A 12 MONTH RANDOMIZED CONTROL TRIAL ASSESSING THE EFFICACY OF AN IRON INGOT TO IMPROVE HEMOGLOBIN CONCENTRATION IN RURAL CAMBODIAN WOMEN.

by

Aviva Rappaport

B.Sc., The University of British Columbia, 2014

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE AND POSTDOCTORAL STUDIES

(Human Nutrition)

THE UNIVERSITY OF BRITISH COLUMBIA

(Vancouver)

October 2016

Abstract

Background: Anemia affects 45% of women of reproductive age in Cambodia. Globally, iron deficiency is thought to be the most common cause of anemia. Iron supplementation is recommended in populations where anemia prevalence is high. However, there are issues of cost, distribution and compliance with iron supplementation. A potential alternative is The Lucky Iron Fish® (LIF®), a reusable fish-shaped iron ingot which, when added to the cooking pot, leaches iron into the boiling water or soup in which it is prepared.

Objective: The primary objective of this study was to determine if there was a difference in hemoglobin concentration among rural Cambodian anemic women (18-49 y) cooking with the LIF or a daily iron supplement compared to control after one year.

Methods: In Preah Vihear, 340 women (18-49 y) with mild or moderate anemia (hemoglobin< 80-119 g/L) were randomized to: 1) a LIF group, 2) a daily 18 mg iron supplement group, or 3) a control group. A venous blood sample was taken at baseline, 6 and 12 months. Blood was analyzed for hemoglobin, serum ferritin and serum transferrin receptor (sTfR). Hemoglobin electrophoresis was used to detect structural hemoglobin variants.

Results: During recruitment, anemia prevalence (hemoglobin<120 g/L) was 45% with the HemoCue 301. At baseline, prevalence of iron deficiency anemia differed by biomarker: 9% with serum ferritin (<15µg/L), and 30% with sTfR (>8.3 mg/L).

At endline, the prevalence of anemia was 61%, 67%, and 62% in the LIF, supplement, and control groups, respectively. There was no significant difference in hemoglobin (mean; 95% CI) between the LIF group (116; 113, 118 g/L), iron supplement group (115; 112, 117 g/L), and control group (115; 113, 118 g/L; p=0.897). Serum ferritin was significantly higher in the iron supplement group (97; 88, 105 μg/L; p=0.002) compared to the LIF (78; 69, 83 μg/L) and control groups (76; 71, 85 μg/L).

Conclusions: Our findings show that in this population with a low rate of iron deficiency, the LIF is ineffective at reducing anemia. Our findings suggest that factors other than iron deficiency are likely responsible for the high rate of anemia in Preah Vihear, Cambodia.

Preface

This thesis was prepared in partial fulfillment of the requirement for the degree of Master of Science in Human nutrition under the mentorship of Dr. Timothy Green. I also received supervision from my committee members Dr. Rickey Yada, and Dr. Gwen Chapman who have all reviewed this thesis. This research received ethical approval from the clinical research ethics board at the University of British Columbia (H14-02551) and from the National Ethics Committee for Health Research in Cambodia (NECHR 0392). This study was supported by NutriFood Cambodia, in Phnom Penh. NutriFood Cambodia assisted with the coordination of research activities, procurement of Cambodia ethical approval, and translation of all data collection.

I played an integral role in leading the randomized controlled trial described in this thesis. In order to conduct this research I spent 10 months in Cambodia. I was involved in all aspects of the research project from study design, to recruitment and implementation, and data analysis. I developed all study materials utilized in this trial. I made the questionnaires, monitoring and evaluation tools, and developed a blood collection and processing protocol. Along with Sok Hoing Ly from Helen Keller International Cambodia, and Kheang Khin Meng from Nutritfood Cambodia I was involved in the training of enumerators and phlebotomists for each of the three separate data collection periods. I worked with the laboratory staff at Siem Reap provincial hospital to process all blood samples during data collection. Tze Lin Chai, an undergraduate research assistant worked with me during the 6-month data collection and assisted with data entry and collection.

I also conducted a method comparison that evaluated the agreement between two different point-of-care hemoglobinometer models to assess hemoglobin concentrations in a rural

setting. I presented these results in the form of a poster at Experimental Biology/American Society for Nutrition Annual Meeting in April 2016 in San Diego, CA (*FASEB J* 2016; 30 (Suppl 1): lb401). These results have also been submitted, and have been accepted for publication in the International Journal for Laboratory Hematology.

Preliminary results related to the randomized controlled trial have been presented at the Land and Food Systems Graduate Student Conference in March 2016 where I won an award for best academic poster. Recently, I have had an abstract accepted for oral presentation at the Micronutrient Forum in Cancun in October of 2016. At this conference I will present final results on the efficacy of an iron ingot to improve hemoglobin concentration in rural Cambodian women. I intend to submit the final results for publication in late 2016 to the American Journal for Clinical Nutrition.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

AGP	α-1-acid glycoprotein
APP	Acute phase protein
APR	Acute phase response
BMI	Body mass index
CI	Confidence Interval
CRP	C-reactive protein
DNA	Deoxyribonucleic acid
EAR	Estimated average requirement
HIV	Human Immunodeficiency virus
ID	Identification
IDA	Iron deficiency anemia
IFA	Iron and folic acid
ITT	Intent to treat
IQR	Inter-quartile range
LIF	Lucky Iron Fish
MCH	Mean corpuscular haemoglobin
MCHC	Mean corpuscular haemoglobin concentration
MCV	Mean corpuscular volume
MMR	Maternal mortality rate
NECHR	National ethics committee for health research
RBC	Red blood cell
SD	Standard deviation
sTfR	Serum transferrin receptor
WHO	World Health Organization

Acknowledgements

First and foremost I would like to thank all of the women who voluntarily participated in this research study and all of the staff at NutriFood Cambodia for making this study possible. A special thank you to my colleagues Mr. Seang Ny, Mr. Kheang Meng Khin, Mr. Mesa Leang, Ms. Sreynet Thy, and Ms. Ratha Khin for your warm welcome into Cambodia and for helping with the data collection process.

Next, I like to extend my thanks to my advisor, Dr. Tim Green. Despite his desire at times, he allowed me to take on this project. Throughout this process I've improved my communication skills in both writing and orally, and critical thinking. You always provide valuable insight, and unconditional support. I would also like to thank my committee members Dr. Rickey Yada, and Dr. Gwen Chapman for supporting me over the last two years. I'm grateful for your support and valuable feedback.

I would also like to take this opportunity to thank my other academic mentors who've provided me with extremely valuable learning opportunities in research Dr. Susan Barr and in teaching Dr. Candice Rideout.

To my extremely supportive lab mates and colleagues Crystal, Kristina, Abeer, Kaela, Kyly, Dayna, Vashti, Phil, Alejandra, Amynah, Lin, Zach, Rebecca, and Allison thank you for making this positive learning experience memorable and fun. I always looked forward to long days in the Green Lab office, or crossing paths in Cambodia.

Lastly to my friends, family, and especially to Allison, thank you for your unconditional support, for always believing in me, and fixing my grammar.

Finally this research would not be possible without the funding provided by the University of Guelph, Grand Challenges Canada and the Danish Red Cross.

Chapter 1: Introduction

Cambodia is located in South-Eastern Asia north of the equator, bordering Lao People's Democratic Republic, Thailand and Vietnam. Cambodia has 23 provinces, and over 14 million inhabitants, with a population density of 85.1 people per square km (1,2). There are 4 different geographical regions in Cambodia: planar region, Tonle Sap region, coastal region, and the plateau and mountainous region (3). The two main water basins are the Mekong River and the Tonle Sap River originating from the South China Sea and Tonle Sap Lake, respectively (4). Generally, the climate in Cambodia is tropical with two distinct seasons: the dry season often referred to as the lean season, from November through April and the wet season where the people are busy harvesting from May thru October (4). In the capital city, Phnom Penh, mean rainfall ranges from 5 mm in January to 250 mm in October. As such, there are drastic seasonal differences in dietary diversity, and food availability.

