. The findings of this study will generate evidence to support development of a modality of providing bioavailable iron that does not or produces less adverse effects in iron-replete individuals. Ultimately, this study aims to contribute to the development of safe and effective forms of supplemental iron to address the global burden of iron deficiency and iron-deficiency anemia.

### Data availability

#### Underlying data

No underlying data are associated with this paper.

#### Extended data

Open Science Framework: Safe and effective delivery of supplemental iron to healthy volunteers. https://doi.org/10.17605/OSF.IO/U6SAZ[21].

The following extended data are available:

- Supplementary File 1. Main Informed Consent Form_ Revised May 10, 2019.docx

- Supplementary File 2. Screening ICF_Revised May 10, 2019.docx

#### Reporting guidelines

Open Science Framework: SPIRIT checklist for "Safe and effective delivery of supplemental iron to healthy older adults: The double-blind, randomized, placebo-controlled trial protocol of the Safe Iron Study". https://doi.org/10.17605/OSF.IO/U6SAZ[21].

Data are available under the terms of the Creative Commons Zero "No rights reserved" data waiver (CC0 1.0 Public domain dedication).

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# Open Peer Review

## Current Peer Review Status: ✓ ✓ ✓

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**Version 2**

Reviewer Report 24 February 2021

https://doi.org/10.21956/gatesopenres.14450.r30390

✓ **Associated Prof. Sukru Gulec** (iD)

Molecular Nutrition and Human Physiology Laboratory, Food Engineering Department, İzmir Institute of Technology, İzmir, Turkey

The authors indicated explanations for my concern about the manuscript. The manuscript provided valuable information using iron supplementation for humans. Excessive iron might lead to toxicity during consumption. Thus, the manuscript gives us data to use an iron at the safety levels. Moreover, ex vivo studies do not cover the in vivo situation; however, I could understand the study's limitation.

*Competing Interests:* No competing interests were disclosed.

*Reviewer Expertise:* Molecular Nutrition

**I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.**

Reviewer Report 17 February 2021

https://doi.org/10.21956/gatesopenres.14450.r30391

✓ **Richard Hurrell** (iD)

Laboratory of Human Nutrition, Institute of Food, Nutrition and Health, ETH Zurich (Swiss Federal Institute of Technology), Zurich, Switzerland

I am not convinced that the primary ex vivo outcomes will provide useful new information.

However, I approve the study for the secondary outcomes of NTBI, iron status, and potential adverse side effects. The secondary outcomes will provide important new information.

***Competing Interests:*** No competing interests were disclosed.

**I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.**

Reviewer Report 17 February 2021

https://doi.org/10.21956/gatesopenres.14450.r30389

✔ **Nana Gletsu-Miller** (iD)

Department of Applied Health Science, School of Public Health, Indiana University Bloomington, Bloomington, IN, USA

This is a good protocol paper that describes a RCT that is rigorous in design and the detail provided ensures that transparency and reproducibility have been considered. Findings should be impactful for prevention or treatment of iron deficiency anemia. My original review was that the manuscript should be accepted with minimal revisions. In the revised manuscript, the authors addressed most of the comments that I suggested. In particular, I now understand their decision to use post-menopausal women as the study population even though children and pre-menopausal women are the population of interest. I defer to the other reviewers to assess the authors' responses to their comments.

***Competing Interests:*** I received funding from Cura Global Health, the manufacturer of Aspiron from Oct 2016 to Sept 2018.

***Reviewer Expertise:*** Nutrition science, iron deficiency, bariatric surgery

**I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.**

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**Version 1**

Reviewer Report 03 September 2020

https://doi.org/10.21956/gatesopenres.14152.r29204

**?** **Associated Prof. Sukru Gulec** [ID]

Molecular Nutrition and Human Physiology Laboratory, Food Engineering Department, İzmir Institute of Technology, İzmir, Turkey

The study aims to develop new iron supplement forms (IHAT and aspiron) and test their physiological features comparing to the $FeSo_4$ form. The study contained two phases to compare two different Fe supplementation amounts, including 60mg/day and 120mg/day for four weeks. The older adults living in the Boston area were selected as the study group. The hypothesis was tested by a randomized, double-blinded, placebo-controlled study.

