EVALUATING THE EFFECT OF 12 WEEKS OF SUPPLEMENTATION WITH

FERROUS SULFATE, FERROUS BISGLYCINATE OR PLACEBO, ON IRON STATUS

AND GUT INFLAMMATION IN CAMBODIAN WOMEN

by

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Evaluating the effect of 12 weeks of supplementation with ferrous sulfate, ferrous bisglycinate or placebo, on iron status and gut inflammation in Cambodian women

Submitted by   Jordie Fischer   in partial fulfillment of the requirements for

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Abstract

The WHO recommends 12 weeks daily iron supplementation for women in countries where anemia prevalence is ≥40%, such as in Cambodia. However, if iron deficiency is not a major cause of anemia, then, at best, untargeted iron supplementation is a waste of resources; at worst, it could cause harm. My aim was to assess the non-inferiority of 12 weeks of ferrous sulfate and ferrous bisglycinate supplementation on ferritin concentrations and the effect on gut inflammation concentrations in Cambodian women, as compared to placebo. A double-blind, three-arm, randomized controlled trial was conducted in Kampong Thom province, Cambodia. Non-pregnant women (*n*=480, 18-45 years) were randomized to receive 60 mg ferrous sulfate, 18 mg ferrous bisglycinate, or placebo. Non-fasting blood and stool samples were collected at baseline and 12 weeks. Ferritin was measured with an ELISA, and values were adjusted for inflammation. Fecal calprotectin was measured as an indicator of gut inflammation with use of an ELISA kit (BÜHLMANN fCAL®). Mixed-effects generalized linear models were used to assess the effect of the two iron interventions on ferritin and fecal calprotectin concentration at 12 weeks, as compared to placebo. A total of 480 women were enrolled with 88% (*n*=421) trial retention at 12 weeks. Our non-inferiority analysis was inconclusive to determine if ferrous bisglycinate was non-inferior to ferrous sulfate, as the CI for our predicted mean difference in ferritin concentrations between the two iron interventions crossed our '*a priori*' defined margin of non-inferiority (20 µg/L). In a secondary analysis with use of a superiority approach, mean ferritin concentration at 12 weeks was significantly higher in the ferrous sulfate group (98.6 [94.7,102.6] µg/L, *P*<0.001) than in the ferrous bisglycinate (84.0 [79.9, 88.2] µg/L) and placebo groups (77.8 [73.9, 81.7] µg/L). No differences in fecal calprotectin were detected across groups at 12 weeks. We were unable to establish non-inferiority between the two iron interventions;

however, we did confirm that 12 weeks of 60 mg ferrous sulfate significantly increased serum ferritin concentrations as compared to 18 mg ferrous bisglycinate or placebo with no differences in gut inflammation across groups in this population of predominantly iron-replete, non-anemic women.

Lay Summary

Global guidelines recommend daily oral iron supplementation for all non-pregnant women in countries where anemia is common, yet little research has been done on the potential harms of blanket iron supplementation programs. In populations where the main cause of anemia is not iron deficiency, such as in Cambodia, these programs may expose women to too much iron. This has the potential to be harmful, as excess unabsorbed iron may travel to the gut and cause inflammation. I assessed the effect of 12 weeks of daily iron supplementation (a 60 mg dose of the standard form), compared to a lower dose (18 mg) of a more bioavailable form of iron, or placebo (no iron), in non-pregnant Cambodian women. Blood and stool samples were collected from women before and after 12 weeks of supplement consumption. The findings from this study will help inform safe and effective iron supplementation policies for women worldwide.

Preface

This thesis is based on the research I conducted under the supervision of Dr. Crystal Karakochuk, submitted in partial fulfillment for a Master of Science in Human Nutrition degree at the University of British Columbia. I received additional guidance from my thesis supervisory committee members, Drs. David Goldfarb and Rajavel Elango, who contributed to the study design, interpretation of results, and review of this thesis. The research presented here is part of a larger project undertaken in the province of Kampong Thom, Cambodia, through a collaboration between the University of British Columbia, Helen Keller International Cambodia, and the Cambodian Ministry of Health. Helen Keller International Cambodia assisted with the Cambodian ethics approval, translated all data collection and training tools and coordinated in-country research activities. Other research partners assisted with laboratory analyses, including the VitMin Laboratory in Willstaett, Germany and the National Institute of Public Health Laboratory in Phnom Penh, Cambodia. Ethical approval for this trial was obtained from the University of British Columbia Clinical Research Ethics Board (H18-02610) and the National Ethics Committee for Health Research in Cambodia (273-NECHR). The trial was registered with ClinicalTrials.gov (NCT04017598).

The design of this study was a collaborative effort between my supervisor Dr. Crystal Karakochuk (principal investigator) and study co-investigators, Dr. David Goldfarb, Dr. S Katyal, Dr. Angela Devlin, Dr, Amee Manges, as well as collaborators, Mr. Hou Krouen from Helen Keller International Cambodia, and knowledge-users, Dr. Sopphoneaery Prak from Ministry of Health Cambodia and Dr. Arnaud Laillou from UNICEF Cambodia. I spent eight months in Cambodia, where I was responsible for oversight of the research trial. I was involved

in all aspects of the project from planning, recruitment, implementation, data collection and management, laboratory analysis, biospecimen shipment and data analysis. I developed the materials used in this trial in collaboration with Helen Keller International's lead project staff and my committee members: questionnaires, monitoring tools, data collection forms, blood and stool collection and processing protocol. Along with the Helen Keller International Cambodia team, I led enumerator and data collector training, oversaw daily field activities of data and biospecimen collection and transportation, and assisted lab staff to process blood and stool samples during the data collection period. Lulu Pei, an undergraduate research assistant, assisted with project implementation for two months in Phnom Penh in preparation for the trial. Finally, I was responsible for completing the fecal calprotectin ELISAs in Dr. Karakochuk's laboratory at the University of British Columbia, conducting the statistical analysis, interpreting the research findings, and thesis writing.

Results of this randomized controlled trial were presented at the Social Exposome Cluster Research Day in November 2020, where I was awarded *Best Master's Level Academic Poster*, BC Children's Hospital Global Health Conference in January 2021, and Healthy Starts Research Day in February 2021, where I was awarded a travel scholarship for *Best Master's Level Poster*. Further, I have submitted abstracts to the Land and Food Science Graduate Student Conference and Canadian Nutrition Society Conference held in March and May 2021, respectively.

A version of chapter 2 has been published: Fischer JAJ, Pei L, Goldfarb DM, Albert AY, Elango R, Hou K, Karakochuk CD. Is untargeted iron supplementation harmful when iron deficiency is not the major cause of anaemia? Study protocol for a double-blind, randomized controlled trial

among non-pregnant Cambodian women [Protocol]. *BMJ Open* 2020; 10(8): e037232. I helped

draft the protocol and prepared the ethics applications and clinical trial registries. Pei L,

Goldfarb DM and Elango R contributed to manuscript edits. Albert AY guided sample size

calculations and provided statistical support. Hou K oversaw Cambodian ethic approval and trial

implementation. Karakochuk CK was the principal investigator on the project, was responsible

for concept formation and final manuscript revisions. I intend to submit a version of chapter 3, as

well as the final results of the full trial, beyond the scope of my thesis objectives, for publication

in a peer-reviewed journal.

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List of Abbreviations

AGP	α-1-acid Glycoprotein
BMI	Body Mass Index
BRINDA	Biomarkers Reflecting Inflammation and Nutritional Determinants of Anemia
C. coli	*Campylobacter coli*
C. jejuni	*Campylobacter jejuni*
CI	Confidence Interval
COVID-19	Coronavirus Disease 2019
CRP	C-Reactive Protein
CV	Coefficient of Variation
DHS	Demographic and Health Survey
DNA	Deoxyribonucleic Acid
DMT1	Divalent Metal Transporter 1
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme-linked Immunosorbent Assay
Fe	Iron
Fe^{2+}	Ferrous Ion
Fe^{3+}	Ferric Ion
Hb	Hemoglobin
Hb E	Hemoglobin E Disease Heterozygous
Hb EE	Hemoglobin E Disease Homozygous
HO-1	Heme Oxygenase 1
ID	Iron Deficiency
IDA	Iron Deficiency Anemia
IFA	Iron and Folic Acid
IFNγ	Interferon-γ
IL-1	Interleukin-1
IL-6	Interleukin-6
IL-10	Interleukin-10

IQR	Interquartile Range
MCHC	Mean Corpuscular Hemoglobin Concentration
MCV	Mean Corpuscular Volume
MNP	Multiple Micronutrient Powder
NaFeEDTA	Ferric Sodium Ethylenediaminetetraacetate
NTBI	Non-Transferrin-Bound Iron
PCR	Polymerase Chain Reaction
P-value	Probability Value
QC	Quality Control
qPCR	Quantitative Polymerase Chain Reaction
RBC	Red Blood Cell
RDW	Red Blood Cell Distribution Width
SD	Standard Deviation
s-ELISA	Sandwich Enzyme-Linked Immunosorbent Assay
sTfR	Soluble Transferrin Receptor
TNF-α	Tumor Necrosis Factor α
UL	Tolerable Upper Intake Limit
UNICEF	United Nations International Children's Emergency Fund
WHO	World Health Organization
WRA	Women of Reproductive Age
ZnPP	Zinc Protoporphyrin

Acknowledgements

Dedication

"But when the kindness and love of God our Savior appeared, **he saved us, not because of righteous things we had done, but because of his mercy***."* Titus 3:4-5

Because of this truth and radical love given to me, everything I do on this earth is to glorify my God and my Saviour. Jesus devoted his ministry to care for the sick, oppressed and vulnerable. May this research bring You glory.

Until the whole world knows.

Chapter 1: Introduction

1.1. Preamble

This literature review discusses anemia, iron deficiency, the causes of anemia in Cambodia and current interventions to treat anemia. A brief explanation of iron metabolism and homeostasis is provided. The role of ferritin, an iron storage protein, is introduced, along with measurement methods and limitations of iron status assessment. I give a review of iron supplementation and its potential harms, focusing on the literature that examines the potential harm of supplementation in iron-replete populations and the effect of iron on gut inflammation. Lastly, I review different iron forms used for supplementation and fortification, their bioavailabilities and side effects.

1.2. Cambodia

Cambodia is an agrarian country located in Southeast Asia, bordering Thailand to the north and west, Laos to the north, Vietnam to the south and east and the Gulf of Thailand to the south and west.[1] Home to 13.4 million people, Cambodia is still overwhelmingly rural, with 80% of the population living in rural areas.[2]

Cambodia has experienced a painful history of political turmoil and civil unrest.[3] Cambodia was declared a constitutional monarchy in 1993, which has resulted in economic progress and governmental stability.[4] Notwithstanding, Cambodia remains one of the least developed nations in Southeast Asia, Cambodia remains one of the least economically developed countries in Asia, with a gross domestic product per capita of ~USD1,088,[5] and in 2012, it was estimated ~19% of the population live below the poverty line.[6]

White rice makes up over 70% of the traditional Cambodian diet, along with other cereals.[7] Fish, fruit, soups and vegetable curries make up the rest of daily energy consumption, which are non-iron-rich food sources.[7] Condiments such as soy sauce, fish sauce, sweet chilli sauce, *prahok*, a fermented fish paste, and *kapi*, a salted fermented shrimp paste, are always found on the table.[8] The Cambodian diet has low dietary diversity and is low in energy, fat, and high bioavailable micronutrient sources, including iron.[7]

Cambodia has 24 provinces, including the capital city of Phnom Penh. The trial described in this thesis was conducted in the rural province of Kampong Thom, shown in **Figure 1**. It is centrally located in Cambodia, situated three hours between the capital city of Phnom Penh and Siem Reap, famously known for the ancient Angkor Wat temple complex, the largest religious monument in the world.

Figure 1. Map of Cambodia
National Institute of Statistics, Directorate General for Health and ICF International. *2014 Cambodia Demographic and Health Survey Key Finding*s, 2015. https://dhsprogram.com/pubs/pdf/SR226/SR226.pdf (accessed January 4, 2021).

1.3. Anemia

The World Health Organization (WHO) states that anemia is a global public health problem impacting both low and high-income countries, affecting 1.62 billion people globally.[9] It can occur at any stage of life, but pregnant women and young children living in Africa and Southeast Asia are at the highest risk for developing anemia.[9] Anemia is a public health issue in Cambodia, with 45% of women of reproductive age reported (WRA) having anemia in the 2014 Demographic and Health Survey (DHS),[1] for which the WHO defines as a "severe" public health problem.[9]

Anemia is a deficiency of healthy red blood cells (RBC), also known as erythrocytes, resulting in a decreased oxygen-carrying capacity of the blood. The WHO defines anemia as a hemoglobin concentration level <120 g/L for non-pregnant WRA.[9,10] Mild, moderate, and severe anemia for non-pregnant WRA is defined by having hemoglobin concentrations ranging from 110-119 g/L, 80-109 g/L, and <80 g/L, respectively.[10] Anemia is characterized by a hemoglobin concentration below a specific threshold established for a population of a particular age, sex, or stage of life. Other factors that influence hemoglobin status that must be considered when using hemoglobin cut-offs to define anemia include sex, age, pregnancy, altitude, cigarette smoking and African ethnicity.[10] Women in this study were similar in age, not pregnant, did not live at high altitudes (>1,000 m), generally do not smoke cigarettes and were of Khmer (Cambodian) ethnicity. For these reasons, altitude, smoking, and ethnicity were not measured in this study or included in the assessment and interpretation of hemoglobin concentrations.

1.3.1 Consequences of Anemia

Anemia is a public health problem of great concern as it is a common disorder, and there are many negative consequences of anemia in women. Most importantly, women with anemia have an increased risk of adverse pregnancy outcomes, such as maternal and child mortality, low birth weight and preterm birth.[9] Women with anemia may have impaired work productivity and exertion capacity due to weakness and fatigue due to less oxygen being transported to the brain and working muscle.[9,11]

In anemia, the number of circulating RBCs is lower than normal, and the oxygen-carrying capacity does not meet one's physiological needs.[12] RBCs contain hemoglobin, an iron-rich protein, which functions to transport oxygen in the body.[13] RBCs are essential for human survival as they provide the sole means for oxygen binding in the lungs, transportation in circulation and tissue delivery through capillaries.[12] Low RBC levels lead to oxygen starvation of body tissues and other consequences, including weakness, fatigue, increased morbidity and mortality rates, and overall decreased quality of life.[14,15] Decreased work productivity and capacity can reduce a household's income and may increase the risk of household-level food insecurity. In Cambodia, where a large portion of employment is in agriculture, requiring physical labour, anemia may negatively affect the country's economic performance.[16,17]

1.3.2 Causes of Anemia

There are many causes of anemia, as numerous factors govern the production, destruction, or loss of RBCs.[12,14] The etiology of anemia can be related to hereditary or developed RBC abnormalities, or it may be a symptom of an underlying condition.[14] As the cause of anemia can

be multi-factorial, it is imperative to test for all factors that may cause anemia in an individual.[14] Although iron deficiency is a major cause of anemia, it is rarely present in isolation, as it frequently co-exists with several other conditions. Other factors contributing to anemia may include genetic hemoglobin disorders, infection, malaria, excessive blood loss (heavy menstruation) or nutritional deficiencies (folate, vitamin B-12, riboflavin and vitamin A).[13]

1.3.2.1 Genetic Hemoglobin Disorders

The hemoglobin molecule contains two α-globin polypeptide chain subunits and two β-globin subunits.[12] Each of these four globin polypeptide chain subunits is a helical protein that encloses a single heme group containing one oxidized iron molecule (Fe^{2+}) that can bind to oxygen.[12] A hemoglobin molecule can bind four oxygen molecules, now functioning as an oxygen transporter.[12,15] Deletions or substitutions of these α- and β-genes results in reduced or abnormal forms of hemoglobin, referred to as genetic hemoglobin disorders.[18,19]

Genetic hemoglobin disorders are disorders of the blood which affect the structure, function or production of hemoglobin.[20] The 2014 DHS reported that approximately 60% of reproductive age women have genetic hemoglobin disorders in Cambodia.[1] The most common variants in Cambodia are α-thalassemia and hemoglobin E (HbE).[1,21,22] With thalassemia, there is a nucleotide deletion in either the α or β-globin chains of hemoglobin, resulting in α or β-thalassemia.[23] In α-thalassemia, mutations involving the nucleotide deletion of one or more genes encoding the α-globin chain of hemoglobin cause impaired globin chain synthesis.[18] Hemoglobin E is caused by a nucleotide substitution in one of the genes encoding the β-globin chains of hemoglobin.[18] α-Thalassemia and hemoglobin E can both be co-inherited in the same

individual. Hemoglobin disorders can result in decreased or ineffective hemoglobin synthesis, leading to an increased risk of anemia, reduced oxygen-carrying capacity and other serious health problems.[18,20]

Genetic hemoglobin disorders range in symptomatic severity and present an array of outcomes, ranging from asymptomatic presentations to severe anemia that may put an individual at risk of death.[18,22] Inheritance of only one abnormal allele is termed as 'heterozygous,' while inheritance of two abnormal alleles is termed as 'homozygous.'[18] The latter is the severe phenotype, resulting in more severe anemia and clinical outcomes. An additional concern with these inherited disorders is that they put women at risk of iron overload, as some of these disorders can also cause altered iron metabolism.[24]

In a cross-sectional study conducted in Prey Veng province, of $n=450$ women aged 18-45 years, it was reported that women who were Hb EE homozygous had significantly lower mean ± SD hemoglobin concentrations (109 ± 7.3 g/L, $P<0.05$), as compared to women with no hemoglobin disorder (130 ± 8.9 g/L); thus, demonstrating the association of Hb EE disease with anemia.[22] Furthermore, the mean ± SD serum ferritin concentrations of women with the homozygous Hb EE disorder (129 ± 91 µg/L, $P<0.05$) were significantly higher than women without abnormal traits (96 ± 56 µg/L).[22] Likewise, women with homozygous Hb EE disorder were shown to have more elevated serum soluble transferrin receptor (sTfR) concentrations than women without a hemoglobin disorder. Zimmermann et al. reported that Thai women who were heterozygous for α-thalassemia had significantly higher median (IQR) serum ferritin concentrations [28 (1,142) µg/L] than women without a genetic hemoglobin disorder [15 (1,148) µg/L, $P<0.01$].[24]

Therefore, genetic hemoglobin disorders are shown to not only cause anemia but also alter some common iron biomarkers, such as ferritin and sTfR, regardless of the individual's actual iron status.

1.3.2.2 Infection and Inflammation

Anemia of chronic disease/inflammation is recognized as the second most common cause of anemia, following iron deficiency anemia.[25] Conditions in which anemia of inflammation is common include cancer, infections, autoimmune diseases, and chronic kidney disease.[26]

Disease and infection can elicit an immune activation response and stimulate production of inflammatory cytokines, such as interferon-γ (IFNγ), tumour necrosis factor α (TNF-α), interleukin-1 (IL-1), interleukin-6 (IL-6) and interleukin-10 (IL-10).[15] Cytokines, such as IL-6, trigger the liver to upregulate hepcidin production, inhibiting macrophages from releasing iron, thus, trapping iron inside the macrophage in its storage form. This significantly decreases the amount of iron available for erythropoiesis. Additionally, the upregulation of hepcidin holds iron in macrophages by degrading ferroportin-1, an iron transporter, and in the same way, hepcidin lessens intestinal dietary iron absorption.[25] IFNγ and TNF-α inhibit erythropoietin production in the kidneys, impeding RBC production in the bone marrow.[15] Further, TNF-α promotes RBC degradation in macrophages via phagocytosis.[15,25] Also, on the surface of the macrophage, IFNγ increases the expression of divalent metal transporter 1 (DMT1), allowing the increased rate of iron uptake into the macrophage, resulting in less available iron for hemoglobin synthesis.[25] These processes cause the sequestration of iron inside macrophages and the inability of iron to be fully absorbed, which results in inadequate iron available for hemoglobin production.

In anemia of chronic inflammation, ferritin usually presents as normal to high, reflecting the fact that iron is being stored in cells and converted to ferritin. Additionally, ferritin, an acute-phase protein, becomes elevated in the presence of inflammatory mediators.[25,27] Under normal circumstances, ferritin is considered a sensitive marker for iron status, but the falsely elevated ferritin concentrations observed in anemia of inflammation may cause the underestimation or misdiagnosis of iron deficiency. To address the confounding factor of inflammation in populations, it is recommended that ferritin be adjusted for inflammation using two acute-phase proteins, α-1-acid glycoprotein (AGP) and C-reactive protein (CRP).[27] The use of this approach may improve the accuracy of iron deficiency diagnosis by measurement of ferritin in the presence of inflammation.

In many countries, malaria is a cause of anemia of inflammation. In response to the WHO recommendation, there was impetus for two large intervention trials coordinated by the WHO to evaluate the safety of untargeted iron supplementation regarding malaria. Trials in Pemba Island, Tanzania and Nepal, evaluated the impact on overall mortality comparing iron+folic acid, iron+zinc, iron+zinc+folic acid, zinc alone, or placebo in infants and young children.[28,29] The trial in Tanzania, now known as the 'Pemba trial,' was stopped early because of a higher incidence of severe adverse events, including hospitalizations due to malaria and other infections and death in the iron+folic acid arm than in the solely zinc and placebo arms.[28] The trial in Nepal, which is not a malaria-endemic area, showed no effect,[29] therefore, concluding the adverse events exposed in the Pemba trial resulted from the interaction between malaria and iron. In 2007, the WHO and the United Nations International Children's Emergency Fund (UNICEF)

issued a joint statement cautioning iron supplementation in malaria-endemic regions and recommending that supplementation be aimed explicitly at anemic individuals or those at risk of iron deficiency.[30] The advice emphasized that iron supplementation programs be accompanied by malaria prevention and treatment initiatives.[30]

In Cambodia, hookworms, parasites and malaria are present and may be contributing to anemia of inflammation. It is known that hookworm infection is common, with 15% of mothers presenting with hookworms in 2014, with a higher prevalence in rural Cambodia (17%) than urban areas (7%).[1] Hookworms attach themselves to small intestine villi and feed on host blood, with this blood loss contributing to anemia.[31] The prevalence of parasitic intestinal infection in women was 19% (including Hookworm, *Hymenolepsis nana*, *Enterobius*).[1] Additionally, a cross-sectional study in Cambodia in 2012 sampling stool from 218 rural individuals revealed 57% had a hookworm infection, with 52% testing positive for *Ancylostoma ceylanicum*.[32] Malaria is more common in the northeastern and southwestern forested Cambodian provinces, and rainy season (September – August) is thought to be when transmission is highest.[33] Our trial setting is not a high-risk malaria province (Kampong Thom is centrally located in Cambodia) and was not conducted during these months.