Anemia remains a severe public health problem in Cambodia, according to the World Health Organization (WHO) and the most recent Demographic and Health Survey (5,6). The authors of the last Demographic and Health Survey reported a national prevalence of anemia (hemoglobin <120 g/L) among women of reproductive age of 45% (6). There are several negative consequences of anemia. For women who become pregnant there is an increased risk for maternal and perinatal mortality (5). In addition, anemia may lead to impaired work capacity and productivity, and may negatively impact cognitive and physical development in children (5). Globally, iron deficiency is believed to be the most common cause of anemia; it is estimated that it causes ~50% of all anemia (5). Iron deficiency is more common during periods of rapid growth such as childhood, adolescence and pregnancy because iron requirements are higher (5). However, it also occurs when dietary intake is low and absorption is poor, such as when

phytates, tannins, or other metal nutrients (i.e. calcium) are consumed in high amounts (5,7). In non-pregnant women of childbearing age, blood loss during menstruation is a major contributing factor leading to iron deficiency.

However, it is important to note that there are other causes of anemia (7). Other nutritional causes of anemia include folate, B-12, or vitamin A deficiency (7). In low income countries, such as Cambodia, parasitic infections such as hookworms can lead to blood loss (5,7). Parasitic infections, tuberculosis, and human immunodeficiency virus (HIV) can also lead to anemia of chronic disease (5,7). Finally, genetic hemoglobin disorders (hemoglobinopathies), which may present as anemia, have a high frequency in parts of Africa and South East Asia (7). Regardless of the cause of anemia, for non-pregnant women of childbearing age, anemia is defined as low hemoglobin concentration <120 g/L (8). Maternal anemia and postpartum hemorrhage during childbirth are the leading causes of maternal mortality globally, which may be an issue in Cambodia as a result of blood loss during childbirth among anemic women (9). The maternal mortality rate (MMR) in Cambodia is high with 170 deaths per 100,000 live births (6).

The WHO strongly suggests blanket intermittent supplementation with IFA in populations where the prevalence of anemia among women of reproductive age is greater than 20% (10). In accordance with this recommendation, the Cambodian government recommends routine iron supplementation for women of reproductive age (11–13). However, past research indicates poor adherence to iron supplements, and there are issues in Cambodia, as elsewhere, with distribution, procurement, and sustainability (12,14). As such, alternative approaches are needed to address iron deficiency anemia (IDA). The Lucky Iron Fish® (LIF®) is an iron ingot used during cooking as an in-home fortification system. The LIF works on the same principle as

cast iron cookware. When the LIF is placed in a pot with mild acid, iron is leached from the LIF into the pot and is believed to increase dietary intake of iron (15). Two randomized controlled trials of the iron ingot were carried out in Kandal Province, Cambodia (16,17) but had limitations and reported inconsistent findings. A larger trial with more robust biomarkers was needed.

This thesis presents the results of a 12-month randomized control trial of the LIF, an iron ingot to improve hemoglobin concentration in rural Cambodian women. This study was conducted in partnership between the University of British Columbia (Vancouver, Canada), University of Guelph (Guelph, Canada) and NutriFood Cambodia (Phnom Penh, Cambodia). This trial took place in Rovieng District of Preah Vihear Province from April 2015 – May 2016. A map of Cambodia with Preah Vihear (the province where this research was conducted) can be found in Figure 1. Women were randomized to daily oral iron (~ 57mg ferrous sulfate, 18 mg elemental iron) supplementation, the LIF, or control (nothing) for 12 months. Data were collected at baseline, after 6 months, and 12 months.

Figure 1: Map of Cambodia and surrounding areas

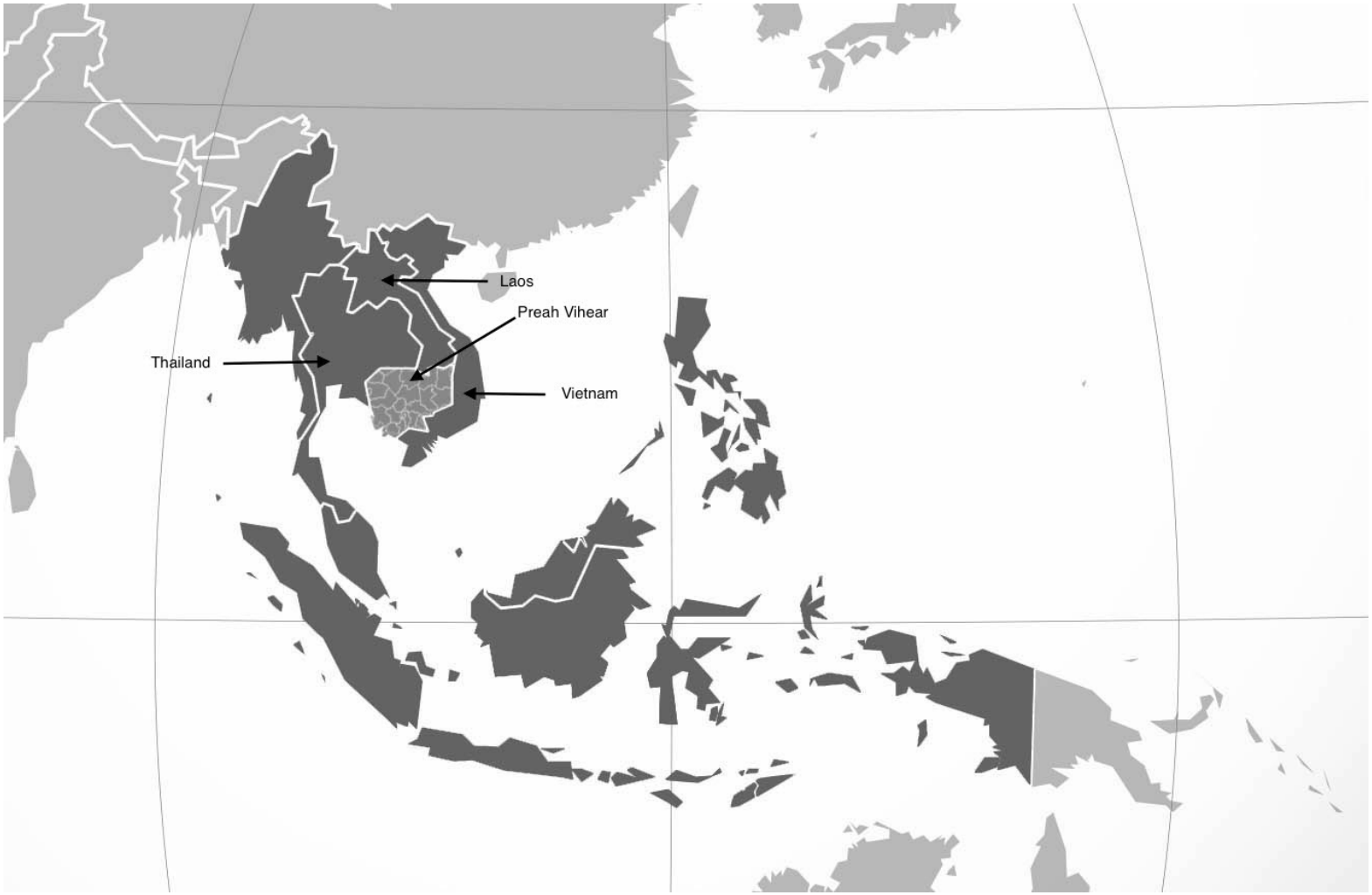


Image by "Creative Commons Southeast Asia (orthographic projection)" by keepscases is licensed under CC BY-SA 3.0. Colours were modified, map labels and provinces in Cambodia were added

Chapter 2: Literature Review

2.1 Preamble

In the literature review I will discuss the current potential causes of anemia in Cambodia. I will focus on nutritional anemia, anemia of chronic disease, and genetic hemoglobin disorders. Information on methods utilized to measure, diagnose, and differentiate between different forms of anemia will be reviewed. As this research is focused on iron deficiency and IDA, current policies and programs around provision of iron will be summarized. The mechanism and research on iron cookware will be reviewed. Past research on the LIF will be examined and a rationale will be provided for this clinical trial.