The main concern about the manuscript:
- The efficacy of IHAT and aspiron were tested on the people from the Boston area. However, one of the hypotheses of the study was malaria infectivity that is the target for the Tanzania population. We see two different populations into the study, so how do the populations model each other for iron supplementation and malaria infectivity experiments? Furthermore, the risk of iron deficiency also commonly seen in young children/childbearing ages and iron supplamentation is also essential for them to reduce iron deficiency anemia. Why did not researchers also focus on those groups?

- The researchers compared daily (60mg iron/day) and weekly (420mg iron/week) iron administration. How do they relate 420 mg/week iron administration to 60mg iron/day in terms of physiological consequences? The older people have a risk for iron overload, so 420 mg iron/day might have a negative effect on them. The also 120 mg iron/day supplementation brings the same risk for the older population.

- The inflammation (mainly systemic) and iron metabolism are closely related via, for instance, IL-6 and hepcidin. Thus, systemic inflammatory markers (IL-6, TNFa) might be important to compare iron supplementation efficacy on the study groups. Moreover, their basal levels must also be considered in the study groups.

- The environmental area might affect the health status of people in Tanzania, and they might have very different physiological situations (inflammatory levels in the body) than the study group. As a result, this might cause a different response to iron metabolism. Thus, iron supplementation strategies might not resemble the real situation in Tanzania.

- How do ex vivo experiments cover the similar responses that are Tanzania population? The authors indicated '...increased severity of clinical malaria among children in Tanzania effectively halted efforts to address iron deficiency in malaria-endemic areas. Lack of a safe and effective treatment leaves large numbers of children iron deficient, many with associated anemia...'. They stated malaria problems in children, but the investigation was performed in older adults. How do researchers conclude that?

**Is the rationale for, and objectives of, the study clearly described?**

Yes

**Is the study design appropriate for the research question?**

Partly

**Are sufficient details of the methods provided to allow replication by others?**

Yes

**Are the datasets clearly presented in a useable and accessible format?**

Not applicable

*Competing Interests:* No competing interests were disclosed.

*Reviewer Expertise:* Molecular Nutrition

**I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.**

Reviewer Report 10 August 2020

https://doi.org/10.21956/gatesopenres.14152.r29198

? **Nana Gletsu-Miller** iD

Department of Applied Health Science, School of Public Health, Indiana University Bloomington, Bloomington, IN, USA

This manuscript describes the rationale and design of a clinical trial to evaluate iron supplementation regimens that are safe and effective. The premise of the study is the oral iron supplementation is recommended for prevention and treatment of iron deficiency, one of the most widespread nutritional disorders worldwide. However, oral iron supplementation is not often effective because the gastrointestinal side effects prevent people from complying with the regimens. Other side effects of oral iron supplementation are systemic oxidative stress and growth enhancement of unfavorable gut and systemic bacteria. The investigators designed the study to address the need for oral iron supplemental regimens that are safe and effective. Ferrous sulfate serves as the standard formulation and it is compared to placebo (corn starch), as well as to two novel iron formulations, providing iron weekly rather than daily, and providing multiple micronutrients along with the iron. The study was designed to have two phases, both of which are well underway and may already be completed by the time of this review. Thus, while there are several strengths of the study, weaknesses of the study design are not modifiable, which is a major criticism of this review.

Strengths:
- The study is rigorously designed to test various hypotheses that the experimental regimens are as safe as ferrous sulfate, but have better safety profiles which are assessed as primary and secondary outcomes (e.g. malaria infectivity, bacterial proliferation, gut inflammation, oxidative stress, gut microbiota profile). This is a randomized, double-blinded, controlled, comparative effectiveness intervention.

- The design is innovative as only the safe and effective regimens from phase 1 will be used in phase 2. Phase 1 will test lower doses of the regimen and Phase 2 will test a dose that may be used therapeutically.

- The description of the design and conduct of the study is thorough allowing for transparency and reproducibility.

- The study is well underway, which demonstrates the feasibility of recruitment and conduct of the protocol.

Major weaknesses:
- Characterizing the phase 1 dose, 60 mg Fe/day as low is debatable, since it is several times higher than the RDA (18 mg/day Fe) and some treatment guidelines recommend this as a treatment dose (e.g., Killip et al, Am Fam Phys, 2007).