1.3.2.3 Blood Loss

In non-pregnant WRA age, heavy blood loss from menstruation is a potential contributor to the development of iron deficiency anemia. Heavy menstrual blood loss is defined as a total blood loss regularly exceeding 80 mL per menstrual cycle.[34] This excessive blood loss may contribute to depleted iron stores. It has been found that in Caucasian women (*n=105*, aged 20-45 years),

those with heavy menstrual bleeding lose five to six times more iron each menses (5.2 mg iron, *P*=0.0001) compared to women with normal blood loss (~0.87 mg iron).[35] With a prolonged loss of blood (such as 7+ days of menses) or moderate blood loss when combined with an iron-deficient diet, women may also be at risk of developing iron deficiency.[34]

1.3.2.4   Other Micronutrient Deficiencies

Anemia can be a result of deficiencies of other micronutrients such as folate, vitamin B-12, riboflavin and vitamin A. Folate and vitamin-B12 are required for DNA synthesis and are necessary for RBC maturation and division. Folate deficiency may result in abnormally large (macrocytic) and immature RBCs (megaloblasts) with shortened life spans.[36] Megaloblastic macrocytic anemia occurs as the number of megaloblastic RBCs increase in circulation and the amount of healthy RBCs are reduced. Further, vitamin B-12 is a co-enzyme with folate, and deficiency, as a result of the body's inability to absorb vitamin B-12, causes pernicious anemia - a type of megaloblastic anemia.[37] Elevated folate concentrations may mask pernicious anemia, which is a concern as deficiency of vitamin B-12 may remain undetected and eventually lead to cognitive issues.[38] The 2014 DHS reported the prevalence of folate deficiency (plasma folate <10 nmol/L) as ~19% in Cambodian mothers aged 15-49 years.[1] This survey reported a very low prevalence (1%) of vitamin B-12 deficiency (plasma vitamin B-12 concentration <150 pmol/L) in the same population.[1]

It is thought that riboflavin deficiency may contribute to anemia by impairing iron mobilization and absorption and RBC synthesis.[39] The prevalence of riboflavin deficiency (erythrocyte glutathione reductase activation coefficient, EGRac ≥1.4) has been reported as high (80%) in

WRA in urban and rural Cambodia.[40] In a study of 515 Cambodian WRA analyzed for biomarker status of riboflavin and genetic hemoglobin disorders, it was found that women with the Hb EE genotype were associated with an 18% (9-28%) higher geometric mean (95% CI) EGRac than women with normal hemoglobin.[41] Finally, vitamin A deficiency has been linked to impaired incorporation of iron into hemoglobin and greater breakdown of malformed RBCs, resulting in anemia.[42] In 2012, a national cross-sectional survey of 2112 Cambodian women of reproductive age found that vitamin A deficiency (inflammation-adjusted retinol binding protein (RBP) <0.70 μmol/L) was <1%.[43]

1.3.2.5 Iron Deficiency and Iron Deficiency Anemia

Iron deficiency, specifically iron deficiency anemia (IDA), is one of the most severe nutritional deficiencies globally.[44] IDA affects all age groups, with young children and women being particularly vulnerable. IDA negatively impacts a child's cognitive development into adolescence, weakening the immune system and correlating with an increased morbidity rate.[45] Throughout pregnancy, IDA may lead to an array of adverse outcomes for mother and baby, including a higher risk of hemorrhage, sepsis, maternal and perinatal mortality and low childbirth weight.[45] Additionally, physical work capacity and quality are impaired in iron deficient and anemic men and women.[17]

Although the terms iron deficiency, IDA and anemia are often used interchangeably, there are different definitions for iron nutritional status. This is likely because anemia is the most common indicator used to screen for iron deficiency, as hemoglobin, the biomarker for anemia, is quick, easy and inexpensive to assess. Iron status is interpreted as a continuum (severely low to severely

high body iron) from iron deficiency with anemia, to iron deficiency not causing anemia, to normal iron status (with varying levels of iron stores), to lastly, iron overload.[45] Characterized by reducing or depleting iron stores, iron deficiency may be detected before clinical iron deficiency anemia, a more severe condition.[46] A long-term negative iron balance (iron loss > absorption) results in iron deficiency. The continual diminishing of an individual's iron stores results in the inability to meet typical iron turnover requirements.[47] Inadequate iron intake (including low dietary intake and poorly bioavailable sources), increased iron requirements and blood loss may lead to the development of iron deficiency.[48] IDA can be considered a subset of iron deficiency, in which it represents the extreme lower end of the spectrum of severity.[45] It is important to note that the extent of the overlap between iron deficiency and IDA, or the extent to which iron deficiency is the cause of anemia, varies widely depending on the population's context, including ethnic, country, gender and age groups.[45] Prevalence rates for a specific subgroup (by ethnicity, gender or age) cannot be used as a proxy for the rest of the population because iron deficiency risks vary widely.

The progression of nutritional iron deficiency can typically be categorized into three stages. In the early stage of iron depletion, iron stores are exhausted, but iron supply for erythropoiesis remains adequate.[42] The status of iron biomarkers at this stage usually present with low ferritin concentration and normal values for sTfR (tissue iron), transferrin saturation and hemoglobin. The second stage is characterized by inadequate iron supply for RBC production. Ferritin concentration remains low, sTfR becomes elevated, indicating tissue iron deficiency, while hemoglobin concentration remains normal. Progressing to the final stage of IDA, where iron stores are depleted (low ferritin concentration), supply to tissues and RBCs is compromised (high

sTfR), and RBC production decreases (low hemoglobin), resulting in anemia.[49] If anemia is a consequence of chronic disease or inflammation, iron is sequestered in the macrophage (as a result of the inhibition of ferroportin expression), resulting in elevated sTfR reflecting the compromised iron supply to RBCs.[25] Interestingly, ferritin concentrations may remain normal or increase as a result of the inflammation, a phenomenon known as functional iron deficiency, as opposed to the commonly understood absolute iron deficiency.[49]

The magnitude of anemia or iron deficiency as a public health problem in a region is defined by prevalence, using population-specific hemoglobin and/or ferritin concentration cut-offs values. Anemia can be classified as a severe, moderate, or mild or no public health problem based on an anemia prevalence of ≥40.0%, 20.0–39.9%, 5.0–19.9% or ≤4.9%, respectively (measured by hemoglobin concentration below the recommended cut-off values).[50] Similarly, to classify iron deficiency as a severe, moderate, mild or no public health problem, iron deficiency prevalence would be ≥40.0%, 20.0–39.9%, 5.0–19.9% or ≤4.9%, respectively (measured by ferritin concentration below the recommended cut-off values).[50]

1.4. Iron

Iron (Fe) is one of the most abundant elements, accounting for 5% of the earth's crust and is an essential component of most biological systems. Iron is a crucial mineral for the human body found abundantly in water, food and soil.[23] It is required for RBC production, oxygen transport, respiration, DNA synthesis, electron transport and other critical biological functions.[42]

1.4.1 Dietary Intake of Iron and Bioavailability

Dietary iron is absorbed in different forms: heme, inorganic and ferritin. The amount of iron absorbed from the quantity consumed is generally quite low but may range from 5-35% depending on ingestion circumstances, the form of iron and the presence of dietary components that may enhance or disrupt iron absorption.[23] Foods with high iron content include red meat, liver, beans, fortified cereal products and leafy greens, but iron absorption varies greatly.

Iron bioavailability describes the amount of iron absorbed from the diet and utilized for normal bodily functions, including being incorporated into hemoglobin, ferritin and iron enzymes.[42] Unique from other minerals, iron has no regulated excretion pathway, so absorbed iron is almost entirely stored or utilized; thus, iron bioavailability encompasses iron used for storage in the body.[42]

The two primary forms of dietary iron are heme iron, found solely in animal products, and non-heme iron, found in both plant and animal food sources.[42] Heme iron, from hemoglobin and myoglobin within meat products, is estimated to provide 10-15% of the daily dietary iron intake in meat-eating populations.[23,51] Heme iron is highly bioavailable and may contribute up to 40% of the daily total absorbed iron due to its greater and less variable absorption.[51] Non-heme iron has a considerable variation in absorption from <1% to >90%, dependent on an individual's iron status and the food matrix, including the presence of iron absorption enhancers or inhibitors.[42] Ferritin iron is found in high quantities in the liver, with plant-based sources, including beans, contain approximately 30% of their iron as ferritin iron.[42]

After consumption, heme iron remains protected within its heme complex, unlike non-heme iron. This complex prevents the oxidation of Fe^{2+} (ferrous state) into Fe^{3+} (ferric state). The iron atom is then transferred from the intestinal lumen into enterocytes by heme oxygenase 1 (HO-1).[23] Once inside the enterocyte, ferrous iron is freed from the heme molecule complex and then bound to ferritin or lasts as free-iron.[23] Therefore, heme iron's bioavailability is affected to a lesser degree by dietary compounds, such as polyphenols and phytates, since the heme complex's structure prevents ferrous iron from being chelated by other food components.[42]

Some dietary components affect the bioavailability of iron by influencing absorption, but these food components do not hinder iron utilization. Nutritional non-heme iron absorption enhancers prevent iron from binding to inhibitory compounds by reducing the highly reactive ferric iron (Fe^{3+}) to its less reactive ferrous state (Fe^{2+}) or securing iron in bioavailable complexes. Vitamin C (found in vegetables and fruits, especially citrus) is the most potent enhancer of non-heme iron absorption; whereas, the most significant inhibitory components are phytic acid (found in grains, beans, seeds) and polyphenols (found in tea, coffee, chocolate and berries).[42] Iron inhibitors bind to non-heme iron in the gastrointestinal tract, preventing its absorption.[42] Phytic acid forms complexes in the gastrointestinal tract with compounds other than iron, including various metals and proteins, reducing their bioavailability.[42] Calcium has demonstrated absorption inhibition on non-heme and heme iron absorption, different from other inhibitors that only affect non-heme iron absorption.[23]

In regions in the developing world, staple foods may not provide sufficient iron to meet daily recommended intakes. Staple foods in low-resource countries are often low in highly bioavailable heme iron (e.g. meat) and high in iron inhibitors (e.g. phytates found in cereals, leafy greens, legumes and nuts).[45] Rice and most staple cereals have low iron levels, as most iron-containing components are lost during grain processing. Populations with monotonous diets consisting mainly of cereals are especially prone to iron deficiency.[23]

Food fortification is an alternative method to increase iron content and overall improve nutritional intakes in populations. Fortification of widely consumed and distributed foods can be a cost-effective method to combat iron deficiency in the most vulnerable individuals. Micronutrients are commonly added to cereal products, milk, dairy and milk-alternative products, condiments and sauces, meal replacements, and infant foods.[52] Preferred forms of iron for food fortification include iron amino acid chelates, electrolytic iron, ferric sodium ethylenediaminetetraacetate (NaFeEDTA), ferrous sulfate, and ferrous fumarate.[53]

1.4.2 Iron Metabolism

Iron metabolism is the process of chemical reactions that maintain bodily iron homeostasis at the systemic and cellular levels.[26] While being essential for many physiological functions, iron can also be toxic in excessive quantities. The risks of iron status are understood as a U-shape curve where both iron deficiency and excess can lead to negative health outcomes; thus, a nutrient balance is needed.[54] Maintaining strict iron levels in the body is a critically important part of many aspects of human health and disease protection.

1.4.3 Iron Homeostasis

The body maintains iron homeostasis by two control systems, systemic and cellular iron homeostasis. In systemic iron homeostasis, iron supply is regulated by maintaining plasma iron levels within a narrow range. At the cellular level, iron is regulated by individual cells, adjusting the amount of iron they import or store. Nearly two-thirds of iron is found in circulating RBCs. Therefore, erythropoiesis has a significant impact on the regulation of iron absorption, transport, and storage.[42]

Most of the iron entering into the plasma (~22 mg/d) comes from the re-processing of the heme component in RBCs that have reached the end of their ~100-120-day lifespan.[42] The iron is either returned to the plasma via ferroportin or stored in ferritin. If in normal iron range, two-thirds is bound to transferrin for transport, but in iron-deficient individuals, almost all the iron is released immediately.[42]

Iron losses are minimal as humans lose only 1-2 mg daily of iron via epithelial shedding and skin peeling.[23,55] Typically, iron loss is only significant if substantial blood loss occurs due to heavy menstruation, pregnancy, or other forms of excessive bleeding, as there is no physiological mechanism for the excretion of excess iron from the body.[23] Systemic iron homeostasis is predominantly regulated at the stage of iron delivery to circulating transferrin. Circulating hepcidin, a peptide hormone secreted in the liver, maintains iron homeostasis by regulating the amount of ferroportin on cell membranes.[56] Ferroportin is a transport protein located on the surface of intestinal enterocytes and macrophages.[15] Hepcidin is an iron-regulating hormone that controls iron absorption, recycling, and the size of iron stores by binding to the extracellular arm

of ferroportin, which then degrades ferroportin, therefore preventing the absorption of iron at the enterocyte or the release of iron from macrophages.[42,57] Hepcidin is a negative regulator of iron absorption; its expression is upregulated when iron stores are adequate or high, or in the event of an inflammatory response as a result of inflammation or injury.[42,58] Hepcidin expression is downregulated (decreased hepcidin release from the liver) in response to low iron stores or anemia, allowing iron to be maximally absorbed and released into the bloodstream.[15,42] Thus, hepcidin is the predominant regulator of iron homeostasis.

1.4.4 Iron Absorption

In the body, iron is meticulously controlled by the regulation of iron absorption from the diet, iron-fortified foods or oral iron supplements at the duodenum and proximal jejunum, as iron excretion is minimal and unregulated; excess iron in the body could be harmful.[23,56] An average North American diet contains ~7 mg Fe/1000 kcal with a healthy man with typical iron stores utilizing ~1-2 mg/day, a small proportion of daily total dietary intake.[59] This amount may increase to ~2–4 mg/day for an iron deficient individual or as low as 0.5 mg/day if iron-replete.[59] Additionally, the utilization of larger amounts of iron is possible if supplemental iron is ingested.[42]

Non-heme iron absorption occurs in the enterocytes by DMT1, a transmembrane protein that mediates proton-coupled ferrous iron uptake, taking place in the duodenum and upper jejunum.[42] DMT1 only transports ferrous iron (Fe^{2+}) to the enterocyte; yet, most dietary entering the duodenum is in the ferric form (Fe^{3+}), as iron exists in the oxidized state at physiological pH. For this reason, insoluble ferric iron must be reduced to absorbable ferrous iron.[55] Gastric acid lowers

the pH environment within the proximal duodenum, reducing Fe^{+3} in the intestinal lumen by duodenal cytochrome B, a brush border ferric reductase enzyme on the enterocyte.[19,28,30] This reduction allows ferrous iron transport across the apical membrane of enterocytes, enhancing ferric iron uptake.[23] If the production of gastric acid is compromised, the absorption of iron is significantly reduced.[23]

Within the enterocyte, ferrous iron can be stored in mucosal ferritin in the cytoplasm or transferred across the basolateral membrane to the blood by ferroportin into systemic circulation.[55] Hephaestin oxidizes ferrous iron to ferric iron, taking place on the basolateral membrane. This enables the transportation of iron by transferrin, the main iron-binding protein in the blood that carries iron throughout the body to the cells or the bone marrow for erythropoiesis, which is the production of RBCs.[23,55] For those that are iron deficient, a feedback mechanism enhances iron absorption via hepcidin, while on the contrary, those who are iron-replete experience dampened iron absorption.[23,42] A generally accepted theory is that iron absorption is regulated by hepcidin binding to, internalizing and degrading ferroportin, which controls the movement of iron from the mucosal cell into the plasma.[23]

1.5. Biomarkers of Hematological Status

There are several biomarkers used to assess iron status and measure iron deficiency. The gold standard method to diagnose iron deficiency is the measurement of iron stores by way of a bone marrow aspiration test, but this is a very invasive procedure.[60,61] Ferritin or sTfR are more commonly used biomarkers for assessing iron status. Assessment of iron deficiency with interpretation of multiple indicators is ideal for in settings where it is feasible to do so.[45] Ideally,

a combination of hemoglobin, serum ferritin and sTfR measurement would be advantageous as it would reflect functional impairment, iron stores and tissue iron deficiency, respectively.[45] In reality, this approach may not be practical in all contexts.[45] The accurate determination of iron status is crucial for clinical diagnosis and population-level guidance of public health interventions.

1.5.1 Hemoglobin

Hemoglobin concentration is a biomarker used to diagnose anemia, and this indicator is often used as a proxy for iron deficiency anemia prevalence rates at the population level. Hemoglobin is a colour pigment, which can be measured by spectrophotometry using an automated hematology analyzer.[62] This method is considered the 'gold standard' because of the machine's standardized quality control checks and calibration methods. Fresh blood (4–6 hours from collection) is required for this analysis, limiting the feasibility of use in field settings where blood may need to be transported long distances to a laboratory.[62] Additionally, these machines are expensive and require trained technicians.

Hemoglobin or hematocrit tests are the only point-of-care tests available that can be easily performed in the field.[45] A portable hemoglobinometer, such as the HemoCue (HemoCue, Angelholm, Sweden), is often used in field settings, as it conveniently measures hemoglobin concentration in a finger prick capillary blood sample. Using hemoglobin data to infer iron deficiency anemia rates may not be appropriate for certain settings, such as Cambodia, where genetic hemoglobin disorders are thought to be the main contributor to the anemia burden.[22]

1.5.2 Ferritin

Recent WHO guidelines indicate that ferritin concentration is a useful biomarker of iron stores and recommend it for the diagnosis of iron deficiency in apparently healthy individuals and to adjust ferritin values for inflammation in populations with inflammation or infection.[27,50]

1.5.2.1 Structure and Function

Ferritin is the body's primary iron-storage protein and is critical to iron homeostasis. The ferritin molecule has an iron core surrounded by an intracellular hollow protein shell composed of 24 subunits.[42] Almost all cells in the body contain iron, but most ferritin is stored in the liver, spleen and bone marrow.[63,64] In the body, small amounts of ferritin are secreted back into the blood circulation by ferroportin when needed.[63] In the absence of inflammation, plasma or serum ferritin concentrations typically reflect an individual's total body iron stores.[65,66] Serum ferritin concentrations increase when iron stores are high and decrease when iron stores are low, making it a good indicator of an individual's iron stores.[66] Serum ferritin is an important biomarker of an individual's iron status, but it only represents a small fraction of the entire ferritin pool, as most ferritin iron is stored intracellularly.[42]

For healthy women, serum ferritin concentrations typically fall within the range of 15-150 µg/L.[42,50] According to the WHO's definition, ferritin levels <15 µg/L in apparently healthy non-pregnant women (age 20-59 years) are indicative of depleted iron stores, referred to as iron deficiency.[50] In individuals with inflammation or infection, a ferritin concentration <70 µg/L in non-pregnant women may indicate iron deficiency.[50] For menstruating women, ferritin levels exceeding 150 µg/L may indicate a risk of iron overload.

1.5.2.2   Measurement

Serum or plasma ferritin is routinely measured manually or with use of an automated high-throughput immunoassay.[50] In 2004, a simple sandwich enzyme-linked immunosorbent assay (s-ELISA) was introduced by Erhardt et al.[67] This s-ELISA conveniently measurements multiple biomarkers of iron, vitamin A and inflammation status, including ferritin, sTfR, CRP and AGP. Ferritin concentration is quantified using specific ferritin detection and capture antibodies.[67] This assay method is highly specific and sensitive compared to traditional detection methods. Moreover, the cost of analysis for all five measurements is $1/sample,[67] making it a popular method worldwide for nutritional biomarker assessment.

This s-ELISA may be advantageous over other methods as multiple biomarkers (ferritin, sTfR, CRP, AGP, retinol bind protein [RBP]) can be measured in one serum or plasma sample. This is useful in gathering information about inflammation markers, as ferritin and RBP are affected by inflammation, allowing for a comprehensive understanding of iron and vitamin A statuses at the population-level. For this method, only a small account of serum or plasma is necessary (50 µL)[42], allowing for a convenient and inexpensive alternative in a low-resource field setting or with children when there are limitations to venous blood collection. Ferritin has also been measured in dried plasma or serum spots in low- and middle-income field settings.[68]

1.5.2.3   Correcting Ferritin for Inflammation

Ferritin is an acute-phase protein and becomes elevated in the presence of inflammation or infection.[27] It is recommended that serum ferritin be measured with the concurrent measurement of inflammation markers.[27,50] The current global consensus is to adjust ferritin concentrations for

levels of inflammation using a linear regression statistical approach. The Biomarkers Reflecting Inflammation and Nutrition Determinants of Anemia (BRINDA) research group have proposed a statistical calculation for this approach with use of α-1-acid glycoprotein (AGP) and C-reactive protein (CRP), acute-phase proteins commonly measured for this adjustment.[27] CRP >5 mg/L is indicative of acute inflammation, and AGP >1 g/L is indicative of chronic inflammation.[69] Alternatively, in a scenario where the prevalence of inflammation is unknown but assumed to be high in WRA, it is suggested to raise the threshold to define iron deficiency from 15 μg/L to 70 μg/L to account for the effect of the suspected inflammation.[70]

1.5.3 Soluble Transferrin Receptor

Transferrin receptor is a protein found outside of cells that regulates iron uptake into the cell. The expression of transferrin receptor is relative to the cell's iron requirements. A soluble form of this receptor, soluble transferrin receptor (sTfR), is found in the serum again at levels relative to the transferrin receptors present in the body.[71] STfR is a biomarker of tissue iron stores reflecting the need for iron or increased erythropoietic activity. While a low ferritin concentration indicates depleted iron stores, conversely, sTfR concentrations increase when the body tissues are deficient in iron.[72] For non-pregnant WRA, a sTfR concentration >8.3 mg/L is reflective of tissue iron deficiency.[70]

As sTfR is a general marker of erythropoiesis, this measurement may be confounded by factors that affect erythropoiesis (other than iron status). In Cambodia, the measurement of sTfR may be confounded by the high prevalence of genetic hemoglobin disorders. It has been shown that Cambodian women with homozygous Hb EE disorder had elevated serum sTfR concentrations

compared to women with normal hemoglobin.[22] This limits the ability of sTfR to accurately reflect tissue iron deficiency.[72] Thus, it is only a reliable measure of tissue iron status when there are no other causes of altered erythropoiesis. Unlike ferritin, sTfR is not as significantly affected by inflammation and can be a useful indicator to distinguish iron deficiency anemia from anemia of chronic disease or inflammation.[72,73]

1.6. Iron Supplementation

There is strong evidence for the efficacy of and rationale for iron supplementation in iron-deplete women.[74] The delivery of iron by supplementation or food fortification has been shown to effectively prevent and treat iron deficiency in both high and low-income countries.[75] However, not all forms of anemia are caused by iron deficiency, and in situations when iron deficiency is not the primary cause of anemia, iron interventions such as supplementation or fortification would not likely be effective in reducing or preventing anemia.