2.2 Anemia

According to the WHO anemia is a global health issue affecting 1.62 billion people in both developed and economically developing countries (5). The WHO also states that children and non-pregnant women of childbearing age living in Africa or Southeast Asia are at greatest risk for developing anemia (5). Anemia is a severe public health problem in Cambodia, with over 45% of women of childbearing age classified as anemic in the 2014 Demographic and Health Survey (5,18).

Anemia is a condition in which circulating erythrocyte concentration is low, or the oxygen carrying capacity of erythrocytes does not meet physiological needs (19). Erythrocytes contain hemoglobin which functions to transport oxygen in the body (20). The hemoglobin molecule is composed of a globin portion, which is four polypeptide chains and a heme group (20) . Normally, globin consists of two identical alpha and two identical beta chains. The other component is a heme group, found within each of the four polypeptide chains (20). An iron

molecule in its oxidized form (Fe^{2+}), is found within each heme group (20). Oxygen binds with this iron, and functions as a transporter of oxygen (20).

Clinically, anemia is diagnosed when the hemoglobin concentration of an individual falls below cutoff values dependent on their age, gender, and pregnancy status (5). The WHO recommends a cutoff of 120 g/L for non-pregnant women of childbearing age (5,8,19). Anemia defined by low hemoglobin concentration is often used as a proxy for iron deficiency (21–23). This is because iron deficiency is often thought to be the most common cause of anemia (19). In populations where iron deficiency is known to be the primary cause of anemia this assumption is generally acceptable (22). However, as will be discussed later, there are other causes of anemia (8). As such, in populations where the causes of anemia are complex, the use of hemoglobin concentration is a poor proxy for iron deficiency (21,24).

2.3 Consequences of anemia

Anemia is considered a severe public health problem due to its negative consequences (5). Pregnant women with anemia are at greater risk of negative pregnancy outcomes including maternal mortality resulting from blood loss during childbirth and infant mortality due to poor iron stores at birth (5,25).

Anemia also leads to a decreased work capacity and productivity due to feeling tired and fatigued (5,25). Feeling tired and fatigued can cause poor endurance, leading to poor efficiency of work output, and ultimately lower socioeconomic status (26). In addition, anemia impacts aerobic capacity, with moderate and severe anemia having a greater impact (26). In countries such as Cambodia, where a majority of the work is reliant on the agricultural industry, and therefore, requires manual labor, there are negative outcomes expressed at a national level on gross domestic product (27). In Bangladesh, a country similar to Cambodia as it has both high

rates of anemia and a high reliance on manual labor, anemia contributed to a loss of 1.5% of the country's total gross domestic product (27).

2.4 Causes of anemia

The causes of anemia can be classified into three broad categories: nutritional, genetics, or infection related (7). However it is also important to recognize that at any given time multiple causes of anemia can co-exist. This section will highlight the causes of anemia and why they are relevant in Cambodia.

2.4.1 Nutritional anemia - iron

IDA is a nutritional anemia and is thought to be the most common form of anemia (5,7,8,19,28). It is caused by a lack of iron in the body resulting from an inadequate dietary intake of iron, impaired absorption of iron, or from a loss of iron from the body (29). IDA will occur when there is an insufficient amount of iron stored in the body to sustain basic physiological functions when dietary intake of iron is low (8). IDA causes insufficient synthesis of hemoglobin, and microcytic cells (low mean corpuscular volume (MCV)) (8,20).

IDA is most common among women and children due to their higher than normal physiological needs (5). Women have a high requirement for iron due to menses: approximately 1.3-1.4 mg of iron is lost in blood per day during menstruation (30). Infants and children have a higher risk of iron deficiency, as breast milk is a poor source of iron and rapid growth is occurring which requires iron for proper and increased rates of erythropoiesis (28,30).

In Cambodia, IDA is believed to be common as polished white rice is a poor source of iron and contributes to upwards of 70% of total energy intake (2,31). In Cambodia there is also a high burden of parasitic infection leading to poor iron absorption and a loss of iron in stool (32). Iron absorption is inversely associated with iron status. However, even when iron status is low,

only a maximum of 2 – 3 mg of iron per day can be absorbed from the diet (33). In Southeast Asia, based on a mixed diet, iron bioavailability is estimated to be approximately 10% (34).

Iron deficiency can exist without anemia. Excess iron in the body is stored as ferritin (8). When dietary intake of iron is low, or absorption is poor, iron from storage is used for erythropoiesis. Over an extended period of time this can lead to IDA (20). Iron deficiency has similar consequences as IDA and can result in negative pregnancy outcomes, delayed or impaired cognitive development in children, and decreased work capacity and productivity (8).

2.4.1.1 Dietary intake of iron

There are two forms of iron in the diet: heme iron is found in animal sources such as beef, fish or poultry only, whereas non-heme iron is in plant-based sources like nuts, vegetables, and grains as well as in animal based sources (30). Heme iron is more readily absorbed than non-heme iron as it is not affected by chelators and ligands (30). In addition, iron competes with other metals for absorption, and its absorption is inhibited by tannins commonly found in coffee or tea, oxalic acid found in spinach or chocolate, phytates found in legumes, and phosvitin common in egg yolks (30). Several factors enhance the absorption of iron (especially non-heme iron): for example, sugars such as fructose, acids like ascorbic acid, mucin, and the consumption of meat, fish, or poultry at the same time (30).

At a population level, estimates of adequate dietary intake are assessed using the Estimated Average Requirement (EAR) (35). The EAR is set at 8.1 mg/day for women between 19 and 50 (35). There is little data on the iron intake of Cambodian women. A recent study conducted in Prey Veng, Cambodia reported that women of childbearing age consumed approximately 11 mg iron/day at baseline, and after 22 months of a homestead food production intervention this increased to 15 mg iron/day (36). A study conducted in Kandal Province

determined that women viewed bioavailable sources of iron such as beef, pork, chicken, and large fish to be expensive (37). Despite this, 98% of women in the study reported consuming small amounts of iron rich foods such as beef, pork, or shellfish in the day preceding the study (37). In addition to consuming animal sources of iron, morning glory (also referred to as kangkong or swamp cabbage) is a dark green vegetable and is a common inexpensive non-heme source of iron that is consumed often in rural Cambodian communities (37). According to the United States Department of Agriculture, in 100g of boiled and drained morning glory there is approximately 1.32 mg iron in the ferric form (Fe^{3+}) (38). While ferric iron is less bioavailable than ferrous, it provides a daily source of iron in the diet. No data on mean daily dietary intake of morning glory for Cambodia could be located. Ground water may be a significant source of iron in Cambodia and may contribute to iron intake. As such, dietary intake of iron in Cambodia may not be as low as originally thought (39,40).

2.4.1.2 Nutritional anemia – folate, vitamin B_{12}, and vitamin A

Anemia can also arise from deficiencies of other micronutrients. Folate and B_{12} deficiency leads to defective deoxyribonucleic acid (DNA) synthesis and therefore poor synthesis of red blood cells (RBCs) (7). Vitamin A deficiency has been linked to decreased incorporation of iron into hemoglobin, and lower levels of iron being released from stores. As such, Vitamin A deficiency can be found in combination with microcytic anemia.