- Choosing post-menopausal women or men for the study population is not well-justified, given this population is least likely to develop iron deficiency, reducing the generalizability of the study's findings. The authors state correctly that women of childbearing age are most at risk for iron deficiency so why not test the regimens in this population?

- Related to the previous point, since post-menopausal women and men are most at risk for iron overload, providing 60mg Fe/day or 120 mg Fe/day for 4 weeks to iron-replete members individuals seems risky. The tolerable upper limit for this population is 45 mg Fe/day.

- The statistical analysis chooses an "as treated approach". Since the high doses may lead to poor adherence to the regimens, it seems that an intention to treat analysis would be less prone to bias.

- Adding the multiple micronutrient to the iron supplement as a treatment regimen is not well justified and seems outside of the scope of the study, per the study's hypothesis: "…examining the safety and efficacy of these novel forms of iron supplement in iron-replete older adults". Similarly, adding an arm to test the weekly versus daily dose of ferrous sulfate is not consistent with the objectives of the study.

- Information on Aspiron regarding NTBI is not complete, should include study - Bries Bries, Curr Develop Nutr, 2019.

Minor comments:
- Statements requiring citations:
    - Introduction – (i.e., 5-20 fold effective levels of heme-iron in foods).

    - Discussion – Rationale for daily intervention doses – WHO recommendation for daily

supplementation for non-anemic pregnant women.
- ○ Typos in Abstract and throughout document.

**Is the rationale for, and objectives of, the study clearly described?**
Partly

**Is the study design appropriate for the research question?**
Partly

**Are sufficient details of the methods provided to allow replication by others?**
Yes

**Are the datasets clearly presented in a useable and accessible format?**
Not applicable

*Competing Interests:* No competing interests were disclosed.

*Reviewer Expertise:* Nutrition science, iron deficiency, bariatric surgery

**I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.**

Reviewer Report 09 December 2019

https://doi.org/10.21956/gatesopenres.14152.r28311

? **Richard Hurrell** (iD)
Laboratory of Human Nutrition, Institute of Food, Nutrition and Health, ETH Zurich (Swiss Federal Institute of Technology), Zurich, Switzerland

**Overview:**

The Safe Iron Study could potentially provide new information that would help prevent the serious adverse events observed with iron supplementation in low-and-middle income countries (LMIC) where poor hygiene and widespread infections are common, and malaria frequently endemic. These serious adverse events, as a result of iron supplementation, need urgent attention as they have restricted iron interventions especially in malaria endemic areas.

**Aim of Study:**

The aim of the Safe Iron Study is to find a way of providing bioavailable iron, in the form of supplements, to iron deficient populations in LMIC without causing adverse side effects, including infectious diarrhea, increased susceptibility to malaria infection, increased inflammation, and detrimental changes to the gut microbiome. It is hoped that this can be achieved by using iron hydroxy adipate tartrate (IHAT) or Aspiron (dried iron-enriched Aspergillus Oryzae biomass) instead of ferrous sulfate as the iron compound for the iron supplements. These novel iron compounds have both been reported to have equivalent iron absorption to ferrous sulfate but to be absorbed more slowly, and they are predicted to have less adverse side effects. More specifically, they are expected to generate less non-transferrin bound iron (NTBI) than ferrous sulfate, and to be less well-metabolized by pathogenic organisms in the hind gut, and thus increasing pathogen numbers in the gut microbiome to a lower extent.

**Proposed Study Protocol:**

The safe Iron Study will compare iron supplements based on IHAT, and supplements based on Aspiron, with ferrous sulfate supplements, given to older (50-80 years old) iron replete adults living in the Boston area. Two feeding studies are planned, (Phase 1 and Phase 2) in which the iron replete older adults are provided with 60mg Fe supplements daily for 4 weeks (Phase 1) or 120mg Fe supplements daily for 4 weeks (Phase 2). Phase 1 has an additional 3 groups. These are a placebo group, a group receiving 60mg Fe as ferrous sulfate plus a micronutrient powder once per day, and a group receiving 420 mg Fe as ferrous sulfate once a week. The additional groups in Phase 1 provide data on a control group with no iron supplement (which can be also be used in Phase 2), and allow an evaluation of whether providing iron within a micronutrient powder, or as a single high dose supplement once a week, changes the observed response to iron supplementation.