In 2009, the WHO recommended intermittent iron and folic acid (IFA) supplementation (60 mg elemental iron weekly) for women of reproductive age in regions of the world where anemia prevalence ≥20%.[76] An updated complementary policy guideline, published in 2016, recommends daily IFA supplementation (30-60 mg elemental iron daily) for three consecutive months of the year among menstruating adolescents and women in regions where anemia prevalence is ≥40%.[77] The WHO bases these recommendations on the widely-accepted assumption that iron deficiency contributes to approximately 50% of the global burden of anemia.[77] As a result of these global policies, iron supplements are widely distributed to many countries across the globe. In 2019, UNICEF alone reported that they provided iron supplements

to ~13.4 million women within 42 countries worldwide (J. Debyser, UNICEF Supply Division Contracts Manager, email communication May 8, 2020).

1.6.1 Iron Supplementation in Cambodia

In 2011, Cambodia's Ministry of Health adopted the 2009 WHO iron supplementation guidelines into the National Policy and Guidelines for Micronutrient Supplementation to Prevent and Control Deficiencies in Cambodia.[78] Thereby recommending weekly supplementation of oral iron and folic acid (60 mg elemental iron and 2.8 mg folic acid) for all non-pregnant women of reproductive age until they become pregnant. Furthermore, in 2012, the Ministry of Planning introduced minimum standards, requiring the fortification of all fish and soy sauce with iron.[79]

Despite Cambodia's adopted policies in an effort to reduce nationwide anemia, the 2014 DHS of 11,000 women reported that only 3% of Cambodian women had iron deficiency, based on ferritin measurements, while 45% of women were anemic based on hemoglobin concentration measured by HemoCue. A cross-sectional survey in Prey Veng determined that approximately 30% of non-pregnant women in Cambodia have anemia, while only 2% have iron deficiency based on ferritin.[22] In a study of predominately anemic Cambodian women by my supervisor, Dr. Karakochuk, only 25% were responsive to 12 weeks of daily oral iron supplementation (60 mg elemental iron), as indicated by a hemoglobin increase of at least 10 g/L.[80] It was concluded that only 10% of women within the broader Cambodian population would benefit from the iron supplementation.[80] Of women enrolled in the study, a total of 78% of women were iron-replete at baseline (ferritin >15 µg/L), however, 74% had a genetic hemoglobin disorder. These are noteworthy findings and require further investigation, as if iron deficiency is not a principal

cause of anemia in Cambodia, then national-level policies and programs for anemia reduction and prevention may need to be reassessed, especially when the population has a high prevalence of hemoglobin disorders.

Based on these recent findings, the Cambodia Ministry of Health decided to put the iron supplementation program on hold, as the data indicates low iron deficiency prevalence amongst non-pregnant Cambodian women and a low proportion of women showing a hematological response to iron supplementation. Thus, there is an urgent need to determine appropriate iron supplementation at the population level and determine if there is a potential for harm when providing untargeted iron supplementation to non-pregnant women when national iron deficiency prevalence is very low.

1.6.2 Potential Harms of Iron Supplementation

If iron deficiency is not a major cause of anemia, then, at best, untargeted iron supplementation is a waste of resources; at worst, it could cause harm. What is key, with regard to iron supplementation, is an individual's iron status at baseline. If an apparently-healthy individual without inflammation is iron deficient, supplementing with iron is likely beneficial to improve iron stores. However, if an individual is iron-replete, has high inflammation levels or a severe genetic hemoglobin disorder, supplementation is not warranted and may be harmful.

As such, given the WHO policy and the massive global scope of implementation, there is an urgent need to determine if there is harm associated with untargeted iron supplementation. Cambodia's anemia prevalence in WRA is higher than 40%, while simultaneously, iron

deficiency prevalence appears to be low.[1] Moreover, the population has a high prevalence rate of genetic hemoglobin disorders.[1]

The Institute of Medicine has established dietary reference intakes (DRIs), intended for Canadian American populations, which indicate that non-pregnant women aged 19-50 years should not exceed a daily intake of 45 mg/day of elemental iron (tolerable upper intake limit [UL]) to safeguard against the experience of adverse side effects such as gastrointestinal pain.[81] To date, there is limited evidence regarding the safety of supplementation higher than the UL, beyond gastrointestinal discomfort. More importantly, most studies assessing the efficacy of iron supplementation in non-pregnant women have failed to evaluate harms beyond gastrointestinal side effects, such as cramping, nausea and diarrhea.

More research is needed to assess outcomes beyond gastrointestinal discomfort, as iron is a catalyst for oxidative and inflammatory reactions.[82,83] Consuming excess iron can result in unbound, free iron, called non-transferrin-bound iron (NTBI). The accumulation of circulating NTBI can increase reactive oxygen species production, leading to oxidative stress[84,85] and DNA and cellular damage.[85–88] Excess iron has additional negative consequences through its interaction with other trace elements (zinc and copper),[89–91] and is associated with diabetes, neuropathy, and some cancers.[92–94] Studies in infants and children have shown decreased growth,[95,96] impaired development,[97–99] and increased morbidity[28,96,100,101] in infants and children. Iron supplementation in malaria-endemic regions increases risks of infection and disease in young children.[28] Excess unabsorbed iron in the colon can also increase susceptibility to pathogen growth.[83,102–104] For example, in Kenyan children, both gut pathogen abundance and

gut inflammation were elevated with consumption of doses of iron that are 1/12 (8%) of the amount currently recommended for women.[83,104]

High-dose oral iron supplementation may pose an even greater risk to individuals with genetic hemoglobin disorders.[24] Iron homeostasis is regulated by a liver peptide called hepcidin.[105] Some types of hemoglobin disorders (such as homozygous hemoglobin EE or HbE/β-thalassemia disorders) cause ineffective RBC production and lower hepcidin expression, resulting in increased iron absorption and ineffective erythropoiesis.[24,106] This outcome of increased iron absorption occurs regardless of iron storage status, placing women with genetic hemoglobin disorders at higher risk of iron overload and toxicity. This is of relevant concern in Cambodia, as approximately 60% of the women are genetic hemoglobin disorder carriers.[1,21,22] Further, women in Cambodia have been exposed to numerous iron sources through untargeted national supplementation for women and the fortification of fish and soy sauce.[42] Therefore, assessing the potential harm of iron supplementation in Cambodian women with a high prevalence of genetic hemoglobin disorders is warranted.

Low et al. conducted a Cochrane review, including ten trials of women of reproductive age (total $n=3,273$) undergoing iron supplementation therapy for a 4-12 week duration. The authors concluded that daily oral iron supplementation reduces the prevalence of anemia (RR: 0.39 [95% CI: 0.25, 0.60]).[74] The findings from this systematic review were used to inform the 2016 WHO guidelines recommending daily iron and folic acid supplementation for menstruating women and adolescents girls for three consecutive months each year in countries with an anemia prevalence $\geq 40\%$.[77] The review failed to examine potential iron supplementation risks (e.g., iron overload,

gut dysbiosis).[74] It is imperative to weigh the evidence for both the benefits and harms of treatment and assessing consequences of deficiency and excess when drafting global guidelines. Iron homeostasis requires a careful balance, as both a deficiency and excess presence of iron can impair host immunity.[58]

1.7. Forms of Oral Iron Supplements

Challenges inherent in iron supplementation programs include those of supplement bioavailability, safety and tolerability.[107,108] This is because there is no standardization of dose or form of elemental iron supplements to prevent or treat iron deficiency and/or anemia. Iron bioavailability studies show that the form of iron supplement is just as important as the dose.[109]

With an increasing awareness of the potentially toxic effects of iron, more research is being devoted to identifying the lowest effective dose to prevent iron deficiency and iron deficiency anemia and investigating novel forms of iron supplements. The bioavailability of oral iron supplements display considerable variation as well as reported side effects. Common types of iron supplements include ferrous sulfate,[110] ferrous fumarate,[111] ferrous ascorbate,[112] carbonyl iron,[113] polymaltose iron,[112] and ferrous bisglycinate.[114]

1.7.1 Iron Salts

Iron salts are common forms of iron supplements used in iron deficiency treatment. Conventional iron salts include ferrous sulfate, ferrous fumarate, ferrous gluconate, ferrous ascorbate and ferrous glycine sulfate. These iron salts contain varying amounts of elemental iron; for example,

ferrous sulfate is 20% elemental iron by weight, ferrous fumarate, 33%; and ferrous gluconate, 12%.[115,116]

1.7.1.1   Ferrous Sulfate

Ferrous sulfate ($FeSO_4$) is one of the most widely available forms of iron supplements. Ferrous sulfate is an inexpensive, inorganic salt commonly used as a fortificant or supplement to prevent or treat iron deficiency.[117] Iron absorption from inorganic salts (including ferrous sulfate) is low; typically, <20% of iron is absorbed in the duodenum. The remaining amount passes unabsorbed into the colon,[118] which can contribute to the virulence and colonization of enteropathogens.[119–121] Ferrous sulfate has also been shown to irritate the stomach lining, causing gastrointestinal side effects, such as cramping, diarrhea, nausea, and constipation,[122,123] thus has the potential to negatively affect the adherence to iron supplementation.

1.7.2   Ferrous Bisglycinate

A chelated form of iron, ferrous bisglycinate, has become increasingly popular, as its' bioavailability is two to four times higher than conventional iron salts,[109,114,124–126] namely, ferrous sulfate. Moreover, ferrous bisglycinate has been associated with fewer gastrointestinal side effects than ferrous sulfate,[127–131] ferrous glycine sulfate,[132] ferrous fumarate,[131,133–135] and iron multi amino acid chelate (IMAAC).[133] This highly stable iron amino acid chelate ($C_4H_8FeN_2O_4$) is formed by binding a ferrous cation to two glycine molecules.[114] Due to its chemical composition (consisting of a covalently bounded iron molecule to an organic ligand, in this case, glycine), it is less prone to bind with common food substances. Therefore, there is less potential for the formation of insoluble compounds with iron absorption inhibitors, such as

metals, dietary fibre, phytates and phenols.[114] Phytates are found in cereal-based foods, including rice, which makes up a large portion of the Cambodian diet. Likewise, the fact that iron is bound to amino acids allows iron to be absorbed intact into the intestinal mucosal cells, where it then effectively disassociates from ferrous bisglycinate for distribution to body tissues.[132] This lessens the adverse side effects associated with direct exposure of iron to the intestinal lumen. Research has confirmed that ferrous bisglycinate is better absorbed in the intestine as compared to ferrous sulfate, ferrous fumarate, IMAAC and polymaltose iron.[133,136–138]

Mexican schoolchildren (n=200; aged 8-13 years) with iron deficiency without anemia (serum ferritin <12 µg/L and altitude adjusted Hb >120 g/L for children 12+ years and Hb >115 for <12 years) were randomized to receive 30 mg elemental iron as ferrous sulfate or ferrous bisglycinate for 12 weeks, with iron status follow-up occurring 1 week and 6 months post-supplementation. While both groups had significant increases in serum ferritin at both time points (P=0.001), ferritin was significantly higher in the ferrous bisglycinate group (P=0.028) than the ferrous sulfate group at 6 months.[114]

In a study by Makled et al. in Eygpt in 2019, 150 pregnant women with iron deficiency anemia (Hb 8-10.5 g/dL, and serum ferritin <15 µg/l) attending an antenatal care outpatient clinic, between 14-18 weeks' gestation, were randomized to 115 mg elemental iron daily as ferrous fumarate or 15 mg elemental iron daily as ferrous bisglycinate for 12 weeks.[135] At week 12, the prevalence of anemia was significantly lower in the ferrous bisglycinate group (16% [n=11/71], P=0.04) than in the ferrous sulfate group (30% [n=21/70]).[135]

Finally, an evaluation of daily iron supplementation in 145 Brazilian pregnant women (<20 weeks' gestation) compared 15 mg of ferrous bisglycinate to 40 mg ferrous sulfate.[128] At endline (30-40 weeks' gestation), iron deficiency prevalence was 31% in the ferrous bisglycinate group (mean ± SD serum ferritin was 14.3 ± 10.7 µg/L) and 55% in the ferrous sulfate group (mean ± SD serum ferritin was 10.8 ± 8.1 µg/L). Approximately 73% (*n=52/71*) of women in the ferrous bisglycinate group had adequate supplement intake (defined as consuming +13 weeks of daily iron supplements), while only 35% (*n=26/74*) of the ferrous sulfate group were considered to have adequate supplement intake. Of the women who did not have adequate intake in the ferrous sulfate group, factors reported to affect their compliance included taste (10% *n=5/48*) and gastrointestinal issues (42% *n=20/48*).[128]

Ferrous bisglycinate has been reviewed and approved for use as a source of iron in foods intended for the general population, including as a food supplement, by the Joint Food and Agriculture Organization/ World Health Organization Expert Committee on Food Additives (JECFA). It was reported as "suitable for use as a source of iron for supplementation and fortification, provided that the total intake of iron does not exceed the provisional maximum tolerable daily intake",[139] a maximum of 0.8 mg/kg body weight, a quantity of which none of the women in our study exceeded. Additionally, ferrous bisglycinate has been tested and proven safe as an iron supplement in Europe, by the European Food Safety Authority,[140] in the United States, by the US Food and Drug Administration,[141] and is listed as an approved form of iron in Health Canada's Natural Health Products Ingredients Database.[142]

1.8. Iron and the Gut

Iron is a growth-limiting nutrient, which is essential for numerous gut bacteria competing for unabsorbed dietary iron.[55,75,121,143] The acquisition of iron plays a vital role in the virulence and colonization of Shigella, Salmonella and E. coli, and other enteric gram-negative bacteria.[119,121,144] Pathogens who require iron for their growth have developed mechanisms for acquiring the metal from their environment by secreting siderophores, which are iron chelators, allowing for transportation across the cell membrane, facilitating iron uptake.[75] In response, humans have acquired mechanisms to stop iron-dependent pathogens from obtaining iron to protect against infection and illness.[75] Good bacteria in the gut, such as *Lactobacillus* species and *Bifidobacterium*, provide a vital 'barrier effect,' protecting against enteropathogens' colonization.[145] Beneficial commensal lactobacilli bacteria do not require iron but instead depend on manganese.[119] Hence, they do not increase at a proportional rate to the pathogenic bacteria in the presence of iron.[146] As only some kinds of bacteria utilize iron, an increase in supplemented iron, passing unabsorbed into the colon, may modify the colon's microbiota. This may also favour the growth of pathogenic bacteria over helpful bacteria.[143] Yet, very few studies have investigated the effect of iron supplementation on gut microbiota dynamics nor investigated different iron compounds with varying bioavailabilities.

1.8.1 Gut Inflammation

Gut inflammation can lead to an altered gut microbiota composition, known as dysbiosis, and fosters an environment where virulent enteropathogens can emerge. Although gut inflammation results from many different conditions, the fundamental environmental changes in the inflamed gut are consistent across different situations. The most widely used biomarkers for measuring gut

inflammation and monitoring inflammatory bowel disease are fecal calprotectin (FC), C-reactive protein (CRP) and fecal lactoferrin. CRP is not a specific marker for gut inflammation; CRP may be elevated for other reasons happening at the systemic level, such as infection or inflammation taking place outside the intestines. Comparatively, fecal calprotectin is specific to the gastrointestinal tract and is appropriate for use as a measure of gut inflammation.[147] Lactoferrin is an iron-binding protein expressed in breast milk and saliva, produced by activated neutrophils.[83] Fecal lactoferrin has been shown to be sensitive and specific for detecting inflammation in chronic inflammatory bowel disease (IBD).[148]

1.8.1.1   Calprotectin

Calprotectin is a 36-kDa calcium and zinc-binding protein found in neutrophils. Increased calprotectin concentrations are observed in the blood and stool in individuals with diseased or inflamed conditions.[149] Calprotectin derives its' name from combining its calcium-binding properties (cal) and antimicrobial activity (protect). During an active inflammatory event in the gastrointestinal tract, neutrophils translocate and migrate to the site of the injury; neutrophils then release calprotectin, resulting in elevated levels in the stool. Fecal calprotectin has a higher specificity for gut inflammation than other systemic inflammatory markers because it solely measures local gut inflammatory processes.[150]

Fecal calprotectin is typically measured via an enzyme-linked immunosorbent assay (ELISA) in stool samples to detect gut inflammation.[150,151] It is stable at room temperature for seven days and stable once frozen for 18 months.[151,152] This makes stool collection and transportation feasible and straightforward, even in rural, low-resource settings. While the detection of elevated

fecal calprotectin is sensitive to intestinal mucosal inflammation, it is not specific. This is because numerous infectious and inflammatory processes may contribute to elevated fecal calprotectin concentrations. In other words, increased fecal calprotectin levels can be interpreted as inflammation-specific but not disease-specific.

High within-day variability has been observed in Ulcerative Colitis patients.[153] Thus, it has been suggested to sample the first bowel movement in the morning.[154] Additionally, in healthy individuals, there appears to be a variation in fecal calprotectin with age, with elderly (60+ years) people having higher levels than individuals aged 10-59 years.[155,156] However, it has been reported that infants and children under ten years of age have higher fecal calprotectin levels than adults.[156,157]

In a multicenter, prospective, case-control study of 478 participants, BÜHLMANN fCAL ELISA showed to be reliable in predicting inflammation (as detected by endoscopy, which is the gold standard for detecting mucosal inflammation).[158] The data support the following recommendation for diagnostic interpretation of fecal calprotectin concentration results: Fecal calprotectin concentration values below 80 µg/g are not indicative of inflammation present in the gastrointestinal tract. Values between 80 and 160 µg/g represent mild inflammation. Lastly, calprotectin values above 160 µg/g are indicative of active disease with gastrointestinal tract inflammation.[151,158] When differentiating IBS (functional disorder) from IBD (disease), the BÜHLMANN fCAL ELISA shows to have high sensitivity (93.3%) with a specificity of 72.3%, at a cut-off of <80 µg/g, while having balanced sensitivity (84.4%) and specificity (85.4%) at a cut-off of >160 µg/g. There is no consensus for appropriate calprotectin cut-offs values, resulting

in a wide variation in calibration between calprotectin kits manufacturers.[158] The manufacturer recommended cut-offs for distinguishing IBS versus IBD range from 50 µg/g – 100 µg/g).[159]

1.8.2 Iron Supplementation and Gut Inflammation

To our knowledge, no studies have reported the effect of iron supplementation on gut inflammation in non-pregnant women; however, the effects of iron fortification on gut inflammation have been examined in children. A systematic review of iron supplementation in children indicates that oral iron supplementation was associated with a small yet significant increase in diarrhea risk.[160] Two double-blind, randomized controlled trials in Kenyan infants (*n*=115; aged six months) were the first studies to investigate the effects of iron-containing micronutrient powders (MNPs) on the infant gut microbiota and inflammation.[83] Infants in the first trial consumed MNP with 12.5 mg iron as ferrous fumarate or a non-iron MNP daily for four months. In the second trial, infants were given an MNP of a highly bioavailable low dose iron (2.5 mg) as NaFeEDTA or a non-iron MNP daily for four months. With study results combined, the infant's microbiomes at baseline were highly colonized with enteropathogens. In the iron groups, there was a significant increase in abundance of enterobacteria (chiefly *Escherichia/Shigella* and pathogenic *E. coli)* compared to the groups that had no iron at four months. There was also a significantly higher concentration of fecal calprotectin at endline in the iron group than the no-iron group.

In a study by Zimmermann et al. in Côte d'Ivoire in 2010, 139 children aged 6-12 years received 20 mg a day iron-fortified biscuits or placebo biscuits over six months.[161] The iron-fortified group exhibited an increase in fecal calprotectin concentrations, which was correlated with an increase in the number of fecal enterobacteria.[161] In 2017, Tang et al. showed that in 33 non- or

mildly anemic Kenya infants aged six months, iron micronutrient powder (MNP) fortification did not significantly impact inflammation markers.[162] Authors state the lack of significant inflammation change may have to do with the high concentrations of inflammation at baseline or a sample size too small to detect change. An investigation of Swedish iron-replete infants (*n=53*; aged six months) given either low iron-fortified formula (1.2 mg Fe/day), iron-rich formula (6.6 mg Fe/day) or iron-free formula with ferrous sulfate liquid drops (6.6 mg Fe/day) for 1.5 months confirmed the findings as discussed above.[163] Although calprotectin did not differ between groups, in the high-iron formula and iron-drops groups, *Clostridium difficile* correlated positively with fecal calprotectin.[163]

Conversely, Dostal et al. conducted a randomized controlled trial in South African children (*n=73*; aged 6-11 years) who were given 50 mg ferrous sulfate for 38 weeks and found that iron supplementation did not affect gut inflammation.[164] The children in this study only had mild iron deficiency, lived in a malaria-free environment and lived in households with access to clean tap water.[164] Further, Tang et al. randomized breastfed infants (*n=44*; aged 9-12 months) from Denver, Colorado to receive iron therapy (6 mg/kg/d) + placebo or iron (6 mg/kg/d) + vitamin E (18 mg/d) for 8 weeks. In this study, iron supplementation did not produce a significant inflammatory response in the gut. The contrasting findings from these two studies in South Africa and the United States compared to the other African and Swedish trials suggest that the local context is critical; the effects of iron supplementation on the gut profile likely depend on environmental factors. The risk of adverse effects of iron supplementation on the gut may increase when hygiene standards are low, and the presence of enteropathogens are high.