Folate is naturally found in plant-based products and some animal products especially organ meats (30). Vitamin B_{12} is only found in animal products, with the best sources in meat products (30). Biochemically, these deficiencies manifest as megaloblastic anemia and are recognized as having elevated MCV and mean corpuscular hemoglobin (MCH) with normal mean corpuscular hemoglobin concentration (MCHC) values (29). Past research in Cambodia

indicates a low prevalence of folate and Vitamin B_{12} deficiencies (36,41). Vitamin A is found in liver, dairy products, and eggs. However, a recent study conducted in combination with the most recent Cambodia Demographic and Health Survey found the prevalence of Vitamin A deficiency to be less than 1% among women (41,42).

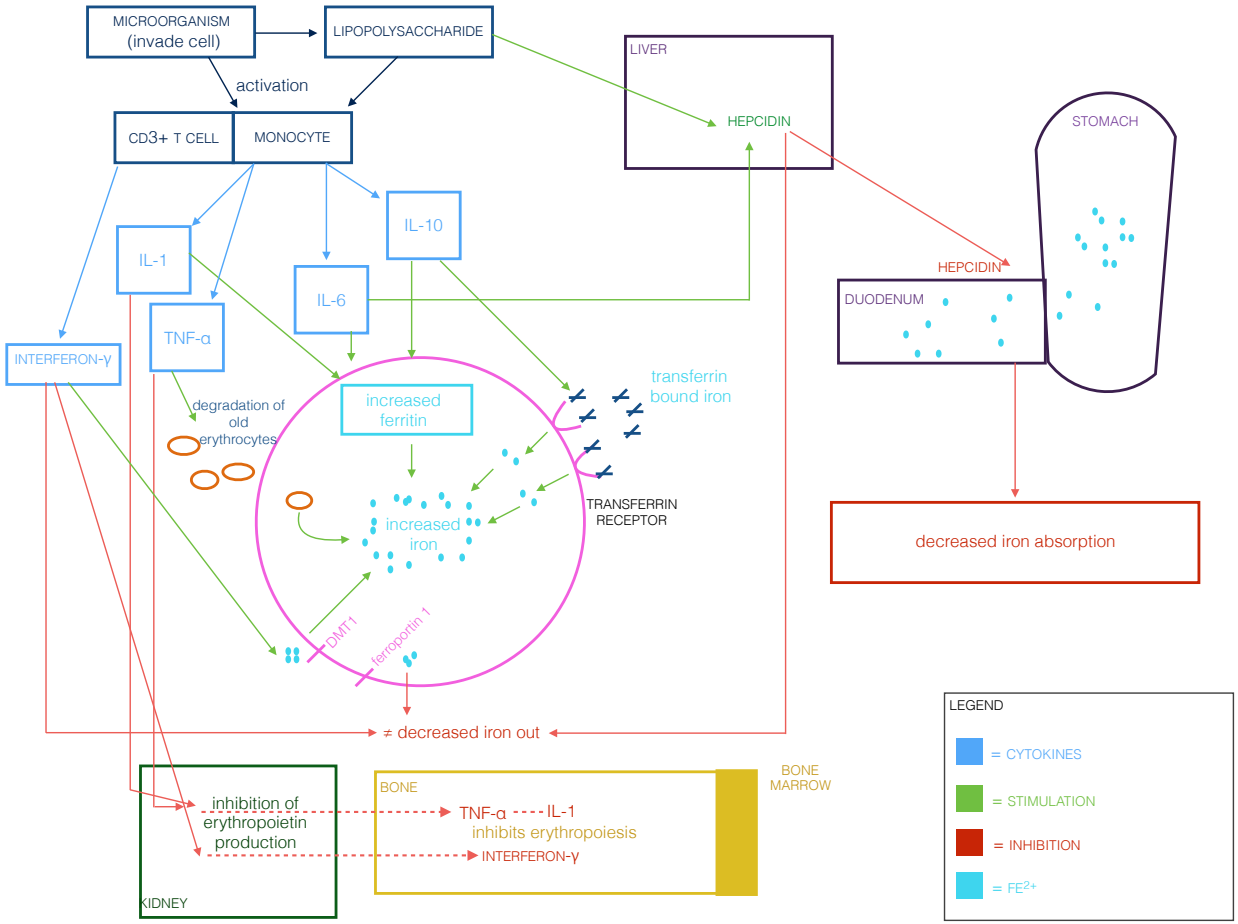
2.4.2 Anemia of chronic disease

Anemia of chronic disease, often referred to as anemia of chronic inflammation, is the second most common cause of anemia globally (43). The causes of anemia of chronic disease are widespread and include viral, bacterial and parasitic infections, cancer, and autoimmune diseases (44). In response to these stimuli the immune system produces cytokines, such as tumor necrosis factor-α, interferon-γ, interleukin (Il)-1, interlueukin-6, and interlueukin-10 (45). Interlueukin-6 causes the liver to produce hepcidin (45), a hormone that is not produced when iron stores are low (30,46). In the absence of infection, low circulating hepcidin levels are indicative of poor iron status (30,46). When iron status is low and hepcidin is absent, iron is released from the macrophages and is used for erythropoiesis or transported into cells (30,46). When iron status is adequate or high, hepcidin is released from the liver and degrades ferroportin on the cell membranes and iron is retained in the macrophages and enterocytes (30).

In the presence of infection, hepcidin is produced by interleukin-6 even if iron status is inadequate or low. The release of interferon -γ causes an increase in the expression of the divalent metal transport 1, which reduces the uptake of iron into the cell (44). Also, interferon-γ acts on the iron transporter ferroportin 1, and inhibits the release of cellular iron (44). Interlueukin-10 acts on the transferrin receptor and increases iron uptake by the cells, and it also acts on the cell and signals it to retain ferritin (44). Tumor necrosis factor- α causes the uptake of iron from degraded erythrocytes into the macrophages (44). In addition, tumor necrosis factor- α,

interferon – γ, and Interlueukin-1 inhibit the production of erythropoietin in the kidney (44). Eryhtropoitein is a hormone that stimulates erythropoiesis (20). The net result is increased iron in the macrophages and decreased iron absorption, circulating iron, and availability of iron for erythroid cells (20,44). As such, the series of stimulation and inhibition mechanisms remove iron from circulation and into macrophages as bacteria require iron for replication (44). When iron is present, but unavailable for erythropoiesis, it is often referred to as functional iron deficiency (29). Figure 2 depicts the stimulation and inhibition pathways involved in anemia of chronic disease.

Figure 2: Simple representation of the stimulation and inhibition pathways involved in anemia of chronic disease. This figure shows the impact of the release of various cytokines on iron.



Note: Figure adapted from Weiss and Goodnough 2005 (44).

IL = interleukin; TNF = tumor necrosis factor; DMT1 = divalent metal transporter 1

Malaria is a common cause of anemia of chronic disease in many countries. Malaria transmission is believed to be highest during the rainy season in Cambodia, with peak transmission in August to October (47,48). The most common malaria causing species in Cambodia is *P. falciparum*, which accounts for 86% of all malaria cases in the country (49). There are other malaria causing mosquitos in Cambodia, and it is estimated that *P. vivax* and *P. malariae* account for ~12% and ~2% of malaria cases, respectively (49). Malaria impacts erythropoiesis and therefore, is believed to be a contributing cause to anemia of chronic disease (44).