In Phase 1 and Phase 2, blood and stool samples are taken at baseline and at 4 weeks. The primary outcomes of the study are malarial infectivity (ex vivo) in the presence of host erythrocytes, bacterial proliferation (ex vivo) in the presence of host plasma, and intestinal inflammation as measured by fecal calprotectin. The secondary outcomes are iron status as measured using a range of iron status biomarkers parameters, NTBI and systemic inflammation biomarkers, all measured in blood; and fecal iron, intestinal inflammation biomarkers and changes in the gut microbiome, all measured in stool.

**Comments on the study design and methodology:**

While I am in strong support of the need for further research that enables iron supplements to be given safely in LMIC, and in support of much of the proposed program, I am not in complete agreement with the need to study the increased susceptibility to malaria infection ex vivo in the host blood, nor do I see much usefulness in measuring bacterial proliferation ex vivo in host plasma. Both measurements are primary outcomes of this study.

1. In relation to the malarial infection, the increased mortality and morbidity reported after iron supplementation (Sazawal *et al.*, 2006[1]) appear to be due to an intensification of the malarial infection, leading to more cerebral malaria and bacteremia, rather than to an increase in the number of malaria infections. The underlying cause of the intensification of a

malaria infection is thought to be the formation NTBI in the blood serum on absorption of the relatively large dose of iron in the supplements (Hurrell, 2010[2] and Hurrell, 2011[3]). The likely explanation for NTBI formation is that the rate of iron influx into the plasma from high-dose oral supplements exceeds the rate of iron binding to transferrin. It is proposed that NTBI increases the severity of malarial infections by increasing the sequestration of malaria-infected erythrocytes in the capillaries of the brain and intestine (Kartikarsari *et al.*, 2006[4]), leading to more cerebral malaria and a breach of the intestinal barrier leading to bacteremia. At the same time, high iron doses in supplements can stimulate the growth of pathogenic bacteria in the stool, further increasing the potential for bacteremia, which is frequently reported in children with severe malaria (Oundo *et al.*, 2002 [5]).

While the usefulness of measuring malaria infectivity ex vivo as a primary outcome can be questioned, the Safe Iron Study is measuring NTBI as a secondary outcome so will compare the iron supplements in relation to their ability to increase the intensity of the malaria infection. NTBI will be measured at baseline and 2h after supplement ingestion. Results from the NTBI measurements are perhaps the most important new outcome from the study and could be upgraded from a secondary outcome to a primary outcome of the study.

2. The second primary outcome of the Safe Iron Study is bacterial proliferation (ex vivo) in the presence of host plasma. The reason for these studies is presumably to compare the risk for bacteremia during malaria infection after ferrous sulfate supplementation with that of the novel iron compounds which are absorbed more slowly. The serum will be tested at baseline and 2h after supplement ingestion. At the 2h time point, the serum taken after ferrous sulfate ingestion would be expected to be higher in iron than the serum taken after ingestion of the more slowly absorbed novel iron compounds. The 2h serum after ferrous sulfate ingestion therefore would be expected to increase pathogen growth to a greater extent.

While the risk of bacteremia may depend to some extent on the iron concentration of the blood plasma, the greater risks for bacteremia appear to be the permeability of the gut mucosa (depending on NTBI formation) and the number of pathogens present in the gut microbiome. Iron supplementation to children living areas of widespread infection has been reported to increase pathogen numbers (Zimmermann *et al.*, 2010[6]) in the stool. In children with severe malaria, it is possible that these pathogens enter the blood stream via the damaged capillaries in the intestinal villi. The pathogens could presumably enter the blood stream at any time, so the ideal times within the Safe Iron Study to take sera for bacterial proliferation measurements are not easy to select. As iron absorption from ferrous sulfate, IHAT and Aspiron is reported to be similar, the total amount of iron entering the plasma over a 24h period between supplement ingestion would be similar, but the pattern of entry would be different.