It should be emphasized that the provision of iron to women who have iron deficiency or IDA has longstanding proven benefits; providing iron to a population with a high prevalence of either condition is assumed to benefit the alleviation of iron deficiency IDA. However, there is emerging evidence showing iron *fortification* in infants and children in developing countries, who are predominately iron-replete, may cause adverse effects in the gut microbiome and increase the presence of enterobacteria and inflammation. There is a lack of high-quality data investigating the potential harms of untargeted iron *supplementation* in all populations, let alone iron-replete people or those with genetic hemoglobin disorders. Untargeted iron supplementation (either if iron deficiency is not the chief cause of anemia or in areas with a high prevalence of genetic hemoglobin disorders) is additionally concerning because the most common form of supplementation, ferrous sulfate, is poorly absorbed.

1.9. Study Aim, Rationale and Significance

This research aims to understand the best form and dose of iron that should be prescribed to women to effectively increase ferritin concentrations and reduce the potential for harm. The primary aim of my thesis is to assess the non-inferiority of 18 mg iron as ferrous bisglycinate (experimental) compared to 60 mg iron as ferrous sulfate (standard treatment) on mean ferritin concentrations in non-pregnant women at 12 weeks. I will also determine if 60 mg iron as ferrous sulfate (as per the 2016 WHO global policy) increases biomarkers of potential harm (gut inflammation, as measured by fecal calprotectin) in women at 12 weeks, compared to placebo or 18 mg iron as ferrous bisglycinate. My goal is that these findings inform WHO guidelines for iron supplementation and contribute to the evidence base for safe and effective supplementation practices for women globally.

1.10.   Research Objectives and Hypotheses

1.10.1   Research Question

Does providing a lower dose of a more bioavailable form of iron (18 mg iron as ferrous bisglycinate) effectively increase ferritin concentrations and reduce inflammation in the gut, compared to the standard 60 mg iron as ferrous sulfate?

1.10.2   Research Objectives, Hypotheses and Outcome Measures

Objective 1: To assess the non-inferiority of 18 mg iron as ferrous bisglycinate (experimental) compared to 60 mg iron as ferrous sulfate (standard treatment), on mean ferritin concentrations at 12 weeks.

- Hypothesis: Women who receive 12 weeks of 18 mg daily oral iron as ferrous bisglycinate will have similar (non-inferiority defined by being within a 20 µg/L margin) ferritin concentrations compared to women who receive 12 weeks of 60 mg daily oral iron as ferrous sulfate.

- Outcome Measure: Serum ferritin concentration (µg/L) at 12 weeks, adjusted for inflammation using α-1-acid glycoprotein (AGP, g/L) and C-reactive protein (CRP, mg/L)[27]

Objective 2: To determine if 60 mg iron as ferrous sulfate (as per the 2016 WHO global policy) increases gut inflammation in women at 12 weeks compared to 18 mg iron as ferrous bisglycinate or placebo.

- Women who receive 12 weeks of 60 mg daily oral iron as ferrous sulfate will have higher

levels of gut inflammation than women who receive 18 mg daily oral iron as ferrous bisglycinate or placebo.

- Outcome Measure: Fecal calprotectin concentration (mg/kg stool) at 12 weeks

Chapter 2: Research Design and Methods

My thesis objectives are the two primary objectives of a 12 week double-blind, randomized controlled trial. In this chapter, a report of the research design and methodology for the trial and a detailed description of my objectives' analysis methods are provided.

2.1. Study Design

This study was a 12 week double-blind, three-arm, randomized, placebo-controlled trial comparing two forms and doses of oral iron supplementation in non-pregnant Cambodian women. The trial was conducted between December 2019 and May 2020. Collaborators and partners in Cambodia include The Ministry of Health, UNICEF, and Helen Keller International. Other key stakeholders involved in this research include international and nutrition organizations, village community members and study participants.

2.2. Study Participants and Setting

The study was conducted in three different health centre catchment areas of Kampong Thom province: Srayov, Prey Kuy and Tboung Krapeu, as seen in **Figure 2**. Kampong Thom was the selected location for this study because of its proximity to the National Institute of Public Health Laboratory for sample processing and the high prevalence of anemia among women as based on the 2014 DHS.[1] In addition, our implementing partner, Helen Keller International Cambodia, has extensive experience working with women of reproductive age on nutrition research and programs in Kampong Thom province and had a strong relationship with the local Health District office.
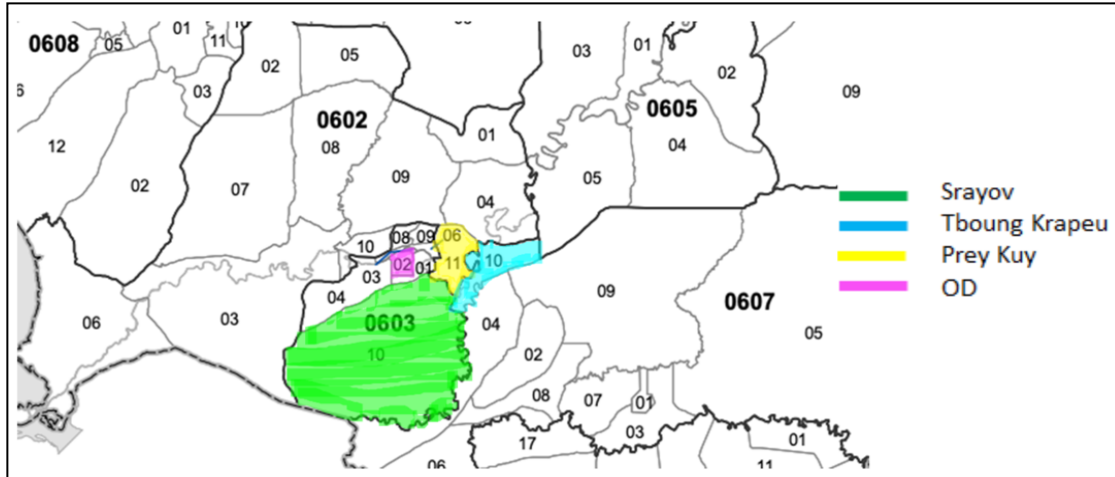
Figure 2. Map of Kampong Thom province divided into health centres with three study health centres highlighted; OD, Occupational District Central Health Centre (study team office base).

2.2.1 Inclusion and Exclusion Criteria

The trial aimed to recruit non-pregnant women of reproductive age in the chosen districts within Kampong Thom. To be eligible to participate in the study, women had to meet the following inclusion criteria:

    i.   Non-pregnant women 18-45 years old

    ii.   Apparently healthy

    iii.   Consent to participate in the study and provide blood and flocked swab fecal samples

    iv.   Expect to reside in the study location for the study period

Study exclusion criteria included:

    i.   Any known illness or disease

    ii.   Pregnancy (self-reported)

    iii.   Taking antibiotics, non-steroidal anti-inflammatory drugs, dietary supplements, or vitamin and mineral supplements in the previous 12 weeks.

2.3. Randomization

A convenience sampling method was used based on the selected villages. Health centre volunteers and study staff informed women about the study. They were invited to attend a small gathering at the health centre if they were interested in hearing more about the study or participating. Also, study staff went to individual homes inviting women to participate in the study. Eligible and consenting women were enrolled, and each participant was assigned a unique identification (ID) number. A total of 480 women were randomized (1:1:1) at the health centre level by a computer-generated random list to one of the three intervention arms in equal allocation (n=160 in each group).

Allocation was concealed from the trial investigators, research staff, and participants to prevent bias. An external elected official kept the allocation sequence concealed and confidential. The allocation codes remained blinded during all stages of the trial until after the analyses of the primary outcome were completed and shared with the research team. Preliminary unblinding of the trial was only to be executed by an independent researcher as deemed necessary in the case of a severe adverse event.

2.4. Interventions

Participants were randomized to one of three treatment arms and instructed to consume one capsule daily for 12 weeks:

    i.   60 mg elemental iron as ferrous sulfate

    ii.   18 mg elemental iron as ferrous bisglycinate

    iii.   placebo (microcrystalline cellulose) containing no elemental iron

Two global WHO policies currently recommend daily oral iron supplementation (60 mg iron as iron salts [e.g., ferrous fumarate or ferrous sulfate]) in women living in areas of high anemia prevalence, such as Cambodia.[77] As ferrous bisglycinate has a higher bioavailability (2-4x greater than iron salts), an 18 mg dose of ferrous bisglycinate (18 mg) one-third the amount of the 60 mg ferrous sulfate was chosen.

2.4.1 Manufacturing

The supplement formulations were manufactured in May 2019 by Natural Factors, Factors Group (Coquitlam, British Columbia, Canada). The supplements were packaged in child-safe screw-cap bottles containing 88 capsules. The gel capsules were identical in size and colour, and they only differed by the supplement container's identification code. The Factors Group Study Coordinator was solely responsible for blinding and did not share the identification code with any study team member until the primary objective's statistical analysis was complete. In both Canada and Cambodia, all investigators, research staff and participants were blinded to the group allocations. The elemental iron content of the supplements was tested as a quality control measure (**Table 1**). Three capsules were analyzed from each bottle, and the mean elemental iron content (mg/capsule) was reported.

Table 1. Elemental iron content of study supplements as per an external laboratory analysis

Blinded Bottle	Mean elemental iron content (mg/capsule)	SD
Ferrous sulfate	57	0.052
Ferrous bisglycinate	17	0.11
Placebo	0	0.00050

2.5. Ethical Approval & Participant Consent

Ethical approval was granted by the University of British Columbia Clinical Research Ethics Board (H18-02610) and the National Ethics Committee for Health Research in Cambodia (273-NECHR). Before the start of recruitment, the trial was registered at clinicaltrials.gov (NCT04017598), and a study protocol was published in a peer-reviewed journal.[165]

2.6. Procedures

One day of training was conducted for the research team before recruitment, and two days of training were conducted to prepare for baseline and endline stages. Four full-time data collectors were instructed during recruitment training on how to screen for eligibility and complete the consent form. For baseline and endline training, and a total of eight data collectors were involved and instructed on how to complete the questionnaire, deliver and provide instruction to participants on how to use the stool collection kits, take anthropometric measurements and collect the stool with the collection kits. The questionnaire was modified and improved during several rounds of trial. One monitoring training session provided guidance on conducting monitoring interviews and completing the monitoring form and capsule count. Training was conducted in English, with Khmer translation offered as needed.

Screening and recruitment began on December 10, 2019, and enrollment continued on a weekly-rolling basis for five weeks, with the first baseline blood collection visit on January 19, 2019. The final data collection was completed on May 10, 2020. Four research staff, who were trained and experienced local Cambodian field staff, were based in Kampong Thom province for the trial duration. These four staff oversaw participants' enrollment, study implementation, data and

sample collection, monitoring and follow-up counselling with participants. The study coordinator and study manager were present for baseline and endline data and specimen collection.

Communication about the study began with consultations with the village chief, followed by community sensitization sessions. Women were recruited by means of convenience sampling and invited to screening to assess eligibility. Women were informed at the initial screening visit about the study protocol, as well as eligibility criteria. Women who were enrolled attended a total of six visits over the course of the study (**Table 2**), including screening, baseline, day 1, 7, 35, and 84 time-points. Research staff and health centre volunteers at the village level conducted regular monitoring visits throughout the 12 weeks, as needed. Adverse events (i.e., a new illness, worsening of a coexisting illness) were monitoring and recorded at each visit.

Table 2. Schedule of assessment time points and study visits

Visit (V)	Screening	V1	V2	V3	V4	V5
		Assessment Time Points				
Time per session, hours		0.25	0.25	0.25	0.25	0.5
Study day		0	1	7	35	84
Enrolment and Randomization						
Eligibility assessment	X					
Randomization			X			
Implementation						
Questionnaire		X				X
Flocked rectal swab & stool collection kit provided		X			X	
Blood collection			X			X
Flocked rectal swab & stool sample collected			X			X
Adverse event reporting				X	X	X
Review symptoms diary				X	X	X
Supplementation						
Capsule distribution			X		X	
Capsule count				X	X	X

2.6.1 Study Visits

Screening Period

The study protocol and consent form were verbally communicated in Khmer (the local language). A printed copy translated in Khmer was also offered to each woman. Women were enrolled once they provided their written signature as consent.

Visit 1 (Baseline, Day 0)

Trained research staff administered the baseline questionnaire via electronic tablets. The baseline questionnaire captured socio-demographic and health data and information on factors associated with hematological and gastrointestinal indicators (e.g., a reported history of infection, diarrhea, enteropathogens and medication, antibiotics or non-steroidal anti-inflammatory drug use). The final baseline questionnaire included participant information, health, food, water and sanitation, anthropometrics sections (**Appendix A).** Height and weight were also collected. Women were provided with a flocked rectal swab and a container for stool sample collection and were asked to return both samples in the collection kit on visit 2. They received instructions on how to collect both samples (via a simple Khmer translated infographic) and were provided printed copies to take home. Find examples of the English version of "How to collect stool" in **Appendix B.** Stool samples were collected within seven days of Day 0 if the sample could not be collected on the actual scheduled visit 2 (i.e., if the woman was unable to pass stool or was not available on the day of the visit).

Visit 2 (Day 1)

Trained phlebotomists from the Cambodian National Institute of Public Health Laboratory in

Phnom Penh were responsible for the collection of the non-fasting venous blood sample (15ml) during the morning visit. Research staff collected the flocked rectal swab and stool sample container. Supplement bottles had labels with a serial number and coloured sticker identifying the treatment (A, B or C), along with instructions and a contact number translated in Khmer. The women received their bottle of capsules and were instructed to consume them daily, with adequate fluid and ideally with dinner. Women were provided with straightforward instructions to lessen the possibility of adverse gastrointestinal side effects. The next visit took place six days later.

Visit 3 (Day 7)

At this visit, the research staff completed the monitoring form, recording intervention adherence by counting the remaining capsules, recorded reported side effects, as large doses of iron often cause gastrointestinal discomfort, and encouraged continued adherence. Additionally, they reviewed the symptoms diary, which was completed if necessary, at visits 3, 4, and any other time participants contacted research staff to meet together because they had any questions or concerns. They returned 28 days later for the fourth visit.

Visit 4 (Day 35)

At this visit, research staff counted the remaining capsules, documented supplement regimen adherence, recorded reported side effects, reviewed the symptoms diary, and encouraged continued adherence. Women were provided with a second flocked rectal swab and container for stool sample collection, of which they were asked to collect both samples 24 to 48 hours before visit 5. Research staff returned 56 days later for the final study visit but additional monitoring

visits were conducted on an as-needed basis to ensure supplementation compliance.

Visit 5 (Day 91, Endline)

Trained phlebotomists collected non-fasting blood samples. Flocked rectal swab samples and stool samples were also collected. Stool samples were received within seven days of Day 84 if the sample could not be collected on the exact day. The research staff were responsible for conducting a capsule count, recording supplement adherence, recording reported side effects, reviewing the symptoms diary, and administering the endline questionnaire to collect additional data from each woman.

Study endline visits took place in April and May 2020, during the coronavirus disease 2019 (COVID-19) pandemic. The study team took every precaution to maintain the health of everyone involved in this research project.

Helen Keller International Cambodia, our NGO partner, implemented various health and safety protocols to ensure the health of study staff, health centre staff, study participants and the community. All participants were provided masks, gloves and hand sanitizer. Social distancing was practiced at the health centre and limited seating was offered, with ample space to stand outside. Home follow-ups were given as an option if women did not want to come to the clinic.

Study End (Day 92+)

After completing the 12-week study, nutrition education was provided in small groups to all

women by trained research staff on good dietary sources of iron and practices to prevent anemia. This educational session was offered to all women, regardless of intervention arm. At each data collection time point, women received a small remuneration, such as a sarong, krama (scarf), laundry soap bar or two cans of fruit juice.

2.7. Blood and Fecal Collection and Processing

Venous blood samples (15 ml total) were collected using three Becton Dickinson vacutainers on day 1 and 84: one 6 mL trace-element free tube and two tubes containing anticoagulant EDTA (6 mL and 2 mL). A flowchart outlining the blood collection and processing protocol for the laboratory staff can be found in **Appendix C**. Blood processing was conducted at Cambodia's National Institute of Public Health Laboratory.

A flocked rectal swab (FecalSwab™, Copan Italia, Italy) and a user-friendly stool sample collection kit were used to collect a stool sample at baseline and endline. The stool specimen was collected into a leak-proof receptacle with a screw cap. At the laboratory, the fresh stool was extracted into the BÜHLMANN CALEX® Cap containing a medium for gut inflammation analyses and stored at -80°C until analysis or shipment.

Blood vacutainers, flocked rectal swabs and stool samples were placed on ice and transported within 4-6 hours to the National Institute of Public Health Laboratory in Phnom Penh for further processing or storage.

2.8. Blood Analyses

Blood was shipped on dry ice to Dr. J. Erhardt's VitMin lab in Germany, where serum samples for serum ferritin (μg/L) and inflammatory markers, α-1-acid glycoprotein (AGP, g/L) and C-reactive protein (CRP, mg/L), were analyzed simultaneously via an established, low-cost competitive sandwich ELISA.[67]

Ferritin concentrations were adjusted for the presence of inflammation based on CRP and AGP concentrations with use of the globally-accepted BRINDA method.[27]

A complete blood count was performed by an automated hematology analyzer (Sysmex XN-1000) at the National Institute of Public Health Laboratory in Phnom Penh on the day the blood samples were brought back from the field to the laboratory. Measured analytes included: hemoglobin (g/L), mean corpuscular volume (MCV, fl), mean corpuscular hemoglobin concentration (MCHC, g/dL), red blood cell distribution width (RDW, %), and reticulocyte count (% of RBC). See **Table 3** for blood analyte methods.

Table 3. Summary of blood analytes and methods of analysis

Analyte	Methods
Serum ferritin	Sandwich-enzyme linked immunosorbent assay (s-ELISA)
α-1-acid glycoprotein (AGP)	
C-reactive protein (CRP)	
Hemoglobin	Automated hematology analyzer (Sysmex XN-1000)
Mean Corpuscular Volume	
Red Cell Distribution	
Reticulocyte Count	

2.9. Fecal Analyses

Stool samples arrived on ice at the National Laboratory in Cambodia and were immediately extracted and frozen at -80 ºC. The samples were extracted from neat stool (a plain sample in no specified medium) using the CALEX® Cap extraction device (Bühlmann Laboratories AG, Schönenbuch, Switzerland), following the manufacturer's instructions, allowing for convenient and efficient extraction of stool specimens and providing high sample stability. The sampling pin of the CALEX® Cap device was dipped into the stool sample and removed 3-5 times in different places on the stool sample to ensure the grooves are filled. In the circumstance of liquid stool or if it would not stick in the grooves of the sampling pin, 10 µL of stool sample was pipetted directly into the device, following the manufacturer's protocol. Next, the sampling pin was reintroduced into the 5mL of extraction buffer and vortexed vigorously, resting and repeating until the grooves were free of stool before proceeding. The CALEX® Cap at 3000g was then centrifuged for 5 minutes. As outlined in the BÜHLMANN fCAL® protocol, extracted calprotectin obtained by the BÜHLMANN CALEX® Cap is stable for three days at room

temperature, six days at 2-8 °C and 18 months at -20°C. Thus, centrifuged extracts were frozen

at -20 °C until shipped to Canada (August 3, 2020) and were frozen immediately upon receipt at

-80 °C in Dr. Karakochuk's UBC laboratory until analysis. The Stool Extraction Protocol

(reproduced from the BÜHLMANN CALEX® Cap instruction manual) provided to laboratory

staff can be found in **Appendix D.**

The BÜHLMANN fCAL® ELISA selectively measures calprotectin in stool extracts by the

sandwich enzyme-linked immunosorbent assay method (Bühlmann Laboratories AG, Basel,

Switzerland) and is FDA 510(k) cleared. The microtiter ELISA plate is coated with a

monoclonal capture antibody highly specific to calprotectin.[151] The microtiter plate wells were

loaded with the stool sample extracts from the CALEX® Caps, controls for determining the

acceptability of the ELISA run, and calibrators. The standard working range of 10-600 µg/g was

chosen as it was suspected that not all samples would have exceptionally elevated fecal

inflammation and possibly no inflammation at baseline. Samples were initially diluted 1/50, and

if calprotectin levels were out of the standard curve range, the samples were analyzed again at

1/400 dilution. After a half-hour incubation at room temperature and washing and shaking steps,

a detection antibody conjugated to horseradish peroxidase (HRP) identified the calprotectin

molecules by binding to the capture antibody. Following further incubation and washing, the

tetramethylbenzidine (TMB) substrate is added (change to blue colour); this is followed by a

stopping reaction (change to yellow colour). The absorption was measured at 450 nm on a

SpectraMax Microplate absorbance reader. Calprotectin concentrations were measured in µg/g of

feces and were determined using the calibration curve generated from the measured calibrator

values.[151]

Quality control (QC) criteria were stated in the datasheet for the kit lot number for the 10-600 µg/g working range were expressed as means: low 38 (3 SD: 21-56) µg/g and high 159 (3 SD: 111-207) µg/g; and 30-1800 µg/g working range: low 114 (3 SD: 63-168) µg/g and high 477 (3 SD: 333-621) µg/g fecal calprotectin. The mean was taken for duplicate samples as long as both values fell within the standard curve. For the lower working range (10-600 µg/g), the intra-assay precision was 1.9-8.0% CV, and within-lab accuracy was 5.5-14.0% CV. For the higher working range (30-1800 µg/g), the intra-assay precision was 1.7-5.8% CV, and within-lab accuracy was 3.1-9.4% CV.