Even though malaria is not common in Cambodia, anemia of chronic disease may be common due to a high rate of other parasitic infections. A cross sectional study conducted in Cambodia in 2012 measured stool samples collected from 218 people from 67 households (32). Almost 30% of participants tested positive for hookworms by microscopic examination and about 57% tested positive for hookworms determined by polymerase chain reaction analysis (32). Those who did not wear shoes had a six-time greater risk of having hookworms than those who wore shoes (32). However, this study did not look at anemia prevalence.

2.4.3 Genetic hemoglobin disorders

Genetic hemoglobin disorders, often referred to as hemoglobinopathies, are recessive conditions inherited from one or both parents who are carriers for the abnormal gene (50). Normal hemoglobin consists of two pairs of unlike globin chains, an α pair combined with a β pair (51). Genetic hemoglobin disorders are caused by mutations that affect the synthesis of the globin chains impacting the hemoglobin molecule (52). Genetic hemoglobin disorders fall into two broad categories. First, structural variants of hemoglobin result from a single amino acid

substitution in the α or β globin chain (51). There are over 700 structural hemoglobin variants but the most common ones are hemoglobin S, hemoglobin C, or hemoglobin E (51). Second, and of more severe consequences than the structural variants of hemoglobin are thalassemias, which result from poorly synthesized and slower production of globin chains (50,53). These are characterized by ineffective and reduced synthesis of hemoglobin leading to anemia and as such, reduced oxygen carrying capacity (53).

The most common structural variant is hemoglobin E (51). In its heterozygous form, (hemoglobin AE) does not impact RBCs and RBC indices; when homozygous (hemoglobin EE), it results in hypochromic microcytic RBCs (54). This is of clinical significance because hemoglobin E is synthesized more slowly than normal hemoglobin (50). In addition hemoglobin E can interact with α or β thalassemia making it clinically relevant (51,54). There are over 200 mutations for the α and β globin chains (51). The severity of the mutations varies from no clinical symptoms to transfusion dependency (51). Hemoglobin E β^o-thalassemia (β^o/β^E) results in moderate to severe anemia, while hemoglobin E β^+-thalassemia (β^+/β^E) results in mild anemia (52).

A recent study conducted in Prey Veng Province, Cambodia found that 54% of women had an abnormal genotype for hemoglobin (41). The most common structural variant was the hemoglobin AE variant, and the most common thalassemia was the $\alpha^{3.7}$ thalassemia trait (41). Following these, the combination of hemoglobin E and the $\alpha^{3.7}$ thalassemia trait was prevalent (41). Likewise, two additional studies conducted in Cambodia found similar results (55,56). The first study conducted in 260 children living in Siem Reap Province determined that approximately 52% of children had a genetic hemoglobin disorder, with the most common being

hemoglobin AE (28.8%) (55). The α-globin gene was also prevalent, specifically $\alpha^{3.7}$ (28.4%) which is similar to the results found in Prey Veng (41,55).

The second study was conducted Cambodia in Battambang, Preah Vihear, and Phnom Penh (56). This study reported data on children with normal hemoglobin, those with hemoglobin AE, hemoglobin EE, and α^{+} thalassemia which occurs in combination with a structural hemoglobin variant (56). This study found that 42% of children had normal hemoglobin, 23% had hemoglobin AE, 14% had α^{+} thalassemia, 8% had hemoglobin E with α^{+} thalassemia, and 5% were hemoglobin EE (56). This blood was further analyzed and reported on in a recent paper (57).

A total of 1631 blood samples were collected from children in Battambang, Preah Vihear, and Phnom Penh (57). They reported the highest prevalence of β thalassemia and hemoglobin AE to be in Battambang and in Preah Vihear (57). Preah Vihear had the highest prevalence of hemoglobin H disease, as well as the highest frequency of hemoglobin Constant Spring (57).

2.5 Diagnosing anemia

2.5.1 Hemoglobin

Hemoglobin is a protein that is synthesized in all RBCs giving them their oxygen carrying capacity (30). There are two fragments in a hemoglobin molecule, the heme component which binds iron, and the globin portion which is a protein involved in oxygen transport (30). Before heme can bind to globin to create hemoglobin, iron is required in the heme molecule to bind nitrogen on the protein globin (30). Therefore, functional iron is required for hemoglobin synthesis, and without it, hemoglobin synthesis is halted, leading to low hemoglobin concentration in the blood (5,8,19,30).

The WHO defines normal, mild anemia, moderate anemia, and severe anemia for non-pregnant women of reproductive age (>15y) as a hemoglobin concentrations of >120 g/L, 110-119 g/L, 80-109 g/L, and <80 g/l, respectively (19). According to the International Committee for Standardization in Hematology, the gold standard for measuring hemoglobin concentration is the cyanmethemoglobin method (58–60). During this method, potassium cyanide and ferricyanide convert hemoglobin in blood into cyanmethemoglobin and absorbance is measured at 540 nm with a spectrophotometer (58,59,61). However, cyanide is toxic reagent that causes pollution, it is difficult to dispose of, and is not practical in field settings (61,62). As such, it is not commonly utilized in practice (8,63). Instead, automated hematology analyzers are commonly utilized in a laboratory setting, and are often referred to as the new gold standard for measuring hemoglobin concentration (8,63,64), as these machines have standardized quality control processes and calibration methods (8,63). Automated hematology analyzers have gained popularity as they often utilize non-cyanide reagents such as sodium lauryl sulfate. Sodium lauryl converts hemoglobin into methamoglobin where it measures hemoglobin concentration at

555nm (65,66). Automated hematology analyzers have been compared to the cyanmethemoglobin method and have proven to be accurate and precise (i.e. measures are close to the true value) (62,66).

Aside from measuring hemoglobin concentration, automated hematology analyzers also measure other parameters of RBCs such as MCV, MCH, and MCHC (8). These parameters can often be used to give indication of genetic hemoglobin disorders. For example, in a healthy adult population, MCV usually falls between 80-100 femtolitres (fL), but in thalassemic individuals or those with IDA, MCV is often decreased to <80 fL (20,67). As such, thalassemia and iron deficiency both present themselves with low MCV, making it challenging to differentiate between the two (67).

However, this analysis must be done on whole blood that is fresh, and in a field setting it is often difficult to transport blood for analysis in the short time frame (8). As such, the portable hemoglobinometers such as the Hemocue® (Hemocue Hemoglobin; Angelholm, Sweden) are commonly used on capillary blood to determine hemoglobin concentration in field settings (4,63).

2.6 Biomarkers of iron status

While hemoglobin concentration is used to diagnosis anemia, and is often used as a proxy for IDA at a population setting, there are cases where this inference should not be made. For example, in Cambodia, there is evidence indicating a high prevalence of infection and genetic hemoglobin disorders that are responsible for the high burden of anemia (32,41,48,55–57). As such biomarkers of iron status are needed. The gold standard method of iron deficiency diagnosis is through a bone marrow aspiration test, but this is expensive, painful, and invasive (69).

There are several biomarkers for iron deficiency and IDA. Reticulocyte hemoglobin content is a sensitive biomarker but cannot not be measured in field settings as it requires a fresh blood sample measured on a specific automatic hematology analyzer (70). In addition, erythrocyte zinc protoporphyrin, is commonly used particularly in children, but usually requires the washing of RBCs (70). Venous blood samples may be collected to measure biomarkers of iron status such as serum transferrin receptor (sTfR), and serum ferritin (71).

2.6.1 Serum ferritin

Almost all cells in the body contain ferritin, a protein found in the liver, bone marrow, spleen, and in reticuloendothelial cells (30,72). In addition, excess iron in the body is stored as ferritin (30,72). Previous research has determined that serum ferritin is a good indicator of body iron stores, as it increases when stores are high and decreases when stores are low (21,30). As such, serum ferritin is the biomarker commonly measured when assessing iron stores as it is less invasive than a bone marrow aspiration test (21,30). According to the WHO, iron deficiency occurs when serum ferritin concentrations fall below 15 μg/L for non-pregnant women of reproductive age, and IDA is defined by serum ferritin <15 μg/L and hemoglobin concentration <120 g/L (8,73).