Increased pathogen numbers in the gut microbiome of children living in areas of poor hygiene and widespread infections is clearly a major safety issue in relation to iron supplementation, and is a major risk for bacteremia during malaria infection, in addition to being a risk for infectious diarrhea. The Safe Iron Study proposes to measure the gut microbiome in older, iron replete adults in Boston after ingestion of IHAT and Aspiron

supplements. The choice of subjects, and the choice of a hygienic environment in which to make the study, can be explained by a need for absolute safety. However, ferrous sulfate supplements are still regularly administered in Sub Saharan Africa to women living in areas of poor hygiene and widespread infections without the serious side effects reported in children, and IHAT and Aspiron administration has caused no serious negative side effects in women living in industrialized countries. Feeding the compounds in Boston, will lead to new information concerning their influence on the gut microbiome. However, it will add little to our knowledge regarding potential adverse events resulting from iron supplementation in countries with poor hygiene and widespread infections. There would appear to be no safety concerns to making a similar Safe Iron Study in young women in sub Saharan Africa study so as to compare the gut microbiome after IHAT, Aspiron and ferrous sulfate supplementation, in a region where ferrous sulfate supplementation is commonly practiced. Such a study would provide vital new information and would be the major first step the evaluation of the safety of IHAT and Aspiron.

3. Another concern relates to the choice of iron replete older adults as the study subjects. This is based on the suggestion from the Pemba study that the serious adverse events occurring with iron supplementation in malaria endemic areas are observed mostly in iron replete children. This "finding" however was not statistically significant (so is really only a suggestion), it has not been confirmed in further studies, and there is no clear mechanism to explain why iron replete subjects have more adverse events. Iron absorption would be extremely low in iron replete subjects with malarial infection and inflammation, and a mechanism for serious adverse events that is linked to absorbed iron would be less likely. Young women of marginal or low iron status may be more appropriate subjects than older iron replete adults as they would be expected to exhibit a higher iron absorption. This would facilitate finding differences between iron compounds in relation to NTBI, systemic inflammation biomarkers and iron status biomarkers. Potential side effects of iron supplementation of women in high income countries and also LMIC, are well known and are not considered a safety issue.

4. The measurement of fecal calprotectin as a primary outcome will generate important new information on IHAT and Aspiron in older adults in a hygienic environment. A similar study may be needed in adults living in a less hygienic environment. An increase in fecal calprotectin has already been reported with iron fortification in children in the Cote d'Ivoire (Zimmermann *et al.*, 2010[6]).

5. The measurement of iron status biomarkers in the blood should confirm the high bioavailability of IHAT and Aspiron, whereas the measurement of systemic inflammation biomarkers in the blood and fecal iron should generate useful new information.

**Conclusion:**

The Safe Iron Study aims to find a way to provide supplements of bioavailable iron to populations living in areas of poor hygiene and widespread infection without increasing infectious diarrhea, the susceptibility to malaria infection, inflammation, and detrimental changes to the gut

microbiome. The approach to compare daily supplements of ferrous sulfate with supplements of IHAT and Aspiron, in iron replete older adults living in Boston in the United States may be overcautious and will provide little new information on the safety of IHAT and Aspiron with respect to infectious diarrhea, inflammation or changes gut microbiome. Important new results on NTBI should help judge their ability to intensify malarial infections, and iron status measurements should confirm their bioavailability. The primary outcomes of malaria infectivity in the presence of host erythrocytes and pathogen proliferation in the presence of host plasma are judged to be of limited usefulness.

Increased morbidity and mortality with iron supplementation of malaria infected children appear to be due to the formation of NTBI. It is proposed that NTBI increases the severity of malarial infections by increasing the sequestration of malaria-infected erythrocytes in the capillaries of the brain and intestine leading to more cerebral malaria and a breach of the intestinal barrier leading to bacteremia. At the same time, high iron doses in supplements can stimulate the growth of pathogenic bacteria in the stool, further increasing the potential for bacteremia. An important first step in the evaluation of IHAT and Aspiron would be to compare their influence on pathogen numbers in the gut microbiome of young women living in Sub Saharan Africa, with the influence of ferrous sulfate.

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**Is the rationale for, and objectives of, the study clearly described?**

Yes

**Is the study design appropriate for the research question?**

Partly

**Are sufficient details of the methods provided to allow replication by others?**

Yes

**Are the datasets clearly presented in a useable and accessible format?**

Not applicable

*Competing Interests:* No competing interests were disclosed.

*Reviewer Expertise:* micronutrient interventions, iron and malaria, iron status

**I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.**