2.10. Data Analysis

2.10.1 Sample Size Calculation

The sample size calculation for this trial was based on our primary objective of a non-inferiority comparison of ferritin concentrations at 12 weeks between the two iron groups in consultation with a biostatistician. WHO has reported that a clinically meaningful change in ferritin concentration in response to an iron intervention would be ±0.2 SD units;[70] and data from Dr. Karakochuk's 2015 trial indicated the SD of ferritin to be ~50 µg/L after 12 weeks of supplementation with 60 mg elemental iron as ferrous sulfate;[80] therefore, a margin of 20 µg/L was chosen to determine non-inferiority. To detect a non-inferiority margin of 20 µg/L for ferritin, with 80% power and $\alpha=0.05$, $n=140$ women in each group were required. To account for a 15% loss to follow-up (a conservative estimate based on our previous trial),[80] we rounded up to $n=160$ women in each group, totalling $n=480$ women.

2.10.2 Statistical Analysis

Data cleaning involved the assessment of the data for abnormalities and unreasonable values (e.g., incorrectly inputted), as well as deviations from the protocol (e.g., missing data). A descriptive analysis was performed to examine participant characteristics, such as the prevalence of iron deficiency and anemia. Descriptive statistics were computed for each outcome at baseline and endline. Mean and standard deviation (SD) for each group are reported for normality distributed continuous data and median and interquartile range (IQR) for skewed continuous data. For categorical data, n (%) was calculated.

The primary outcome was serum ferritin concentrations (µg/L) at 12 weeks. Serum ferritin was corrected for inflammation based on CRP and AGP inflammation markers.[27] The primary analysis was based on a non-inferiority framework and compared mean serum ferritin concentrations (95% CI) between the two iron intervention groups, ferrous sulfate and ferrous bisglycinate, at 12 weeks. A margin of 20 µg/L was used to define non-inferiority. A generalized linear mixed-effect model was used to predict the mean ferritin concentrations (95%CI), controlling for baseline ferritin (fixed effects) and health centre clusters (random effects).

An intention-to-treat (ITT) analysis of the data was used for all outcomes. All participants were analyzed according to their allocated treatment group, regardless of supplementation protocol compliance. A 'per-protocol' analysis was performed on a subset of women who completed the 12-week trial, had baseline and endline samples available for analysis, and were adherent to the supplement regime (consumed ≥80% of capsules over the trial period).

We also tested for an interaction effect to determine if a woman's baseline iron status modified the effect of the iron interventions on ferritin concentrations in our generalized linear mixed-effect model (interaction terms: baseline inflammation-adjusted serum ferritin concentration and treatment). A *P*-value >0.05 defined a significant interaction effect.

Our secondary outcome was fecal calprotectin concentrations. In this analysis, we compared mean fecal calprotectin concentrations at 12 weeks across the three groups using a generalized linear mixed-effects model, controlling for baseline fecal calprotectin and health centre clusters.

We also tested for an interaction effect to determine if a woman's baseline gut inflammation status modified the effect of the iron interventions on 12 week fecal calprotectin concentrations in our generalized linear mixed-effect model (interaction terms: baseline gut inflammation status and treatment). A *P*-value >0.05 defined a significant interaction effect.

Tests were two-tailed, and values were considered statistically significant at *P*-values <0.05. All statistical analyses were performed using STATA IC v.16.0 (StataCorp., Texas, USA).

Chapter 3: Results

3.1. Recruitment and Follow-up

During recruitment, 1,286 women were screened for eligibility from a total of 25 villages within three health centre districts. Of these 1,286 women, $n=577$ did not meet the inclusion criteria: $n=213$ reported consumption of iron-containing contraceptives, $n=104$ were not within the age range of 18-45 years, $n=51$ were pregnant, and $n=48$ had plans to migrate outside of the province during the study. A total of $n=229$ declined to participate. The $n=480$ women who were deemed eligible and consented to participate were randomized; $n=140$ were from Prey Kuy health centre, $n=187$ were from Srayov and $n=153$ were from Tboung Krapeu, as shown in **Table 4.**

A total of $n=441$ (92%) women remained in the study until completion at 12 weeks: $n=421/480$ (88%) women provided a blood sample and $n=434/480$ (90%) women provided a stool sample at 12 weeks. The attrition rate at 12 weeks ranged from 6-10% and did not significantly differ across groups. Reasons for loss to follow-up were that women moved to work in a different province, refused blood collection due to fear, or chose to discontinue the intervention. Participant flow, follow-up and attrition are depicted in **Figure 3**. Primary outcome data (ferritin concentration) were available for $n=421/480$ (88%) of the women at 12 weeks. Samples for the second outcome (fecal calprotectin) were available for $n=385/480$ (80%) of the women at 12 weeks; unfortunately, this was limited by the number of BÜHLMANN CALEX® caps that were available in Cambodia for this collection method.

Table 4. Enrollment at the health centre and village level

Health Centre	Village	*n*
Prey Kuy	Bendey	12
Prey Kuy	Kampong Krabao	2
Prey Kuy	Pren	16
Prey Kuy	Prey Kuy	18
Prey Kuy	Prey Kuy (K)	30
Prey Kuy	Sambour	15
Prey Kuy	So Chey	43
Prey Kuy	Svay Klok	4
Srayov	Chambak	37
Srayov	Kampong Samroung	10
Srayov	Kamraeng	23
Srayov	Por Sen Snay	13
Srayov	Por Ta Un	13
Srayov	Pramat Dei	3
Srayov	Srayov Cherng	26
Srayov	Srayov Tboung	42
Srayov	Trapaing Veng	20
Tboung Krapeu	Aom Pus	10
Tboung Krapeu	Chong Da	9
Tboung Krapeu	Kal Meak	24
Tboung Krapeu	Mneav	27
Tboung Krapeu	Panha Chi	10
Tboung Krapeu	Pok Yuk	14
Tboung Krapeu	Por Khav	14
Tboung Krapeu	Roka	45
Total in all three health centres:		**480**