Ferritin is an acute phase protein (APP) and is elevated in the presence of infection limiting the sensitivity of the measure (74). Fortunately, there are established methods to adjust serum ferritin measurements in populations with high rates of infection. There are two APPs which can be used to adjust ferritin in the presence of infection; C-reactive protein (CRP) and α-1-acid glycoprotein (AGP) (75). The first APP to react is CRP within the first 10 hours of infection onset (74,75). CRP reaches its maximum within 24 to 48 hours (74,75). AGP responds more slowly with its initial elevation in concentration beginning after twenty-four hours, and

AGP takes between 3 to 5 days to reach its maximum, therefore making it more indicative of chronic infection (74,75). While the cut-offs for infection are not widely agreed upon, cut-off values of >5 mg/L for CRP, >1 g/L for AGP are commonly utilized in practice (75,76). In this study it is important to measure the prevalence of inflammation using APP such as AGP and CRP as the prevalence of infection in Cambodia is high (32,48,77).

Thurnham *et al.* (78) suggest that iron deficiency is underestimated by 14% when serum ferritin values are not corrected for infection (78). There are a variety of methods suggested to address serum ferritin in populations with elevated CRP and AGP levels. For children under five years of age, the WHO recommends increasing the cut off value for depleted iron stores assessed using serum ferritin, however, an increased cut-off for serum ferritin has not been established for women of childbearing age (28).

Despite this, in a meta-analysis, Thurnham *et al*. (78) conclude that the increase in ferritin concentration during the acute phase response (APR) is proportional to the ferritin concentration before the onset of inflammation, and is dependent on the state on inflammation (78). Thurnham *et al.* (78) suggest correction factors for serum ferritin based on the state of infection into four different groups: a reference group with no elevated APPs, a group in the early stage of inflammation with elevated CRP (>5mg/L) (incubation), a group in early convalescence that has elevated CRP and AGP (>5 mg/L and >1g/L), and finally a group who is in late convalescence with elevated AGP (>1g/L) only (78). The results from this meta-analysis confirm that ferritin concentrations are quickly elevated at the onset of inflammation and are positively correlated with CRP concentrations (78).

2.6.2 Serum iron

Serum iron is an indicator of iron that is bound to transferrin in the blood (8). It is advantageous as it measures the iron supply of the bone marrow and various tissues, but is limited by its diurnal variability, change after a meal, and it is affected by chronic disease (8). Specifically, infection and inflammation decrease serum iron (8).

2.6.3 Serum transferrin receptor

Transferrin receptor is a protein found on the exterior of all cells and functions to regulate iron uptake into cells (29). The expression of transferrin receptor is proportional to the cell's iron needs (29). A soluble form of transferrin receptor, sTfR is found within the serum at levels proportional to the body's cells transferrin receptors (29). When ferritin drops below 15 μg/L, sTfR concentrations increase rapidly as the cells compete for iron (75). There is a lack of consensus about the most appropriate cutoff for sTfR. Tissue iron deficiency is believed to occur when sTfR values are >8.3 mg/L (29). Similar to these suggested cut-off values, a study found the mean sTfR concentration for middle aged healthy males and females to be 5.6 mg/L and ranged from 2.8 mg/L to 8.5 mg/L (79).

Unlike serum ferritin, sTfR is not an APP and is not affected by the presence of anemia of chronic disease, making it advantageous when trying to distinguish between IDA and anemia of chronic disease (29).

Because 75% of transferrin receptor is produced in the bone marrow, changes in erythropoiesis can affect sTfR concentrations. In Cambodia, due to the known high prevalence of genetic hemoglobin disorders, changes in erythropoiesis in this population affect sTfR concentrations and falsely elevate these values, limiting its use as a biomarker in Cambodia and other contexts where genetic hemoglobin disorders are prevalent (29,80). For example, a recent

study conducted in Cambodian women found that those with the hemoglobin EE genotype had a 50% and a 51% mean ferritin and mean sTfR concentration, respectively, compared to women with normal hemoglobin (80).

2.7 Treatment of anemia

2.7.1 Treatment of anemia in Cambodia

Cambodia has several strategies and policies to address anemia. In 2007, the Ministry of Health acted to address anemia by implementing an iron and folate supplementation program for pregnant and post-partum women (11). These guidelines include 90 iron and folic acid (IFA) tablets during pregnancy, and 42 IFA tablets postpartum (11). In both cases the tablets include 60 mg of iron and 400 μg of folic acid. The guidelines also recommend a single 500 mg dose of mebendazole de-worming treatment after the first trimester of pregnancy, and an additional dose during lactation (11). The last Demographic and Health Survey in Cambodia reported that 76%, 72%, and 49% took iron supplements during pregnancy for at least 90 days, took deworming during their last pregnancy, and took iron supplements postpartum, respectively (6).

In 2012, the Ministry of Health and the National Nutrition Program expanded this supplementation program. The expansion focused on improving the micronutrient status of women and children. For non-pregnant women of reproductive age without anemia, 60 mg of iron and 2.8 mg of folic acid are recommended weekly (12). For non-pregnant women of reproductive age with anemia, 60 mg of iron and 400 μg of folic acid are recommended twice daily for three months (12). Similarly, in 2012 the Ministry of Planning proclaimed minimum standards for the fortification of fish sauce and soy sauce with iron (81).

The current program by the Ministry of Health and National Nutrition Program is not consistent with the WHO's recommendation of intermittent IFA supplementation when the anemia prevalence is above 20% and daily blanket IFA supplementation when the anemia prevalence is above 40% (10,82).

2.7.2 Iron supplementation in Cambodia

A study was conducted in Siem Reap and Kampong Cham Provinces to determine factors associated with adherence to IFA supplements (14). Siem Reap and Kampong Cham have high poverty rates, and rely heavily on agriculture (14). In Siem Reap Province, there was only 25% adherence to IFA supplements (14). The Siem Reap and Kampong Cham study determined that education and knowledge about anemia and antenatal care are important determinants of adherence. Age and socioeconomic status had no impact on adherence to the supplements (14). With low compliance rates, and high costs associated with them, iron supplements may not be the most effective means to address IDA in Cambodia (27,83). Therefore, with the high national prevalence of anemia in Cambodia, and evidence of poor adherence to iron supplements in the country, other approaches to address IDA are needed, if warranted (5,14).

2.8 Food fortification

Due to poor adherence to iron supplements and issues associated with distribution and procurement, an alternative approach is necessary to address purported high rates of iron deficiency and IDA in Cambodia. A simple alternative is to increase the dietary intake of iron. Food fortification may be a potential cost effective method to address iron deficiency (82).

2.8.1 Iron fortified fish sauce and soy sauce

Iron fortified fish and soy sauce are one potential approach; costing approximately 0.02 USD more per litre than non-iron products, making this more cost effective than iron

supplements (84). In Cambodia staple condiments such as fish sauce or soy sauce are good vessels for fortification as they are consumed daily by a majority of the population (84). However, even though fortification has been in place since 2011, in the most recent Demographic and Health Survey in Cambodia in 2014 anemia rates were no lower than the previous surveys (6,84).

The form of iron used to fortify the fish sauce and soy sauce is sodium iron ethylenediaminetetraacetate (NaFeEDTA). NaFeEDTA is expensive, but is known to be a bioavailable, water-soluble form of iron (85). It has been reported that the median daily consumption of fish and soy sauce is about 11 mL and 8 mL, respectively (84). In a recent study the average iron content found in iron fortified products in 2012-2014 was 300 mg of iron per kg of fish or soy sauce (86). Therefore, if reported intakes are accurate, the estimated median daily consumption of iron from fortified products would be 5.9 mg if all fish and soy sauce consumed was fortified. However, only 25% of the fish and soy sauce produced in Cambodia is fortified with iron (86). In addition, the population that these products can reach is diminished, because poorer households are more likely to make their own fish sauce rather than purchasing it.

Despite the benefits and cost reduction associated with the use of iron fortified fish and soy sauce to increase dietary intake of iron, these products are not widely utilized, marketed, or accepted by consumers in Cambodia (84).

2.8.2 Cast iron and steel cookware and utensils

Another simple alternative to iron supplements is using cast iron utensils during cooking (87). This may be more effective than iron supplements, as it requires no behavior change. However, they have poor acceptance in developing countries because they are heavy and more expensive. Early research with animal models and laboratory analyses of foods determined the

potential impact when cooking with metal such as steel to increase the dietary intake of iron. However, these findings are not consistently supported with results from clinical trials.

2.8.3 Laboratory analysis of foods cooked in iron cookware

Multiple studies have confirmed that iron can successfully be transferred from iron cookware to foods. All studies confirmed that acidic cooking environments and cooking time, particularly longer duration, positively impact the iron level in foods when prepared in iron cookware (88–91). These studies and their results are summarized in Table 1. The greatest transfer of iron was seen in applesauce cooked in cast iron with 6.20 mg of iron per 100 mg of applesauce (92).

Table 1: Summary of evidence of laboratory analysis of foods cooked in iron cookware

Authors	Year	Ingredients	Materials and Methods	Results
Mistry, A. N. Brittin, H. C. Stoecker, B. J.	1988	Cooked lean ground beef, fried potatoes, cornmeal pancakes, applesauce, and vanilla pudding	*Materials:* 26.7 cm iron skillets and 22.9 cm corning ware glass dishes were used for cooking *Methods:* Iron content was analyzed in the non-iron and iron utensils	*Non-significant results ($p>0.05$)*: Lean ground beef, fried potatoes, cornmeal pancakes, vanilla pudding had no significant differences when cooked in iron and glass. *Significant results ($p<0.01$)*: Applesauce 5.20mg/100g food compared to 0.13mg/100g in glass. All foods cooked together 1.67 mg/100g in iron compared to 0.54 mg/100g in glass.
Cheng, Y. J. Brittin, H. C.	1991	Applesauce and spaghetti sauce	*Materials:* 26.7 cm iron skillets and 22.9 cm corning ware glass dishes were used for cooking *Methods:* Food was analyzed raw, and after cooking in the iron and glass cookware. Fifty samples of each sauce were analyzed in both types of cookware	Applesauce cooked in iron (6.26 mg iron/100g of food) had significantly more iron ($p<0.05$) than raw applesauce (0.26 mg/100g) and applesauce cooked in glass (0.18 mg/100g). Spaghetti sauce cooked in iron (2.10 mg iron/100g of food) had significantly more iron ($p<0.05$) than raw spaghetti sauce (0.22 mg/100g) and spaghetti sauce cooked in glass (0.44 mg/100g). The number of cooking uses did not change the iron content of the two foods. Excluding the first two uses, iron content of the foods was consistent among uses ($p>0.05$).
Brittin, H.C. Yang-Dong, Z.	1994	10 different foods (i.e. eggs, sweet and sour sauce, stir-fried pork, stir fried green beans)	*Materials:* Steel Wok, 22.9 cm corning ware glass. *Methods:* raw food, food cooked in a wok, and foods cooked in glass were analyzed	All foods except eggs cooked in the steel wok contained higher iron than in the glass utensil ($p>0.05$). The food with the highest iron content was the sweet and sour sauce ($p<0.05$).
Brittin, H. C. Kollipara, U. K.	1996	Tomato chutney, stir-fried green beans, and chicken curry, chapatis, scrambled eggs, and dosa	*Materials*: Iron karahi (pot), iron tavas (pan), stainless steel karahi and glass pots *Methods:* Food was analyzed raw, and after cooking. Three replications were done	All foods cooked in the iron karahi and iron tavas contained more iron than foods cooked in glass and foods cooked in stainless steel. ($p<0.05$). Tomato chutney cooked in the iron karahi high the highest iron content (50.03 mg/100g food) compared to tomato chutney cooked in stainless steel karahi (1.78 mg/100g of food) and in glass (1.64 mg/100g of food) ($p<0.05$).

2.8.4 Animal models with iron and steel cookware

The bioavailability of iron in foods prepared with a source of iron was confirmed by Rosanoff and Kennedy (93) in an animal model (93). Sixty-eight rats were placed on an iron deficient diet for 23 days; hemoglobin concentration was less than 6.0 g/dL in all rats after this period (93). The rats were then randomly assigned to one of eight groups, four groups received a basal diet and apples cooked with steel nails at varying levels, and four groups received a basal diet and varying levels of ferrous sulfate (93). For the group receiving medium amounts of ferrous sulfate, and the group with medium iron from apples cooked with steel, mean ±SD hemoglobin concentration improved by 0.73±0.16 g/dL and 1.3±0.16 g/dL, respectively at 12 months (93). Similar trends were reported among all groups consuming added iron (93). The results of this study showed the potential to improve hemoglobin concentration in rats by increasing dietary intake of iron when iron was added during the cooking process (93).

A follow-up study conducted in rats by Martinez and Vannucchi confirmed these results in iron deficient rats. One group received a control diet with 36 mg/kg of iron as ferrous sulfate, a second group received a basal diet similar to the control without iron, and finally a third group received the basal diet cooked in an iron pot (94). After four weeks, hemoglobin concentration (mean ±SD) was not statistically significant between the control group receiving ferrous sulfate 12.8 ±1.3 g/dL and the basal group receiving food cooked in an iron pot 13.9 ± 0.9 g/dL (94). The group consuming ferrous sulfate, and the group consuming a diet cooked with iron had significantly higher hemoglobin than the group consuming the basal diet ($p<0.05$) (94). These studies suggest that iron leached during cooking from iron cookware may be a useful means of preventing or treating iron deficiency (93,94).

2.8.5 Iron cookware clinical trials

Several clinical trials have evaluated the efficacy of cast iron cookware to improve hemoglobin status. However, results from these trials are not as promising as laboratory and animal models suggest.

A trial in Ethiopia measured the impact of iron cookware versus aluminum pots on hemoglobin concentration in 407 children after one year (95). Differences in hemoglobin concentration at 12 months, adjusted for baseline hemoglobin value, between the two groups were significantly different (mean (95% confidence interval (CI)) (1.0 (0.9-1.4) p = 0.008) (95).

A randomized trial conducted in Malawi enrolled 322 people over the age of one to compare hemoglobin concentration in a group randomized to an iron pot compared to an aluminum pot group over 20 weeks (96). The results from this study showed an improvement in mean ± (SD) hemoglobin concentration after 20 weeks of consistent iron pot use in those older than 12 years (5.3 ± 8.7 g/L) compared to an aluminum pot group (-2.2 ± 13.2 g/L) $p<0.05$ (96). These findings were not found for participants less than 12 years, nor in those who did not use their pot regularly (96).