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                    ┌─────────────────────────────────────┐
                    │ Assessed for Eligibility (n=1,286)   │
                    └─────────────────────────────────────┘
                                     │
                                     │        ┌──────────────────────────────────┐
                                     │        │ Excluded (n=806)                 │
                                     ├───────▶│  • Not meeting inclusion         │
                                     │        │    criteria (n=577)              │
                                     │        │  • Declined to participate       │
                                     │        │    (n=229)                       │
                                     │        └──────────────────────────────────┘
                                     ▼
                    ┌─────────────────────────────────────┐
                    │ Enrolled and Randomized (n=480)      │
                    └─────────────────────────────────────┘
```

Assessed for Eligibility (*n*=1,286)

Excluded (*n*=806)
- Not meeting inclusion criteria (*n*=577)
- Declined to participate (*n*=229)

Enrolled and Randomized (*n*=480)

Allocated to 60 mg Ferrous Sulfate group (*n*=161)
Blood (*n*=161)
Stool *(n*=153)

Allocated to 18 mg Ferrous Bisglycinate group (*n*=158)
Blood (*n*=158)
Stool *(n*=148)

Allocated to placebo group (*n*=161)
Blood (*n*=161)
Stool *(n*=155)

Lost to follow-up (*n*=6)
- Migrated (5)
- Refused sample collection (1)

Discontinued intervention (*n*=7)
- Withdrew (7)

Lost to follow-up (*n*=12)
- Migrated (10)
- Refused sample collection (2)

Discontinued intervention (*n*=4)
- Withdrew (3)
- Pregnant (1)

Lost to follow-up (*n*=7)
- Migrated (4)
- Refused sample collection (3)

Discontinued intervention (*n*=3)
- Withdrew (3)

Completed 12 week trial (*n*=148)
Blood (*n*=145)
Stool (*n*=147)

Completed 12 week trial (*n*=142)
Blood (*n*=131)
Stool (*n*=139)

Completed 12 week trial (*n*=151)
Blood (*n*=145)
Stool (*n*=148)

Figure 3. Participant flow chart

3.2. Baseline Participant Characteristics

Table 5 presents the baseline characteristics of enrolled study participants. Overall, women had a mean ± SD age of 33.6 ± 7.3 years. Overall, 87% (*n=416*) of women were married and 52% (*n=251*) completed primary school as their highest education level. Eleven percent (*n=55*) of women were nulliparous and the mean ± SD number of children a woman had was 2.1 ± 1.3. Mean ± SD body mass index (BMI) among women in this study was 23.5 ± 3.8, with 60% (*n=286*) having a normal BMI (18.5-24.9 kg/m^2) and 25% (*n=118*) of women were classified as obese (≥30.0 kg/m^2). The mean ± SD household size was similar across the three groups, with 4.7 ± 1.5 people living in a home.

In our study population, 24% (*n=113/480*) were breastfeeding and 40% (*n=190/480*) were currently taking birth control to prevent pregnancy. Of the 425 women who had previously given birth, 93% (*n=396*) reported they had consumed iron and folic acid supplements during their previous pregnancy for any duration. Of the 396 women who reported consuming IFA during their previous pregnancy, 71% (*n=283*) reported consuming ≥90 tablets, which is the current Cambodian public health recommendation.[78] Overall, 44% (*n=213/480*) of women had taken antibiotics in the past year, with 61% (*n=130/213*) of these women consuming them 3+ in the past year. Nearly all women (99%; *n=474/480*) reported to consume fermented fish paste (*prahok*), and 43% (*n=207/480*) used iron-fortified fish sauce. Baseline characteristics among women did not significantly differ across groups for any of the reported variables.

Table 5. Baseline participant characteristics of enrolled Cambodian women by intervention group

	Ferrous Sulfate	Ferrous Bisglycinate	Placebo
Total enrolled, *n* (%)	161 (33.5%)	158 (33%)	161 (33.5%)
Woman's age, y, mean ± SD	33.8 ± 7.1	33.7 ± 7.0	33.4 ± 7.8
Household size, mean ± SD	4.7 ± 1.5	4.8 ± 1.6	4.6 ± 1.4
Marital status			
Single	13/161 (8%)	8/158 (5%)	20/161 (12%)
Married	137/161 (85%)	142/158 (90%)	137/161 (85%)
Widowed	6/161 (4%)	3/158 (2%)	1/161 (<1%)
Separated/divorced	5/161 (3%)	5/158 (3%)	3/161 (2%)
Completed education			
None	19/161 (12%)	13/158 (8%)	10/161 (6%)
Primary	81/161 (50%)	81/158 (51%)	89/161 (55%)
Lower secondary	39/161 (24%)	46/158 (29%)	33/161 (21%)
Upper secondary	17/161 (11%)	15/158 (10%)	23/161 (14%)
Higher education/university	5/161 (3%)	3/158 (2%)	6/161 (4%)
BMI (*kg/m^2*)	23.4 ± 3.8	23.4 ± 3.8	23.7 ± 3.9
Underweight <18.5	13/161 (8%)	15/158 (10%)	10/161 (6%)
Normal weight 18.5-24.9	97/161 (60%)	97/158 (61%)	92/161 (57%)
Overweight 25-29.9	1/1611 (7%)	9/158 (6%)	18/161 (11%)
Obese ≥30	40/161 (25%)	37/158 (23%)	4/161 (26%)
Parity			
0	20/161 (12%)	13/158 (8%)	22/161 (14%)
1-2	97/161 (60%)	83/158 (53%)	82/161 (51%)
3-4	41/161 (25%)	57/158 (36%)	52/161 (32%)
≥5	3/161 (2%)	5/158 (3%)	5/161 (3%)
Currently breastfeeding	40/141 (28%)	43/145 (30%)	30/139 (22%)
Currently use birth control	56/161 (35%)	70/158 (44%)	64/161 (40%)
Reported consuming IFA during last pregnancy[1]	129/141 (91%)	134/145 (92%)	133/139 (96%)
Took antibiotics in last year	66/161 (41%)	69/158 (44%)	78/161 (48%)
Use iron-fortified fish sauce	61/161 (38%)	77/158 (49%)	69/161 (43%)
Eat fermented fish paste (*Prahok*)	159/161 (99%)	156/158 (99%)	159/161 (99%)

Total *n=480*. Values are n (%) or mean ± (SD). IFA, iron and folic acid supplementation.
[1]IFA consumption for any duration or dose. Of the women who reported parity ≥1, 71% (*n=283/396*) reported consuming ≥90 tablets, as per Cambodia's current recommendations.

A total of 14% ($n=65/480$) of women experienced daily symptoms of diarrhea or any form of

gastrointestinal upset, with 11% ($n=51/480$) reporting episodes once per week and 29%

($n=138/480$) once per month (**Table 6**). Lastly, 47% ($n=226/480$) of women reported to

never experience these adverse symptoms. The most commonly experienced symptom across

all three arms was stomach pain (68%; $n=173/254$).

Table 6. Baseline participant gastrointestinal characteristics of Cambodian women by intervention group

	Ferrous Sulfate	Ferrous Bisglycinate	Placebo
Total enrolled, n (%)	161 (33.5%)	158 (33%)	161 (33.5%)
Experience gastrointestinal upset			
Everyday	22/161 (14%)	24/158 (15%)	18/161 (11%)
Once a week	29/161 (18%)	19/158 (12%)	29/161 (18%)
Once a month	49/161 (30%)	50/158 (32%)	61/161 (38%)
Never	61/161 (38%)	65/158 (41%)	53/161 (33%)
Diarrhea[1]	19/81 (23%)	26/82 (32%)	22/91 (24%)
Constipation[1]	14/81 (17%)	9/82 (11%)	11/91 (12%)
Stomach pain[1]	50/81 (62%)	59/82 (72%)	64/91 (70%)
Bloating[1]	27/81 (33%)	31/82 (38%)	38/91 (42%)
Nausea[1]	44/81 (54%)	38/82 (46%)	51/91 (56%)
Vomiting[1]	19/81 (23%)	12/82 (15%)	22/91 (24%)
Pain passing stool[1]	7/81 (9%)	5/82 (6%)	9/91 (10%)
Blood in stool[1]	8/81 (10%)	3/82 (4%)	3/91 (3%)

Total $n=480$. Values are n (%).
[1]Of the 254 women who reported to experience gastrointestinal upset at least once a month.

A total of 93% (*n=444/480*) of women had at least one animal living in the home; the most

common animal was a dog (86%; *n=382/444*) (**Table 7**). Fifty-eight percent (*n=278/480*) of

participants had one or more animals living outside of the home, with the most common

reported as chickens (77%; *n=214/278*). Regarding household water access, 54%

(*n=257/480*) of women reported a hand pump as their main source, and most (91%;

n=437/380) household toilet facilities flushed to a septic tank.

Table 7. Baseline household characteristics of Cambodian women by intervention group

	Ferrous Sulfate	Ferrous Bisglycinate	Placebo
Total enrolled, *n* (%)	161 (33.5%)	158 (33%)	161 (33.5%)
Household water source			
Hand pump	84/161 (52%)	85/158 (54%)	88/161 (55%)
Ring well	40/161 (25%)	38/158 (24%)	36/161 (22%)
Pond/river	16/161 (10%)	19/158 (12%)	20/161 (12%)
Bottled water	20/161 (12%)	14/158 (9%)	15/161 (9%)
Household toilet facility			
Flush to septic tank	151/161 (94%)	142/158 (90%)	144/161 (89%)
No facility (bush or field)	9/161 (6%)	14/158 (9%)	16/161 (10%)
Animal(s) living in the home	144/161 (89%)	147/158 (93%)	153/161 (95%)
Dog[1]	125/144 (87%)	128/147 (87%)	129/153 (84%)
Cat[1]	44/144 (31%)	41/147 (28%)	43/153 (28%)
Chicken[1]	103/144 (72%)	108/147 (74%)	118/153 (77%)
Animal(s) living outside the home	94/161 (58%)	89/158 (56%)	95/161 (59%)
Chicken[2]	69/94 (73%)	71/89 (80%)	74/95 (78%)
Cow[2]	37/94 (39%)	33/89 (37%)	28/95 (30%)
Duck[2]	9/94 (10%)	14/89 (16%)	13/95 (14%)

Total *n=480*. Values are n (%).
[1]Of the 444 women who had animals living inside their home.
[2]Of the 278 women who had animals living outside their home.

Baseline hematological, nutrition and inflammation markers are presented in **Table 8.**

Baseline mean ± SD hemoglobin concentration was 128.9 ± 11.8 g/L. Baseline median (IQR)

unadjusted serum ferritin concentration was 80.7 (43.1, 117.3) µg/L; inflammation-adjusted

serum ferritin concentration was 69.5 (39.2, 105.1) µg/L. Baseline median (IQR) unadjusted

serum sTfR concentration was 5.6 (4.7, 6.8) mg/L; inflammation-adjusted serum sTfR

concentration was 5.4 (4.6, 6.4) mg/L. Lastly, the baseline median (IQR) fecal calprotectin

concentration was 67 (30, 174) µg/g.

Table 8. Baseline hematological, nutrition, inflammation markers in enrolled Cambodian women by intervention group

	Ferrous Sulfate	Ferrous Bisglycinate	Placebo
Total enrolled, *n* (%)	161 (33.5%)	158 (33%)	161 (33.5%)
Hematological indicators			
Hemoglobin, g/L	128.6 ± 11.0	128.7 ± 12.5	129.5 ± 11.9
MCV, fL	81.2 ± 7.9	82.0 ± 8.0	81.8 ± 8.0
MCHC, g/dL	32.9 ± 11.0	32.9 ± 12.5	32.9 ± 10.1
RDW, %	13.5 ± 1.8	13.7 ± 1.9	13.5 ±1.6
Ferritin, µg/L,			
Unadjusted Ferritin, µg/L	88.7 (44.1, 116.1)	74.1 (40.6, 121.0)	80.1 (44.2, 119.4)
Adjusted Ferritin[1], µg/L	74.1 (40.4, 106.1)	65.6 (36.7, 105.0)	66.5 (41.8, 104.1)
sTfR, mg/L			
Unadjusted sTfR, mg/L	5.7 (5.0, 6.7)	5.8 (4.6, 7.0)	5.5 (4.7, 6.5)
Adjusted sTfR[1], mg/L	5.6 (4.7, 6.4)	5.5 (4.6, 6.8)	5.2 (4.5, 6.2)
Systemic inflammation markers			
AGP, g/L	0.57 (0.46, 0.75)	0.60 (0.44, 0.76)	0.61 (0.49, 0.83)
CRP, mg/L	0.48 (0.11, 1.42)	0.43 (0.13, 1.23)	0.57 (0.05, 2.34)
Gut inflammation marker			
Fecal calprotectin µg/g[2]	67.1 (28.1, 171.1)	63.9 (30.0, 149.8)	72.4 (32.2, 221.2)

Total *n*=480. Values are mean ± SD or median (IQR). AGP, α-1-acid glycoprotein; CRP, C-reactive protein; Hb, hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; RDW, red cell distribution width; sTfR, soluble transferrin receptor.
[1] Serum ferritin and sTfR values were corrected for inflammation using Namaste et al. regression methodology.[69]
[2] *n*=456

Table 9 presents the frequencies of iron deficiency, anemia, systemic and gut inflammation among non-pregnant Cambodian women of reproductive age. Anemia prevalence was 17% at baseline (*n=80/480*), based on the cut-off for non-pregnant women (hemoglobin <120 g/L). Of those women who had anemia, 69% (*n=55/80*) had mild anemia (hemoglobin 110-120 g/L), 29% (*n=23/80*) had moderate anemia (hemoglobin 80-110 g/L), and 2% (*n=2/80*) had severe anemia (hemoglobin <80 g/L). Among the 80 women with anemia at baseline, 29% (*n=23/80*) had normocytic anemia (hemoglobin <120 g/L and MCV 80-98 fL) and 71% (*n=57/80*) had microcytic anemia (hemoglobin <120 g/L and MCV <80 fL). Further, 62.5% (*n=50/80*) of women presented with hypochromic cells (MCHC <33 g/dL) and 37.5% (*n=30/80*) presented with normochromic cells (MCHC 33-36 g/dL).

Iron deficiency prevalence was 6% (*n=30/480*) based on inflammation-adjusted serum ferritin <15 µg/L and 9% (*n=40/480*) based on inflammation-adjusted soluble transferrin receptor (sTfR) >8.3 mg/L. Moreover, only 3% (*n=16/480*) of women had iron deficiency anemia defined as inflammation-adjusted ferritin and hemoglobin <120 g/L, and 5% (*n=22/480*) defined as inflammation-adjusted sTfR and hemoglobin <120 g/L.

Fifty-five percent of women (*n=252/456*) had no gut inflammation (fecal calprotectin <80 µg/g), 18% (*n=83/456*) had moderate inflammation (80-160 µg/g), and 27% (*n=121/456*) had high inflammation (80-160 µg/g).

Table 9. Baseline prevalence rates of anemia, iron deficiency, systemic inflammation and gut inflammation by intervention group

	Ferrous Sulfate	Ferrous Bisglycinate	Placebo
n	161 (33.5%)	158 (33%)	161 (33.5%)
Anemia prevalence			
Anemia, Hb <120 g/L	23/161 (14%)	26/158 (16%)	31/161 (19%)
Anemia severity, among those with anemia:			
Mild, 110-119 g/L	17/23 (74%)	17/26 (65%)	21/31 (68%)
Moderate, 80-110 g/L	5/23 (22%)	8/26 (31%)	10/31 (32%)
Severe, <80 g/L	1/23 (4%)	1/26 (4%)	0/31 (0%)
Anemia type, among those with anemia:			
Microcytic Hypochromic	15/23 (65%)	11/23 (43%)	13/31 (43%)
Microcytic Normochromic	5/23 (22%)	7/23 (27%)	6/31 (19%)
Normocytic Hypochromic	1/23 (4%)	4/23 (15%)	6/31 (19%)
Normocytic Normochromic	2/23 (9%)	4/23 (15%)	6/31 (19%)
Iron deficiency prevalence			
ID, ferritin[1] <15 μg/L	8/161 (5%)	12/158 (8%)	10/161 (6%)
ID, sTfR[1] >8.3 mg/L	17/161 (11%)	15/158 (10%)	12/161 (7%)
Iron Deficiency Anemia prevalence			
IDA, ferritin[1] <15 μg/L and Hb <120 g/L	4/161 (3%)	8/158 (5%)	4/161 (3%)
IDA, sTfR[1] >8.3 mg/L and Hb <120 g/L	10/161 (6%)	7/158 (4%)	5/161 (3%)
Systemic Inflammation			
Acute inflammation, CRP >5 g/L	6/161 (10%)	8/158 (13%)	24/161 (15%)
Chronic inflammation, AGP >1 g/L	15/161 (9%)	12/158 (8%)	25/161 (16%)
Gut Inflammation, fecal calprotectin μg/g			
No inflammation, <80	86/153 (56%)	84/148 (57%)	82/155 (53%)
Moderate inflammation, 80-160	27/153 (18%)	29/148 (19%)	27/155 (17%)
High inflammation, >160	40/153 (26%)	35/148 (24%)	46/155 (30%)

Total *n*=480. Values are n (%). AGP, α-1-acid glycoprotein; CRP, C-reactive protein; Hb, hemoglobin; ID, iron deficiency; IDA, iron deficiency anemia; sTfR, soluble transferrin receptor.
[1] Serum ferritin and STfR values were corrected for inflammation using Namaste et al. regression methodology.[69]

3.3. Adherence

On each follow-up visit, research staff conducted a capsule count to measure compliance. At the final visit, the team tallied the number of capsules that were remaining in the bottles. Compliance was calculated by dividing the number of reported capsules consumed by the total number of capsules that women were asked to consume over the 12 weeks of the study. Women were defined as adherent if they consumed ≥80% of the capsules. Adherence rates were 60%, 61% and 64% for ferrous sulfate, ferrous bisglycinate and placebo, respectively and did not differ by intervention group (chi-square, P=0.725).

3.4. Adverse Side Effects

Over the 12 weeks of the trial, a total of 17% ($n=73/441$) of women reported adverse side effects; with 16% ($n=23/148$) for ferrous sulfate, 16% ($n=22/142$) for ferrous bisglycinate and 19% ($n=28/151$) for the placebo group, as presented in **Table 10**. The proportion of women who reported any adverse side effects at 12 weeks did not differ by intervention group (chi-square, $P=0.72$). Of the 73 women who reported any adverse side effects, 22% ($n=16/73$) reported fever, 19% ($n=14/73$) reported cramping, 11% ($n=8/73$) reported nausea, and 7% ($n=5/73$) reported diarrhea (defined as three or more loose bowel movements in 24 hours). When non-gastrointestinal side effects were excluded, such as fatigue and fever, only 6% ($n=28/456$) of women overall reported side effects directly related to gastrointestinal side effects, with 6% ($n=9/148$) for ferrous sulfate, 8% ($n=11/142$) for ferrous bisglycinate and 5% ($n=28/151$) for the placebo group.

Table 10. Adverse side effects reported by enrolled Cambodian women by intervention group

Adverse Events	Ferrous sulfate	Ferrous bisglycinate	Placebo
n, (%)	148 (34%)	142 (32%)	151 (34%)
Women who reported any adverse effects	23/148 (16%)	22/142 (16%)	28/151 (19%)
Type of adverse side effect, among those who reported any adverse effects:			
Stomach cramping	4/23 (14%)	6/22 (27%)	4/28 (17%)
Constipation	0/23 (0%)	0/22 (0%)	1/28 (4%)
Diarrhea	1/23 (4%)	2/22 (9%)	2/28 (7%)
Nausea	4/23 (17%)	3/22 (14%)	1/28 (4%)
Headache	1/23 (4%)	1/22 (5%)	6/28 (21%)
Fatigue	5/23 (22%)	3/22 (14%)	5/28 (18%)
Fever	6/23 (26%)	4/22 (18%)	6/28 (21%)
No gastrointestinal side effect	125/148 (93%)	131/142 (92%)	143/151 (95%)

Total $n=441$. Values are n (%).

3.5. Baseline and 12 Week Ferritin and Change in Iron Deficiency and Iron Deficiency Anemia Prevalence Over 12 Weeks

At baseline, median (IQR) inflammation-adjusted serum ferritin concentrations in the ferrous sulfate, ferrous bisglycinate and placebo groups were 74.1 (40.4, 106.1) µg/g, 65.6 (36.7, 105.0) µg/g and 66.5 (41.8, 104.1) µg/g, respectively (**Table 8**).

At 12 weeks, median (IQR) inflammation-adjusted serum ferritin concentrations in the ferrous sulfate, ferrous bisglycinate and placebo groups were 100.5 (69.3, 133.9) µg/L, 75.9 (46.1, 113.3) µg/L and 70.5 (42.4, 110.7) µg/L, respectively.

Overall, iron deficiency (inflammation-adjusted serum ferritin <15 µg/L) decreased from 6% (*n*=30/480) to 2% (*n*=10/421) at 12 weeks. In the ferrous sulfate group, the prevalence of iron deficiency decreased from 5% (*n*=8/161) to 1% (*n*=1/141) over the 12 weeks. For ferrous bisglycinate, the prevalence of iron deficiency decreased from 8% (*n*=12/158) to 1% (*n*=1/131) at 12 weeks. In the placebo group, the prevalence of iron deficiency did not change, from 6% (*n*=10/161) at baseline to 6% (*n*=8/146) at 12 weeks.

Overall, IDA (inflammation-adjusted ferritin <15 µg/L and hemoglobin <120 g/L) decreased from 3% (*n*=16/480) to 2% (*n*=7/421) at 12 weeks. In the ferrous sulfate group, the prevalence of IDA decreased from 5% (*n*=4/161) to 0% (*n*=0/144) over the 12 weeks. For ferrous bisglycinate, the prevalence of IDA decreased from 5% (*n*=8/158) to 1% (*n*=1/131) at 12 weeks. Whereas, in the placebo group, the prevalence of IDA increased from 3% (*n*=4/161) to 4% (*n*=6/146) over the 12 weeks.

3.6. Serum Ferritin Concentrations at 12 Weeks

3.6.1 Primary Outcome Non-Inferiority Analysis

The primary outcome was a non-inferiority comparison of mean inflammation-adjusted serum ferritin concentrations between the two iron intervention groups (ferrous sulfate and ferrous bisglycinate) at 12 weeks. A generalized linear mixed-effect model (intention-to-treat) was used to predict marginal means of inflammation-adjusted ferritin concentrations (95% CI), controlling for baseline ferritin (fixed effects) and health centre clusters (random effects).

Adjusted mean differences between the two iron intervention groups found for 12 week inflammation-adjusted serum ferritin concentrations are presented in **Table 11**. The mean difference (95% CI) in predicted marginal mean ferritin concentrations for 60 mg ferrous sulfate group compared to 18 mg ferrous bisglycinate was 14.6 µg/L (7.6, 21.6; P<0.0001). Additionally, the mean difference (95% CI) for ferrous sulfate vs placebo was 20.8 µg/L (14.0, 27.7; P<0.0001) and the mean difference (95% CI) for ferrous bisglycinate vs placebo was 6.2 µg/L (-0.7, 13.2; P=0.1). Results from the non-inferiority per-protocol analysis (where only those who completed the trial and consumed >80% of capsules were included) did not differ from the intention-to-treat analysis with the ferrous sulfate group having a predicted marginal mean ferritin concentration of 18.0 µg/L (8.7, 27.3; P<0.0001) greater than the ferrous bisglycinate group. Therefore, the intention-to-treat and per-protocol analyses had similar outcomes.

Table 11. Adjusted mean difference (95% CI) in inflammation-adjusted serum ferritin concentrations between ferrous sulfate and ferrous bisglycinate

Comparison	n	Adjusted mean difference (95% CI), µg/L	P-value
Intention-to-treat, ferritin,[1] µg/L			
Ferrous sulfate vs. ferrous bisglycinate	480	14.6 (7.6, 21.6)	<0.0001
Per-protocol analysis,[2] ferritin,[1] µg/L			
Ferrous sulfate vs. ferrous bisglycinate	263	18.0 (8.7, 27.3)	<0.0001

A generalized linear mixed-effect model was used to predict marginal mean difference of inflammation-adjusted ferritin concentrations (95% CI) in the two iron interventions, controlling for baseline ferritin (fixed effects) and health centre clusters (random effects). Post-hoc (Bonferroni) adjusted P-values for multiple comparisons are reported.

[1]Serum ferritin values were corrected for inflammation using Namaste et al. regression methodology.[69]

[2]Per-protocol analysis where dropouts and those who consumed <80% of capsules were excluded

The mean difference (95% CI) in predicted marginal mean ferritin concentrations for ferrous sulfate vs ferrous bisglycinate (14.6 [7.6, 21.6] µg/L, *P*<0.0001) is depicted in **Figure 4**. Here, the lower bound of the mean adjusted mean difference confidence interval (CI) (7.6 µg/L) is to the right of zero and the upper bound of the CI (21.6 µg/L) crosses Δ (the non-inferiority margin; 20 µg/L). In this circumstance when assessing the non-inferiority of ferrous bisglycinate, our findings are declared as inconclusive (non-inferiority cannot be confirmed).[166] In other words, the CI of the mean difference in ferritin concentrations between the two iron intervention arms cannot exclude the possibility that the true treatment difference is less than Δ.[167]

95% CI for the difference in means of serum ferritin concentration µg/L
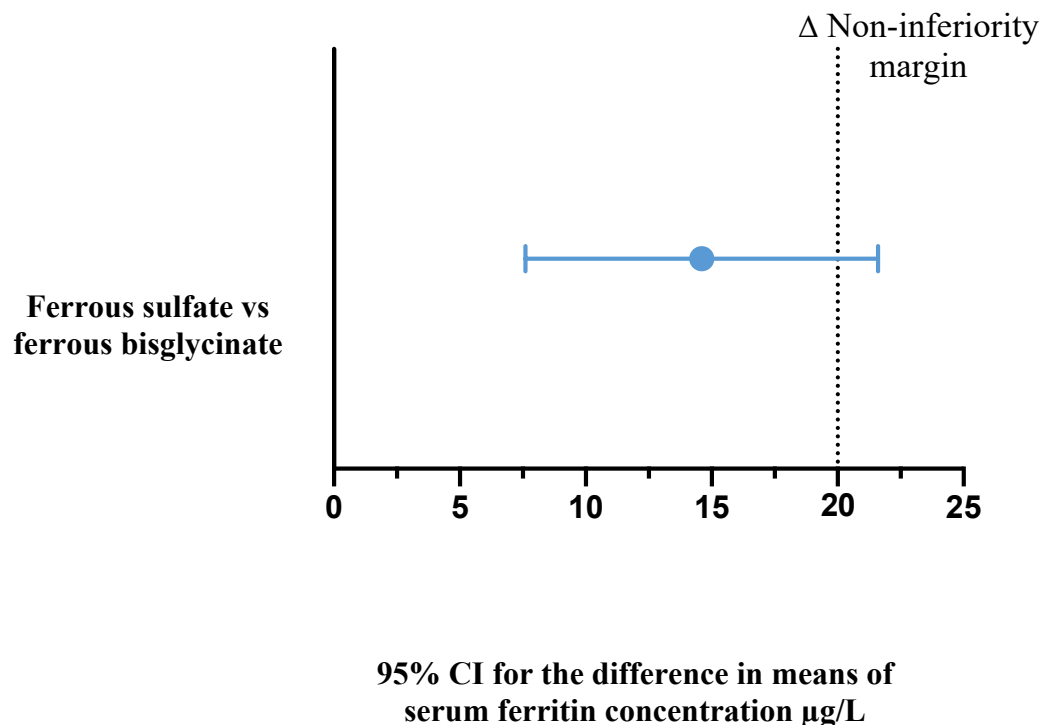
Figure 4. Difference in adjusted means (CI 95%) in predicted mean serum ferritin concentrations between the two iron interventions (ferrous sulfate vs ferrous bisglycinate)

3.6.2 Serum Ferritin Superiority Analysis

I also examined mean ferritin concentrations at 12 weeks across the three interventions using a superiority approach. Again, a generalized linear mixed-effect model (intention-to-treat) was used to predict marginal means of inflammation-adjusted ferritin concentrations (95% CI), controlling for baseline ferritin (fixed effects) and health centre clusters (random effects). Bonferroni-adjusted pairwise comparisons were made across the three groups. Mean (95% CI) serum ferritin concentration at 12 weeks was significantly higher in the ferrous sulfate group (98.6 [94.7,102.6] µg/L, $P<0.001$) as compared with both ferrous bisglycinate (84.0 [79.9, 88.2] µg/L) and placebo groups (77.8 [73.9, 81.7] µg/L); ferrous bisglycinate and placebo groups were not statistically different from each other (**Table 12**). There was no significant interaction between baseline iron status and treatment group found for 12 week serum ferritin status ($P>0.05$) (interaction terms: baseline inflammation-adjusted serum ferritin concentration and treatment).

Using the superiority approach, I also conducted a per-protocol analysis which restricted the analysis to only women who were 80% adherent to the trial supplement regime. Results from the per-protocol analysis were similar to the intention-to-treat analysis with women in the ferrous sulfate having significantly higher serum ferritin concentrations (102.5 [97.2, 107.8] µg/L, $P<0.001$) at 12 weeks than ferrous bisglycinate (84.5 [79.0, 90.0] µg/L) and placebo (77.2 [72.1, 82.3] µg/L) groups.

Table 12. Inflammation-adjusted serum ferritin concentrations at 12 weeks in enrolled Cambodian women by intervention group

	n	Ferrous sulfate	Ferrous bisglycinate	Placebo	*P*-value
Primary outcome, intention-to treat analysis, ferritin,[1] µg/L					
Baseline ferritin, median (IQR)	480	74.1 (40.4, 106.1)	65.6 (36.7, 105.0)	66.5 (41.8, 104.1)	0.69
12-week ferritin, median (IQR)	421	100.5 (69.3, 133.9)	75.9 (46.1, 113.3)	70.5 (42.4, 110.7)	<0.001
12-week GLM-adjusted ferritin	421	98.6 [94.7, 102.6] [a]	84.0 [79.9, 88.2] [b]	77.8 [73.9, 81.7] [b]	<0.001
Per-protocol analysis,[2] ferritin,[1] µg/L					
12-week GLM-adjusted ferritin	263	102.5 [97.2, 107.8] [a]	84.5 [79.0, 90.0] [b]	77.2 [72.1, 82.3] [b]	<0.001

All values are median (IQR) or marginal means [95% CI]. GLM, generalized linear mixed-effects model. A generalized linear mixed-effects model was used to predict marginal means [95% CI] of ferritin concentration at 12 weeks for each group with adjustments for baseline ferritin values and health centre clusters. Post-hoc (Bonferroni) adjusted *P*-values for multiple comparisons are reported.
[a-b] Values with a different superscript letter in each row are statistically different (P<0.05).
[1] Serum ferritin values were corrected for inflammation using Namaste et al. regression methodology.[69]
[2] Per-protocol analysis where dropouts and those who consumed <80% of capsules were excluded.

3.7. Baseline and 12 Week Fecal Calprotectin and Change in Gut Inflammation Prevalence Over 12 Weeks

At baseline, median (IQR) fecal calprotectin concentrations in the ferrous sulfate, ferrous bisglycinate and placebo groups were 67 (28, 171) µg/g, 64 (30, 150) µg/g, and 72 (32, 221) µg/g, respectively (**Table 8**).

At 12 weeks, median (IQR) fecal calprotectin concentrations in the ferrous sulfate, ferrous bisglycinate and placebo groups were 56 (23, 80) µg/g, 50 (22, 96) µg/g, and 48 (22, 138) µg/g, respectively.

Overall, the prevalence of women with gut inflammation (fecal calprotectin >80 µg/L, indicating moderate or elevated inflammation) decreased from 45% (n=204/456) to 31% (n=120/382) from baseline to the end of the 12 week intervention period. Whereas the rate of those with low/no inflammation detected in the gut (fecal calprotectin <80 µg/L) increased from 55% (n=252/456) at baseline to 69% (n=262/382) at 12 weeks.

Within the intervention groups, the prevalence of gut inflammation (fecal calprotectin >80 µg/L, indicating moderate or elevated inflammation) for the ferrous sulfate group decreased from 44% (n=67/153) to 26% (n=34/133), the ferrous bisglycinate group prevalence decreased from 43% (n=64/148) to 34% (n=41/122) and decreased for the placebo group from 47% (n=73/155) to 35% (n=45/127). Whereas the prevalence rates of those with low or no inflammation detected in the gut (fecal calprotectin <80 µg/L) for the ferrous sulfate group increased from 56%

(n=86/153) to 74% (n=99/133), ferrous bisglycinate group increased from 57% (n=84/148) to 66% (n=81/122) and increased for the placebo group from 53% (n=82/155) to 65% (n=82/127).

3.8. Fecal Calprotectin Concentration at 12 Weeks

The secondary outcome in this trial was a comparison of mean fecal calprotectin concentrations across the three groups at 12 weeks. A generalized linear model was used to predict marginal mean (95% CI) fecal calprotectin concentrations across the three intervention groups at 12 weeks, controlling for baseline fecal calprotectin concentrations (fixed effects) and health centre (random effects). Bonferroni-adjusted pairwise comparisons were made across groups.

Marginal mean (95% CI) fecal calprotectin concentrations at 12 weeks were not significantly different across the three intervention groups: 153 (96, 210) µg/g, 137 (76, 197) µg/g, and 135 (76, 193) µg/g, in the ferrous sulfate, ferrous bisglycinate and placebo groups, respectively (**Table 13**).

Table 13. Fecal calprotectin concentrations at 12 weeks in enrolled Cambodian women by intervention group

	n	Ferrous sulfate	Ferrous bisglycinate	Placebo	*P*-value
Secondary outcome, intention-to treat analysis, fecal calprotectin, µg/g					
Baseline fecal calprotectin, median (IQR)	456	67 (28, 171)	64 (30, 150)	72 (32, 221)	0.38
12-week fecal calprotectin, median (IQR)	382	56 (23, 80)	50 (22, 96)	48 (22, 138)	0.78
12-week GLM-adjusted fecal calprotectin	382	153 [96, 210] [a]	137 [76, 197] [a]	135 [76, 193] [a]	1.00
Per-protocol analysis,[1] fecal calprotectin, µg/g					
12-week GLM-adjusted ferritin	238	175 [94, 257] [a]	143 [61, 226] [a]	161 [82, 240] [a]	1.00

All values are median (IQR) or marginal means [95% CI]. GLM, generalized linear mixed-effects model. A generalized linear mixed-effects model was used to predict marginal mean (95% CI) fecal calprotectin concentrations at 12 weeks for each group with adjustments for baseline values and health centre clusters. Post-hoc (Bonferroni) adjusted *P*-values for multiple comparisons are reported.

[a] Values were not statistically different across treatment groups (*P*<0.05).

[2] Per-protocol analysis where dropouts and those who consumed <80% of capsules were excluded.

A significant interaction between baseline fecal calprotectin concentration and treatment group (on fecal calprotectin concentrations) was detected for the 18 mg ferrous bisglycinate group (P=0.02) but not for the 60 mg ferrous sulfate group (P=0.18) (interaction terms: baseline fecal calprotectin concentration and treatment). There were no significant interactions detected between baseline serum ferritin status and treatment group (on fecal calprotectin concentrations) (P>0.05) for any of the groups (interaction terms: baseline inflammation-adjusted serum ferritin concentration and treatment).

A per-protocol analysis was conducted which restricted the analysis to only women who completed the trial and were 80% adherent to the trial supplement regime. Results from the per-protocol analysis were similar to the intention-to-treat analysis with no significant differences across the three intervention groups: 175 (94, 257) µg/g, 143 (61, 226) µg/g, and 161 (82, 240) µg/g, in the ferrous sulfate, ferrous bisglycinate and placebo groups, respectively.

Chapter 4: Discussion

In this section, I discuss the key findings of my research in Cambodia. I compare my results to the current published literature and discuss the strengths and limitations of my research project. I then summarize the significance of my research and how it contributes to the current body of published literature. Lastly, I suggest future research directions.

4.1. Non-inferiority of Ferrous Bisglycinate to Ferrous Sulfate

My primary objective was to assess the non-inferiority of 18 mg iron as ferrous bisglycinate (experimental) compared to 60 mg iron as ferrous sulfate (standard treatment), on inflammation-adjusted mean ferritin concentrations at 12 weeks. I hypothesized that women who received 12 weeks of 18 mg daily oral iron as ferrous bisglycinate would have similar ferritin concentrations as women who received 12 weeks of 60 mg daily oral iron as ferrous sulfate. This hypothesis was based on previous randomized controlled trials conducted worldwide that observed a 2-4x greater bioavailability of ferrous bisglycinate than ferrous iron salts.[109,114,124–126] However, our non-inferiority analysis was inconclusive to determine if ferrous bisglycinate was non-inferior to ferrous sulfate, as the CI for our predicted mean difference in ferritin concentrations between the two iron interventions crossed the margin of non-inferiority (20 μg/L).

A recent cross-sectional analysis of 71 articles revealed that non-inferiority trials with inconclusive results are often inappropriately described and misinterpreted with the use of vague language to report their findings.[167] Therefore, to reiterate, based on our inconclusive findings, we were unable to establish if ferrous bisglycinate was non-inferior to ferrous sulfate in our trial.

Ultimately, there are four factors that may have contributed to our inability to detect non-inferiority between the two iron interventions in our trial: (1) our population had a very low prevalence of iron deficiency and anemia; (2) the dose of ferrous bisglycinate used in our trial may have been too low; (3) our sample size may have been too small; and (4) our '*a priori*' estimation of the non-inferiority margin may have been too conservative. I will elaborate on these factors in the following sections.

4.1.1 Iron Deficiency and Anemia Prevalence in our Study Population

4.1.1.1 Iron Deficiency Prevalence

At baseline, the prevalence of iron deficiency in women enrolled in our trial in Kampong Thom was 6%, based on inflammation-adjusted serum ferritin <15 µg/L. This is comparable to other surveys conducted in Prey Veng province,[22] a cross-sectional nation-wide study,[43] and the national DHS in 2014,[1] all of which reported a low iron deficiency prevalence among women of reproductive age (2%, 8% and 3%, respectively). Research in Prey Veng has shown that anemic women with certain genetic hemoglobin disorders have elevated ferritin concentrations; and hemoglobin disorders are known to be common in Cambodia.[22] Additionally, high iron levels in groundwater in Cambodia have been reported, possibly contributing to the low prevalence of iron deficiency observed in these aforementioned studies. The contribution of groundwater iron to ferritin stores has also been observed in other populations in Asia.[168]

Based on our study exclusion criteria, women had not taken iron-containing supplements for the 12 weeks prior to beginning the study intervention or during the trial. The traditional Cambodian diet is thought to be low in iron, consisting largely of rice with limited sources of iron-rich

animal foods.[7] Therefore, iron intakes were unlikely a contributor to the high baseline ferritin concentrations observed in our trial. The low prevalence of iron deficiency in this trial is in line with past findings and questions whether untargeted iron interventions are needed in Cambodia.

It is well known that iron absorption is regulated by iron status (which is regulated by hepcidin expression); iron absorption is considerably lower in those with normal or high iron stores.[42,125,169] Although ferrous bisglycinate has been deemed "highly bioavailable," most of this research has been conducted in individuals with low iron stores.[114,129,132,135,137] It is possible that ferritin concentrations among the women in our trial were too high to observe a response to ferrous bisglycinate supplementation.

On account of the different chemical structure of ferrous bisglycinate, it has been queried if it has a different absorption mechanism than other forms of supplemental iron, perhaps one that is independent of iron stores. To investigate this, Bovell-Benjamin et al. prepared radioiron solutions of ferrous bisglycinate, ferrous ascorbate and ferrous trisglycinate in distilled water given to 10 fasted, iron-sufficient adult males.[125] Sixteen days after consuming the radioiron solutions, analysis of incorporated red blood cell radioactivity analysis was conducted, and iron absorption from all iron sources was found to be strongly inversely correlated with serum ferritin concentrations.[125] Further, Olivares et al. used a double-isotopic method to measure iron absorption of ferrous ascorbate and ferrous bisglycinate in water and milk in 14 women after sixteen days and similarly found iron absorption of both forms to be strongly related to serum ferritin concentrations (r=0.7, $P<0.006$ and r=-0.6, $P<0.03$, respectively).[170] These studies

confirm that the absorption of ferrous bisglycinate is indeed influenced by bodily iron stores, though these trials were done via food fortification, not supplementation.

Conversely, in our trial, we did not detect a significant interaction between treatment group and baseline iron status (iron-replete vs iron-deplete) on 12 week ferritin concentrations; thus, we conclude that in our trial baseline iron status did not mediate the response of the intervention on ferritin concentrations at 12 weeks. This was unexpected because of the well-documented inverse relationship between iron absorption and serum ferritin concentrations. It may have been that we were underpowered to detect such an association. Nonetheless, it is quite well-established that iron stores regulate iron absorption in healthy individuals.

4.1.1.2 Anemia Prevalence

At baseline, the overall prevalence of anemia among women was 17%, based on the cut-off for non-pregnant women (hemoglobin <120 g/L). This anemia prevalence is significantly lower than the prevalence rate of 45% that was previously reported in Kampong Thom province in the 2014 DHS.[1] We suspect our finding of low anemia prevalence in our study may be due to a difference in hemoglobin measurement methods. In the nationally representative survey conducted in 2014, hemoglobin concentration was measured in a capillary blood sample from the finger with use of a portable HemoCue device.[1] In our trial, hemoglobin concentration was measured in a venous blood arm sample using an automated hematology analyzer.

Research has shown considerable variation in and poor agreement between hemoglobin concentrations measured with these two methods (venous vs. capillary blood samples and

HemoCue vs. automated hematology analyzers).[62] Currently, there is no global consensus on the systematic bias observed with the use of the HemoCue device, just that there is substantial variability in measurement, as both under- and over-estimations of capillary-sampled hemoglobin concentrations have been reported.[62] One factor that may be contributing to this inaccuracy when collecting capillary blood is the "milking" of the finger to quicken blood flow following the finger prick, which is thought to result in excess plasma being pushed onto the microcuvette tip. This excess plasma can cause a dilution effect on hemoglobin concentration, resulting in a lower hemoglobin concentration (thus, an over-estimation of anemia prevalence). For these reasons, prevalence rates of anemia that have been collected with capillary blood samples may be overestimated. This finding of low anemia prevalence in our study population is important as the WHO recommendation for blanket iron supplementation is focused on areas where the prevalence of anemia is ≥40%.

4.1.2 Dose of Ferrous Bisglycinate Used in Our Trial

Secondly, the dose of ferrous bisglycinate (18 mg) may have been too low in our trial, leading to our inconclusive findings in the non-inferiority analysis. We chose a dose of ferrous bisglycinate at 18 mg, approximately one-third the standard WHO dose of ferrous sulfate dose (60 mg) based on current literature conducted in iron deficient individuals (as is typical among many iron supplementation trials). However, our study population was predominantly iron-replete; thus, we speculate that the bioavailability of ferrous bisglycinate may have been compromised in this population (due to the established mechanism of hepcidin down-regulation and decreased iron absorption in healthy individuals). On the contrary, ferrous sulfate did elicit a significant increase in ferritin concentrations at 12 weeks, even though women in this group were also predominantly

iron-replete; however, this group received a 60 mg elemental iron dose of ferrous sulfate. We speculate that our chosen dose of 18 mg ferrous bisglycinate was too low to observe a response in 12 weeks that may have confirmed non-inferiority in our comparison of the two iron interventions.

4.1.3 Sample Size

A larger sample size may have provided us with more power, leading to a more precise estimate of the mean difference in ferritin concentrations with more narrow CIs. If we had a greater sample size, and therefore, higher power, a narrower CI around the mean difference may have been observed, potentially allowing us to conclude non-inferiority or inferiority of the ferrous bisglycinate intervention. To conclude non-inferiority, the upper bound of the CI would have to lie to the left of the margin of non-inferiority (Δ, 20 µg/L) and include zero, meaning the ferrous bisglycinate intervention is non-inferior but not shown to be superior to ferrous sulfate.[166] Additionally, if the CI lies entirely to the left of the margin of non-inferiority (Δ, 20 µg/L) and the right of zero (within 0-20 µg/L), it is indicative of non-inferiority, yet the ferrous bisglycinate intervention would have been considered to be significantly worse than the ferrous sulfate intervention.[166] The latter outcome is considered to be rare as it often requires a very large sample size to produce a narrow enough CIs where the lower and upper bound CI lie between zero and the margin of non-inferiority (Δ).

4.1.4 Predetermined Non-Inferiority Margin for Ferritin Concentration

A predetermined non-inferiority margin is often derived from previous superiority study estimates of the standard treatment compared to placebo. Our '*a priori*' defined margin of non-

inferiority, Δ, of 20 µg/L, was based on a WHO report that analyzed data from 10 studies assessing the predictive ability of hematological biomarkers on iron status changes; an arbitrary change of ≥ 0.2 SD was defined as indicative of a successful response to the iron intervention.[70] The analysis concluded that serum ferritin had a 90% success of detecting changes in iron status (using a change of ≥ 0.2 SD).[70] Using the 0.2 SD value and data from Dr. Karakochuk's previous Cambodian trial (the SD of serum ferritin was ~50 µg/L after 12 weeks of iron supplementation with 60 mg elemental iron as ferrous sulfate), a 20 µg/L margin of non-inferiority (Δ) was chosen. Had we specified the margin to be merely 5 µg/L greater than was initially chosen (25 µg/L; which would have still been a clinically important difference), then the CI would lie fully to the right of the margin, and our comparison of the two iron interventions would be deemed non-inferior. Ultimately, this is why it is imperative in these types of trials that the margin of non-inferiority is determined and confirmed '*a priori*'. Of note, experts are likely to have differing opinions on what quantifies a clinically important margin. Therefore, it is clear that our predetermined margin of non-inferiority was a subjective estimate, which was potentially too conservative, and this estimate heavily influenced our non-inferiority comparison, and ultimately, the conclusion of our primary outcome analyses.

4.2. Secondary Superiority Analysis of the Effect of Iron at 12 Weeks

Our primary non-inferiority comparison was inconclusive; we were unable to determine if ferrous bisglycinate was non-inferior to ferrous sulfate. However, in a secondary analysis with use of a superiority approach, mean ferritin concentration at 12 weeks was significantly higher in the ferrous sulfate group (98.6 [94.7,102.6] µg/L, $P<0.001$) than in the ferrous bisglycinate (84.0 [79.9, 88.2] µg/L) and placebo groups (77.8 [73.9, 81.7] µg/L). Even though our primary

analysis was unable to declare ferrous bisglycinate neither inferior nor non-inferior, this superiority analysis proves that 60 mg ferrous sulfate is more effective than 18 mg ferrous bisglycinate in increasing ferritin concentrations after 12 weeks.

Ultimately, both groups achieved mean (95% CI) ferritin sufficiency (>15 µg/L) after the supplementation period. With an overall iron deficiency prevalence of only 6% in this population, the question of whether this population should be receiving blanket supplementation program appears to be more prominent than the form or dose of iron used.

4.3. Effect of Iron on Fecal Calprotectin at 12 Weeks

My second research objective was to determine if 60 mg elemental iron as ferrous sulfate increases gut inflammation in women, compared to 18 mg elemental iron as ferrous bisglycinate or placebo at 12 weeks. I hypothesized that women who received 12 weeks of 60 mg ferrous sulfate would have more gut inflammation than women who received 18 mg ferrous bisglycinate or placebo. This hypothesis was based on published research in infants and children, which has found detrimental effects of iron fortification on gut inflammation.[160] To our knowledge, no studies have investigated this in adults or non-pregnant women. The current study did not show any differences between groups for fecal calprotectin values at 12 weeks; rather, median calprotectin surprisingly decreased in all groups over the intervention period.

Four factors that may explain the discrepancy between our present findings and the published literature: (1) fecal calprotectin is a highly variable biomarker; (2) fecal calprotectin should be interpreted alongside other changes in the gut microbiome; (3) environmental variables (e.g.,

water, sanitation, or enteropathogen burden) may modulate the effects of iron on gut

inflammation; and (4) the unprecedented COVID-19 pandemic and subsequent social behaviour

changes. I will elucidate these factors in the following sections.

4.3.1   Variability of Fecal Calprotectin

Firstly, the within-day and day-to-day variability of fecal calprotectin should be addressed, as

fecal calprotectin values showed large variation. The baseline and endline median (IQR) fecal

calprotectin concentrations were 67 (30, 174) µg/g and 53 (22, 98) µg/g, respectively.

Fecal calprotectin is a highly variable biomarker that can increase and decrease both day-to-

day[153,171,172] and within-day.[153,173,174] Generally, it is recommended to analyze stool from the first

bowel movement in the morning and for multiple locations in the stool to be sampled, which was

the protocol we followed in our study. Even still, an observational case-control study found the

median CVs of two stool samples 1-5 days apart to be 36% (range: 0–123 µg/g fecal

calprotectin), significantly higher than the intra-stool variability (from single measures between

three punches on a single stool sample) median CV of 17% ($P<0.01$).[171] To understand the

effects of iron supplementation on gut inflammation and appropriately answer our research

question, a single measurement is not likely sufficient as it does not consider this variability. It

would be ideal to measure multiple samples over the day and across days to reduce variability,

however, this was not logistically possible in our trial.

Several factors may affect fecal calprotectin values, including physical activity, age, fibre intake,

non-steroidal anti-inflammatory drug use, or an episode of gastroenteritis.[175,176] Of note, all

women in our study were 18-45 years of age, and the use of non-steroidal anti-inflammatory drugs was indicated as one of our exclusion criteria. One or more factors may have triggered an acute inflammatory response in the days around study stool collection. On account of its high day-to-day variation, we believe fecal calprotectin has low sensitivity to evaluate the relatively marginal impact of iron on the gut. This is especially the case for rural Cambodia, where acute gastrointestinal episodes may substantially bias fecal calprotectin values. Ultimately, we feel that this biomarker should not be interpreted in isolation but rather alongside other markers of potential gut injury, such as the presence of pathogenic bacteria.

4.3.2 Interpretation of Fecal Calprotectin with Other Gut Microbiome Data

Based on the literature revealing the variability of calprotectin, the interpretation of fecal calprotectin should ideally occur in conjunction with other gut microbiome assessments. Many of the aforementioned studies in infants and children measured both 16S rRNA pyrosequencing and targeted real-time quantitative PCR (qPCR) as their primary outcome measure and fecal calprotectin as their secondary outcome measure. We compared fecal calprotectin concentrations across groups at 12 weeks, but it may be useful to explore the changes in gut inflammation at the individual-level, specifically among only those women with a large enteropathogen burden. This will help to understand if iron supplementation results in greater harm to those who have a high prevalence of pathogens and/or parasites at baseline.

4.3.3 Environmental Context

Recent research suggests that environmental variables can modulate the effects of iron on the gut microbiota and gut inflammation; the effects are more prominent in poor hygiene settings and if

the microbiome is likely to be habitually populated by pathogenic bacteria. This was illustrated in a trial investigating the effect of iron fortification on the gut. Children aged 6-14 years (n=139) in Côte d'Ivoire were randomized to 20 mg iron-fortified biscuits or non-fortified biscuits 4x/week for six months.[161] At baseline, this population showed substantial colonization of potential enteropathogens, which was further increased with iron fortification. A significant increase in the number of enterobacteria (P<0.005) and a decrease in the number of lactobacilli (P<0.0001) was reported in the iron group as compared to the control group. Further, the iron group saw a significant increase in fecal calprotectin (P<0.01), which was correlated with the greater abundance of enterobacteria (P<0.05).[161] Similar findings have been demonstrated in a comparable context with a large enterobacteria burden, including two trials in Kenya.[83,103]

On the contrary, Dostal et al. explored the effects of 50 mg ferrous vs placebo on gut inflammation and gut microbiota in South African children iron deficient children (n=49) aged 6-11 years. These children lived in a malaria-free environment and in households with access to clean tap water. At baseline, there was a low burden of enteropathogens; at 38 weeks of intervention, no significant group differences were detected for microbiota makeup or fecal calprotectin.[164] Similar results have been shown in Swedish iron-replete infants,[163] with these studies being implemented in regions with better hygiene practices and improved water supplies. This suggests the risk of adverse effects of iron supplementation on the gut may increase when hygiene standards are poor and the presence of enteropathogens are high.

In Cambodia, improvements are being made to water, hygiene and sanitation practises, yet 20% of rural Cambodians use a non-improved drinking water source during the dry season, and 40%

do not have access to basic handwashing facilities.[177] In our population, 91% of women reported their households had a flush to a septic tank toilet; 54% access their household water from a hand pump and 24% from a ring well. We know enteropathogens are common in Cambodia from research in three provinces (Battambang, Kampong Cham and Kampot) where 12% of participants (*n=82/681*) tested positive for either *C. jejuni* (*n=66*) or *C. coli* (*n=16*).[178] Given these contradictory findings of the effect of iron on the gut microbiota and gut inflammation seen in high and low-resource settings, we would expect the enterobacteria burden to be high in Kampong Thom and the impact of iron to be more pronounced on the gut. Future research is needed to determine the enterobacteria burden in this population and how these variables modify the effects of iron on gut inflammation.

4.3.4 COVID

The COVID-19 pandemic has changed the way most people live and has undoubtedly impacted the health of individuals in numerous ways. The sharing of micro-organisms between humans helps to establish the human microbiome and is important for maintaining human health.[179] Quarantine measures and adherence to social distancing has limited the interaction among individuals and is essential to contain the spread of COVID-19; however, it may come at a microbial cost by decreasing the acquisition of microbes.[179] Changes in the balance of the gut microbiome (known as dysbiosis) are associated with a greater susceptibility to disease and opportunistic infections due to the decrease in the protective microbial load of beneficial bacteria.[180]

There is no data yet on the effect of human behaviour (such as social distancing and hygiene measures) in response to the COVID-19 pandemic on the gut microbiota and gut inflammation in those who did not contract the virus.[179] We suspect that women in our study practiced social distancing (in accordance with public health guidance in Cambodia) with a greater emphasis on regular and proper hand washing practises in the short term (from March 2020 to endline data collection in April/May 2020); this may have resulted in less exposure to pathogenic bacteria, thereby lowering gut inflammation among women in our trial. Ultimately, it is very difficult to quantify or estimate the potential effect of the global COVID-19 pandemic on our research findings, but it is very possible that our research was affected by it.

4.4. Adverse Side Effects

Based on self-reported data, there were no differences in the occurrence of gastrointestinal events across trial arms at 12 weeks. Overall, the incidence of gastrointestinal side effects was low (6%, *n=28/456*), and all interventions appeared to be similarly tolerated. This is surprising, as there is a mounting body of evidence that shows that iron given in the form of ferrous bisglycinate elicits few adverse effects as compared to iron salts[130,138] In contrast, adherence to ferrous sulfate supplementation is often limited due to gastrointestinal adverse effects.[122,123,138]Adherence would not have affected the presentation of adverse side effects, as there was no difference in the proportion of adherent women (defined as women who consumed 80% or more of their daily capsules) as measured by three capsule counts over the 12 weeks, across the three intervention groups.

The frequency of self-reported gastrointestinal side effects in the ferrous bisglycinate at 12 weeks was relatively low (8%), which was not surprising. More puzzling is the equally low frequency of self-reported gastrointestinal side effects in both the ferrous sulfate and placebo groups (5% for both). Gastrointestinal symptoms and complaints are frequently observed in women consuming high-dose iron supplements, and the frequency and severity of these complaints are often related to the size of dose. Ultimately, our findings that show adverse side effects to be low and similar across all groups is unexpected.

Based on baseline self-reported data, 53% (n=254/480) of women experienced gastrointestinal side effects at least once a month. Further, 45% (n=204/456) of women had gut inflammation present at baseline, based on fecal calprotectin >80 µg/g. There is a possibility that daily iron supplementation did not change the symptoms experienced by Cambodian women because gastrointestinal complaints were already very common, and there was preexisting gut inflammation in nearly half the study population. Though this data was participant self-reported and therefore limited by response bias. We conjecture that in a context with better sanitation with lower rates of baseline gastrointestinal symptoms and elevated gut inflammation, differences in the reporting of adverse side effects may have been more clearly seen across groups.

4.5. Strengths and Limitations

4.5.1 Strengths

Strengths of this research include the rigorous study design of a double-blind, randomized, placebo-controlled trial. The diligent work of our experienced field research staff was key to building rapport with study participants and ensuring frequent follow-up visits. This resulted in a

high study retention rate (92% at 12 weeks), notwithstanding the challenges that the COVID-19 pandemic brought to the trial.

Our biochemical outcomes were assessed with 'gold standard' methods for hemoglobin and iron status. Hemoglobin was measured using an automated hematology analyzer with standardized quality control and calibration methods. Biomarkers of inflammation, CRP and AGP, were measured and used to adjust ferritin concentrations to ensure ferritin levels were not falsely elevated by inflammation. We did not restrict our recruitment of women based on iron or anemia status, allowing this cohort to be generalizable to the broader province of Kampong Thom and beyond.

4.5.2 Limitations

One limitation is the low iron deficiency prevalence observed in our study cohort. This limited our ability to assess the response of iron on ferritin concentrations and gut inflammation. Although our aim was to demonstrate that a daily dose of ferrous bisglycinate, one-third the dose that the WHO recommends of ferrous sulfate, would have a comparable increase on ferritin concentrations at 12 weeks, while also being safer on the gut in iron-replete individuals, it may have been that the 18 mg dose was too low, as those who are iron-replete (with higher iron stores) are less responsive to oral iron supplementation.

Our non-inferiority sample size calculation was based on estimates from published literature and was determined in consultation with a biostatistician. However, our trial may have been underpowered to detect non-inferiority between the two iron interventions in our primary

analysis.[181] With a larger sample size, our results might have produced a tighter CI around the true mean difference between the two iron intervention groups. Our '*a priori*' margin of non-inferiority of 20 µg/L may have also been too conservative and limited our ability to obtain conclusive results in our non-inferiority comparison.

Thirdly, we observed considerable variation in fecal calprotectin at both baseline and 12 weeks. With the significant intra-individual sample variability, a single measurement of fecal calprotectin may not be best practice; instead, sampling throughout intervention (possibly once every week, if resources allow) may have been more appropriate.

Another limitation is that we did not genotype women for genetic hemoglobin disorders, though it is widely documented that these are highly prevalent in Cambodia and throughout Southeast Asia.[22] An unforeseen reason for the exclusion of many women from our trial was the use of contraceptives that contained iron (this made up 37% of women who did not meet study inclusion criteria [*n*=213/577]). This skewed the prevalence of women taking birth control, possibly limiting our generalizability to the Kampong Thom population, where some are consuming iron-containing contraceptives.

4.6. Research Significance

Current WHO guidelines recommend daily oral iron and folic acid supplementation for menstruating women and adolescent girls for three consecutive months each year in countries with an anemia prevalence ≥40%. In Cambodia, anemia rates have been previously reported to reach this anemia prevalence threshold (~45%), yet iron deficiency prevalence is very low.

Simultaneously, genetic hemoglobin disorders are highly prevalent, placing the safety and necessity of untargeted iron supplementation in question. My research aimed to investigate if a lower dose of a highly bioavailable form of iron (18 mg ferrous bisglycinate) was just as effective as the standard 60 mg ferrous sulfate dose on ferritin concentrations and if it was associated with less gut inflammation after 12 weeks of iron supplementation compared to placebo. Our non-inferiority analysis was inconclusive to determine if ferrous bisglycinate was non-inferior to ferrous sulfate, as the CI for our predicted mean difference in ferritin concentrations across the two iron interventions crossed the margin of non-inferiority (20 μg/L). However, in a secondary analysis with use of a superiority approach, mean ferritin concentration at 12 weeks was significantly higher in the ferrous sulfate group (98.6 [94.7,102.6] μg/L, $P<0.001$) than in the ferrous bisglycinate (84.0 [79.9, 88.2] μg/L) and placebo groups (77.8 [73.9, 81.7] μg/L). These are promising findings given that 60 mg ferrous sulfate is the current WHO guideline, and it shows to be effective at increasing ferritin concentrations.

Concerning gut inflammation, we observed that fecal calprotectin concentration marginal means at 12 weeks were not different across groups among women in our trial. There was no evidence of increased gut inflammation in either iron intervention compared to placebo. We conclude that fecal calprotectin may not be specific enough to detect inflammatory gut changes due to oral iron supplements; further analyses of gut pathogen abundance and gut microbiome makeup are potential areas that require more investigation.

At this time, untargeted oral iron supplementation does not appear to be necessary for women in Cambodian because of the low prevalence of both anemia and iron deficiency. Ultimately, we

found no increased risk of gut inflammation associated with this unnecessary supplementation. Despite this, untargeted oral iron supplementation is a potential waste of resources in this setting.

4.7. Future Research

Our trial showed no sign of overall increased adverse effects, as measured by gastrointestinal side effects or gut inflammation, in the 60 mg ferrous sulfate group as compared to the 18 mg ferrous bisglycinate and placebo groups. However, these are only two of many possible indicators of potential harm to investigate. It would be interesting and important to examine gut pathogen growth/abundance or changes in the gut microbiome after 12 weeks of iron supplementation. It would be interesting to only examine changes in gut inflammation in those women with a high prevalence of enteropathogens present, as it may be that iron is only detrimental to those who had a high prevalence of enteropathogens at baseline. 16S rRNA pyrosequencing and/or targeted real-time qPCR analyses of stool samples could be completed alongside gut inflammation measurement to assess these factors further. One could also assess the correlations between abundance of bifidobacteria, lactobacilli or present enterobacteria and fecal calprotectin.

Point of care testing for iron status is not available in low-resource settings. This would be incredibly helpful to determine which individuals may be most likely to respond to iron interventions. Currently, there is ongoing work in the development of these point of care devices. Future research could assess the accuracy and utility of novel point-of-care devices diagnosing iron deficiency in the field, such as iCheck Anemia (BioAnalyt, Berlin, Germany), which determines zinc protoporphyrin (ZnPP).[182] A convenient device, at a fraction of the cost of

traditional laboratory techniques, could be a promising method of diagnosing iron deficiency in low-resource settings, allowing individualized iron interventions to be targeted only to those who would likely benefit.

Ferrous bisglycinate is highly bioavailable when given to iron deficient individuals, but our study of iron-replete women did not demonstrate this. More research is needed to understand how the bioavailability of various forms and doses of iron supplements (beyond just ferrous sulfate and ferrous bisglycinate) differ in populations with diverse iron statuses, especially in those who are iron-replete.

Lastly, the use of iron-containing supplements in Cambodia should be further explored. We had a surprisingly high number of women in Kampong Thom who reported consuming contraceptives with the placebo tablets containing iron ($n=213/1,286$ women screened). The use of iron-containing contraceptives in South East Asia or other countries has yet to be described in the literature. Based on this wide-spread use in Kampong Thom and possibly throughout Cambodia, research is warranted to investigate the use of iron-containing oral contraceptives in this population and beyond, as well as measuring the effect of iron-containing oral contraceptives on anemia and iron status.

Chapter 5: Conclusion

In 2016, the WHO introduced a global policy that recommended 60 mg daily oral iron supplementation for 12 consecutive weeks per year in non-pregnant women and adolescents where anemia prevalence is ≥40%, such as in Cambodia.[77] This policy is based on the assumption that approximately 50% of anemia is due to iron deficiency and the well-established benefits of iron supplementation for iron-depleted women.[77] Yet, a nationally-representative survey revealed only 3% of non-pregnant Cambodian WRA had iron deficiency while 45% of women were anemic.[1] Further, 60% of WRA had a genetic hemoglobin disorder, which can place women at risk of iron overload, as some of these disorders cause altered iron metabolism.[1,24] If iron deficiency is not a major cause of anemia, then at best, untargeted iron supplementation is a waste of resources; at worst, it could cause harm.

Blanket iron supplementation (particularly in the absence of iron deficiency and in populations where genetic hemoglobin disorders are highly prevalent) is additionally concerning because the most common forms of iron supplements, iron salts, are poorly absorbed. Typically, <20% of iron from iron salts is absorbed in the duodenum,[118] and the remaining passes unabsorbed into the colon where it can increase susceptibility to pathogen growth and lead to gut inflammation.[119] A novel form of iron, ferrous bisglycinate, has shown to have a 2-4x times greater bioavailability than other iron salts,[124,183] and has demonstrated fewer gastrointestinal side effects.

To date, there is a lack of high-quality data investigating the potential harms of untargeted iron supplementation in WRA, particularly in iron-replete individuals or those with genetic

hemoglobin disorders. Research is warranted to understand if a lower dose of a more bioavailable form of iron may be effective at raising iron levels while simultaneously causing less harm. It was hypothesized that women who received 12 weeks of 18 mg ferrous bisglycinate would have a similar increase in serum ferritin concentrations as women who received 60 mg ferrous sulfate. However, our non-inferiority comparison in this trial was inconclusive (we could not confirm if ferrous bisglycinate was non-inferior to ferrous sulfate). There are four likely explanations for this inconclusive finding: Firstly, the prevalence of iron deficiency (6%) in the study population was very low, thus women were less likely to respond to the iron interventions. Secondly, the dose of ferrous bisglycinate may have been too low to elicit a response in this iron-replete population. Thirdly, we may have been underpowered to detect non-inferiority in our primary analysis. Lastly, our '*a priori*' chosen non-inferiority margin estimate may have been too conservative. Even though we were unable to establish non-inferiority between the two iron interventions, in a secondary superiority analysis, we demonstrate that 60 mg ferrous sulfate more significantly raises serum ferritin levels after 12 weeks of daily iron supplementation than 18 mg ferrous bisglycinate and placebo among predominately iron-replete non-pregnant Cambodian women. This is the first study to compare these forms and doses of iron in a study population of non-pregnant women.

Additionally, we concluded that iron supplementation was not significantly associated with a change in gut inflammation after 12 weeks as compared to placebo. We hypothesized that women who received 12 weeks of 60 mg daily oral iron as ferrous sulfate would have higher levels of gut inflammation than women who received placebo or 18 mg daily oral iron as ferrous bisglycinate. Our null finding may result from the high day-to-day and within-day variability of

fecal calprotectin and due to confounding variables, such as COVID-19 hygiene practises, that

may have more strongly influenced the gut microbiota than our iron interventions. We believe

calprotectin concentrations should be interpreted alongside other measurements, such as gut

pathogen growth/abundance or changes in the gut microbiome, as this would strengthen the

interpretation and conclusions in regard to the potential harms of untargeted iron

supplementation.

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Appendices

Appendix A: Baseline Questionnaire (Original English Version)

BASELINE QUESTIONNAIRE
Effects of iron supplementation in iron replete populations. A 12 week RCT in Kampong Thom, Cambodia

CONFIDENTIAL:
All information collected is strictly confidential.

INTERVIEWER INFORMATION:
Name: _____   Signature: _____
Date of interview (DD/MM/YYYY):   ___ ___ / ___ ___ / ___ ___ ___ ___

PARTICIPANT ID:	GROUP:
___ ___ ___	A B C

MODULE 1: PARTICIPANT INFORMATION	
1. How old are you?	___ ___ years
2. What is your marital status?	1 = Not married 2 = Married 3 = Widowed 4 = Separated or divorced ___ 8 = Don't know
3. How many people currently live in your household (defined as eating from the same pot each day)?	___ ___ people
4. Have you completed any schooling?	1 = YES → continue to Q5 2 = NO → **skip to Q6** ___
5. What is the highest level of school you attended?	1 = Primary school 2 = Lower Secondary school 3 = Upper Secondary school ___ 4 = Higher education 8 = Don't know

MODULE 2: HEALTH	
6. Have you ever given birth?	1 = YES → continue to Q7 2 = NO → **skip to Q12** ___
7. How many children have you birthed <u>in total</u>?	_____ children
8. How many months ago did you <u>last</u> give birth?	_____ months
9. Did you take iron and folic acid supplements during your <u>last</u> pregnancy (for any duration)?	1 = YES → continue to Q11 2 = NO → **skip to Q15** ___

	8 = Don't know → **skip to Q15**
10. How many days of iron and folic acid supplements did you take <u>in total</u> during your <u>last</u> birth?	1 = all 90 days 2 = between 70-90 days 3 = between 50-70 days 4 = between 30-50 days 5 = between 10-30 days 6 = less than 10 days 8 = Don't know ___ ___
11. Do you currently take any birth control or oral contraceptive pills (to prevent getting pregnant)?	1 = YES 2 = NO 8 = Don't know ___
12. How many children live in your household?	_____ children
13. How many adults live in your household, <u>including yourself</u>?	_____ adults
14. Do you experience gastrointestinal upset?	1 = YES, everyday 2 = YES, once a week 3 = YES, once a month 4 = NO
15. What type of gastrointestinal upset do you experience? (Choose all that apply)	1 = Diarrhea 2 = Constipation 3 = Stomach pain 4 = Bloating 5 = Nausea 6 = Vomiting 7 = Pain passing stool 8 = Blood in stool 9 = Other ___
16. Have you had an illness with diarrhea (three loose bowel movements in 24 hours) or any gastrointestinal upset?	1 = YES, in the past 24 hours 2 = YES, in the past week 3 = YES, in the past month 4 = NO
17. Have you taken antibiotics before?	1 = YES → continue to Q18 2 = NO → **skip to Q19** 8 = Don't know → **skip to Q19** ___
18. How many times have you consumed antibiotics in the past year?	1 = Once 2 = Twice 8 = 3 or more times ___
19. Have you taken pain medication?	1 = YES → continue to Q20 2 = NO → **skip to Q22** 8 = Don't know → **skip to Q22** ___
20. How many times have you consumed pain medicines in the past year?	1 = Once 2 = Twice 8 = 3 or more times ___
21. What is the medication?	_____

MODULE 3: FOOD	
Now I would like to ask about the consumption of other foods in the last one-week period.	
22. Do you cook with iron-fortified fish sauce?	1 = YES → continue to Q23 2 = NO → **skip to Q24** 8 = Don't know → **skip to Q24** ___

23. How many times in one week do you eat <u>iron-fortified fish sauce</u>?	1 = Once 2 = Twice 3 = Three times 4 = More than four times 8= Don't know ___
24. Do you use an <u>iron pot</u> for cooking?	1 = YES 2 = NO 8 = Don't know ___
25. Do you eat fermented fish paste *(Prahok)*?	1 = YES → continue to Q26 2 = NO → **skip to Q27** 8 = Don't know → **skip to Q27** ___
26. How many times in one week do you eat fermented fish paste?	1 = Once 2 = Twice 3 = Three times 4 = More than four times 8= Don't know ___

MODULE 4: WATER AND SANITATION

27. What is the <u>main</u> source of <u>drinking water</u> for members of your household?	1 = Hand pump 2 = Ringwell 3 = Pond/river 4 = Rainwater 5 = Bottled water 7 = Other → **specify:** _____ 8 = Don't know ___
28. Where is the drinking water source located?	1 = At own household 2 = At others household 7 = Other → **specify:** _____ 8 = Don't know ___
29. Do you treat your water in any way to make it safer to drink?	1 = YES → continue to Q30 2 = NO → **skip to Q31** ___
30. What do you <u>usually</u> do to the water to make it safer to drink (single answer)?	1 = Boil 2 = Use water filter 3 = Add bleach/chlorine 4 = Stand and settle 7 = Other → **specify:** _____ 8 = Don't know ___
31. Do you have a water filter at your household?	1 = YES → continue to Q32 2 = NO → **skip to Q34** 3 = **Don't know → skip to Q34** ___
32. What kind of water filter is it?	1 = Sand 2 = Ceramic 3 = Composite 7 = Other → **specify:** _____ 8 = Don't know ___
33. Do you <u>usually</u> use the water filter (more than half the time)?	1 = YES 2 = NO ___
34. What is the <u>main</u> source of <u>cooking water</u> for members of your household?	1 = Hand pump 2 = Ring-well 3 = Pond/river ___

	4 = Rainwater
	5 = Bottled water
	6 = Hand dug (no ring)
	7 = Other → **specify:** _____
	8 = Don't know
35. What kind of toilet facility do members of your household usually use?	Flush / pour flush 　　1 = Flush to piped sewer system 　　2 = Flush to septic tank 　　3 = Flush to pit latrine 　　4 = Flush to somewhere else 　　5 = Flush to unknown place / not sure Pit latrine 　　6 = Ventilated improved latrine 　　7 = Pit latrine with slab 　　8 = Pit latrine without slab / open pit 9 = Composing toilet 10 = Bucket 11 = Hanging toilet/latrine 22 = No facility / bush / field

How to Collect Stool

Nutrition for Women – Iron Study

Collect first stool in morning and give to study nurse on the same day**

1. Put on gloves

2. Place plastic bag under bottom

DO NOT ALLOW PLASTIC BAG TO TOUCH TOILET WATER

3. Poop into plastic bag

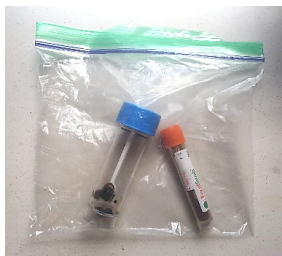**4.** Open stool container tube

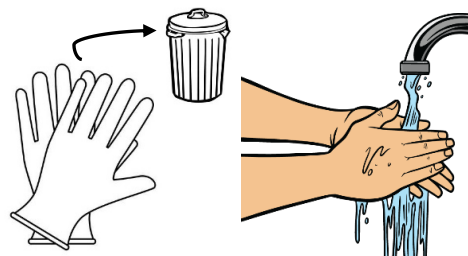**5.** Use scoop to collect portion of stool from plastic bag

6. Place stool sample and scoop into stool collection tube and seal securely
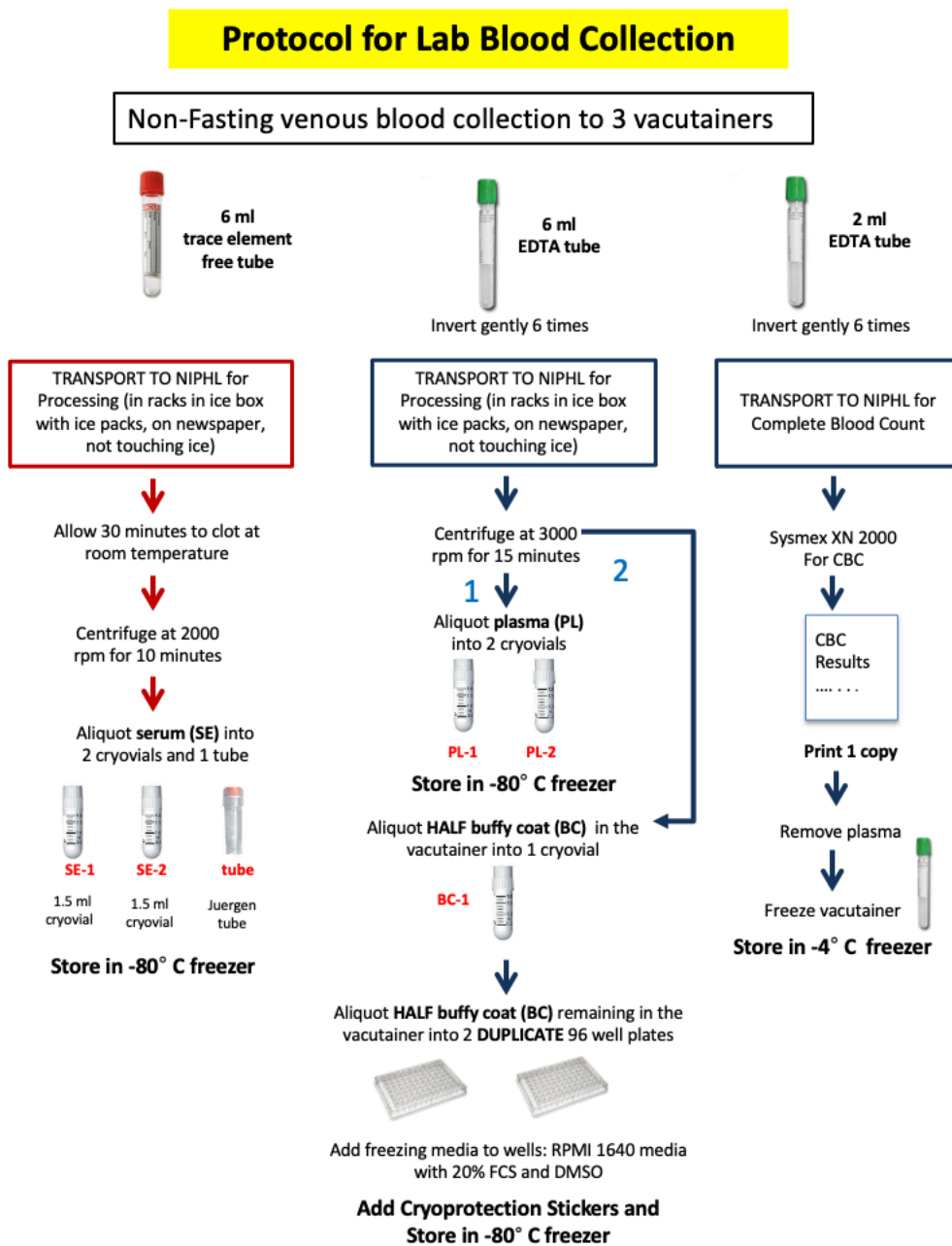

7. Place collection tube into "stool collection kit" along with fecal swab tube

8. Throw away gloves and wash hands

Appendix C: Blood Collection Protocol for Baseline and 12 Weeks

Protocol for Lab Blood Collection

Non-Fasting venous blood collection to 3 vacutainers

6 ml trace element free tube

6 ml EDTA tube — Invert gently 6 times

2 ml EDTA tube — Invert gently 6 times

Column 1 (trace element free tube):
- TRANSPORT TO NIPHL for Processing (in racks in ice box with ice packs, on newspaper, not touching ice)
- Allow 30 minutes to clot at room temperature
- Centrifuge at 2000 rpm for 10 minutes
- Aliquot **serum (SE)** into 2 cryovials and 1 tube
  - SE-1 — 1.5 ml cryovial
  - SE-2 — 1.5 ml cryovial
  - tube — Juergen tube
- **Store in -80° C freezer**

Column 2 (6 ml EDTA tube):
- TRANSPORT TO NIPHL for Processing (in racks in ice box with ice packs, on newspaper, not touching ice)
- Centrifuge at 3000 rpm for 15 minutes
- **1** → Aliquot **plasma (PL)** into 2 cryovials
  - PL-1   PL-2
  - **Store in -80° C freezer**
- **2** → Aliquot **HALF buffy coat (BC)** in the vacutainer into 1 cryovial
  - BC-1
- Aliquot **HALF buffy coat (BC)** remaining in the vacutainer into 2 **DUPLICATE** 96 well plates
- Add freezing media to wells: RPMI 1640 media with 20% FCS and DMSO
- **Add Cryoprotection Stickers and Store in -80° C freezer**

Column 3 (2 ml EDTA tube):
- TRANSPORT TO NIPHL for Complete Blood Count
- Sysmex XN 2000 For CBC
- CBC Results …. . . .
- **Print 1 copy**
- Remove plasma
- Freeze vacutainer
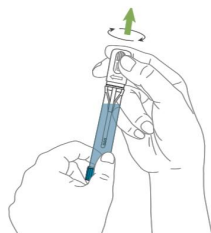- **Store in -4° C freezer**

Appendix D: Stool Extraction Protocol for Baseline and 12 Weeks

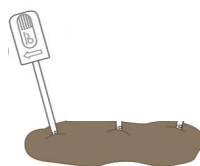### Stool Extraction Protocol: BÜHLMANN fCAL® CALEX® Cap device

National Institute of Public Health Laboratory

1. Hold Calex Cap upright and remove **WHITE** sampling pin from Calex Cap by turning and pulling it upwards
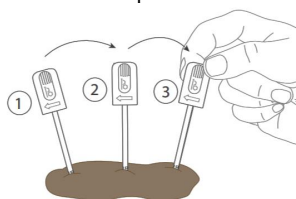
DO NOT unscrew the **BLUE** cap

2. Dip **WHITE** sampling pin tip into stool sample and twist before removing from stool sample*****
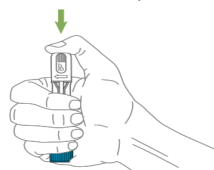
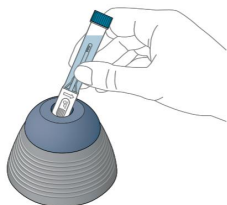**3.** Repeat Step 2 at three to five different positions on the stool sample

Make sure grooves on sampling pin tip are completely filled with stool

4. Put sampling pin back into Calex Cap and push in to lock (you will feel and hear two clicks)

5. Vortex Calex Cap with white head down vigorously on vortex mixer for 30 seconds

6. Allow Calex Cap to stand for 10 minutes on the blue cap

7. If the stool does not completely come off the grooves on the sampling pin, repeat Steps 5-6

Make sure grooves are completely free of stool before proceeding

8. Centrifuge Calex Cap for 5 minutes at 500-3000 × g

9. Freeze Calex Cap at -20℃

*Procedure for liquid stool samples: pipet 10 μL of stool sample directly into Calex Cap. Continue with Steps 4-9.