In Benin, Zlotkin *et al.* (97) compared the use of cooking with a cast iron pot, to a blue steel pot (similar to forged iron but is a lighter alternative) to a control group who received iron supplements or multiple micronutrient powders depending on their age (97). This cluster-randomized control included 161 households and 339 participants including a population with ~50% anemia in young children, adolescents, and women of reproductive age (97). The duration of this clinical trial was one year (97). There were no significant differences between groups in hemoglobin concentration after 12 months (p=0.5) (97). After 12 months, the control group

consuming multiple micronutrient powders or iron supplements had significantly higher serum ferritin (median interquartile ranges (IQR)) 59 (3.4 – 440) than both the iron cookware groups; cast iron was 37 (2.6 – 296) and blue steel: 44 (6.3 – 241) $p<0.001$ (97) The differences in serum ferritin at 12 months for the cast iron and blue steel groups were non-significant $p =0.99$ (97). Even though baseline concentrations of serum ferritin in this study were high, this study does suggest that serum ferritin can be improved by iron supplements and multiple micronutrient powders, but not by iron cookware. More details on these studies can be found in Table 2.

Therefore, iron pots and pans for use during cooking do not appear to be a suitable alternative to improve hemoglobin concentration for women in low- and middle-income countries like Cambodia. It is unclear if this has to do with adherence, bioavailability of the iron provided, duration of cooking or the acidity of the foods being cooked in the cookware as laboratory studies prove they leach iron into the food and can increase dietary intake of iron in animal models.

2.8.6 Acceptability of iron cookware

An acceptability study conducted in Malawi found that cast iron pots and pans were not widely accepted (98). In fact, they were used less frequently than their aluminum counterparts, and 100% of respondents reported that they were too heavy (98). Another study in Tanzania found similar results, showing that respondents preferred stainless steel over mild steel, blue steel, and cast iron (99) . Similarly and most important to this research, their acceptance in Cambodia is low (16). Iron pots are more expensive, less available, rust faster, and are heavier than aluminum pots (16). There is evidence indicating that less than 1% of households in Prey Veng Cambodia are using iron pots (personal communication, May 2015).

Table 2: Summary of iron cookware trials in developing countries

Authors	Year	Sample Size and Design	Outcomes	Methods	Results
Adish, A. A. Esrey, S. A. Gyorkos, T. W. Jean-Baptiste, J. Rojhani, A.	1999	*Sample:* 407 non-anemic and anemic children aged 2 – 5 *Randomized trial*	Hemoglobin concentration by Hemocue from capillary	Comparing hemoglobin concentration after 1 year in children randomized to an iron pot or aluminum pot	There was a significant difference in haemoglobin concentration in children consuming food from the iron pot compared to the aluminum pot. Data are reported as differences in hemoglobin between groups and adjusted for baseline hemoglobin mean (95% CI). 3 months: 1.2 (0.9-1.5) 12 months: 1.2 (1.0 – 1.4)
Geerligs, P. P. Brabin, B. Mkumbwa, A. Broadhead, R. Cuevas, L. E.	2003	*Sample:* 322 non-anemic and anemic older than 1 year *Randomized trial*	Hemoglobin concentration by Hemocue from capillary	Comparing hemoglobin concentration in children randomized to and iron pot or aluminum pot after 20 weeks	No significant differences in mean hemoglobin at 20 weeks for those less than or ≥ 12 years. Consistent use (food consumed at least 7 times from the pot in the last week) did not have an effect on mean haemoglobin change in children under 12, but did in those ≥ 12 years. Consistent users at 20 weeks <12: Mean ± (SD) p>0.05 Iron pot: 6.8 ± (19) Aluminum pot: 4.0 ± (14.2) Consistent users at 20 weeks ≥12: Mean ± (SD) p>0.05 Iron pot: 5.3 ± (8.7) Aluminum pot: - 2.2 ± (13.2)
Sharieff, W. Dofonsou, J. Zlotkin, S.	2007	*Sample:* 161 households with infants (6-24 mos) adolescent girls (11-15y), women (15-44y) *Cluster randomized trial*	Hemoglobin concentration by Hemocue from venous and SF	Interventions: Cast iron, blue steel, and a control group which got MNP for infants, and supplements for women	There were no significant differences between groups in hemoglobin concentration at endline or baseline. After 12 months, the control had significantly higher SF than the iron cookware groups. Median (min - max) - Cast iron: 37 (2.6 – 296) - Blue steel: 44 (6.3 – 241) - Control: 59 (3.4 – 440)

Hemoglobin = hemoglobin, SF = serum ferritin, MNP = Micronutrient Powders

2.9 The Lucky Iron Fish

2.9.1 History of the LIF

As there is evidence of poor acceptability of iron cookware and poor adherence to iron supplements in Cambodia an alternative approach may be needed. This was the impetus for the development of the LIF, an iron ingot used during cooking as an in-home fortification system for iron transfer. The use of the LIF is simple and requires little behavior change as it is placed in an aluminum pot during cooking.

The LIF was designed in collaboration with village elders and community members to ensure it would be accepted in Cambodia (16). The iron ingot resembles a local fish believed to be lucky among villages in Cambodia, contributing to the acceptability of the ingot (16).

2.9.2 Iron released from the LIF

Local scrap metal from recycled car parts is used to produce the LIF in Cambodia. Quality assurance tests are conducted on the raw iron, the LIF, and on the water that the LIF has been boiled in. These tests are conducted on a regular basis at the Royal University of Phnom Penh in accordance with Hazard Analysis & Critical Control Points. These tests are audited at the University of Guelph to ensure the product's safety and that the results are verified (15). Levels of manganese, arsenic, nickel, mercury, copper, zinc, lead, iron and magnesium are measured in the water to ensure that the levels of metal contaminants fall within the WHO drinking water guidelines (15). Data obtained from these tests have shown that the water boiled with the LIF met these standards (15,100).

A laboratory test conducted at the University of Guelph also determined the amount of iron that the LIF leaches (15). The iron content of food and water prepared using the LIF was

determined through laboratory analysis of five samples at the University of Guelph (15). The five samples were analyzed in both glass and aluminum pots and are listed by decreasing pH; pure distilled water, saline distilled water, Khmer pork soup, Khmer fish soup, and weakly acidic distilled water (15). Three replicates of each of the five samples were prepared and analyzed (15). There were trace amounts of iron found in the glass and aluminum pots boiled without the LIF and no significant differences were found between these two vessels (15). Results show significantly more iron leached ($p<0.001$) during cooking in the acidic cooking environments compared to saline water, and pure distilled water (15). However, it is unclear why the less acidic pork soup resulted in significantly more iron leached compared to the Khmer fish soup which has higher acidity reported ($p<0.001$) (15). Results can be found in Table 3.

Table 3: Iron content of foods cooked with the LIF at differing pH

	Glass pot with LIF (mg iron/L)	Approximate intake*	Aluminum Pot with LIF (mg iron/L)	Approximate intake*
pH 7.06	<0.40 (0)	0.22%	<0.40 (0)	0.22%
pH 5.80	<0.40 (0)	0.22%	<0.40 (0)	0.22%
pH 4.58	41.3 (7.5)	22.9%	27.7 (11.72)	15.4%
pH 4.45	4.1 (3.01)	2.3%	2.8 (1.7)	1.6%
pH 3.20	84.7 (15.04)	47.1%	68.3 (18.82)	37.9%

Notes: Data are reported as mean (SD)
Table is adapted from Charles, C. V. *et al* (2011) (15)
*Approximate intake is expressed as a percent of the recommended dietary allowance for individual non-pregnant women of childbearing age (35), assumed 10% bioavailability of iron and 1 L was consumed (34).

2.9.3 Previous trials of the LIF

The efficacy of LIF was first examined in a three-arm randomized control trial conducted in Kandal Province Cambodia (16). The three groups participated in the six month trial: a group who received the LIF with an introductory session on how to use it, and three weekly follow-up

sessions (n=60); a group who received the LIF with an introductory session but no follow-up (n=60); and a control group (n=60) (16). There was a statistically significant improvement in hemoglobin concentration at three months but not at 6 months for the LIF group who received follow-up sessions (13.02 ± 0.95) compared to the control group (12.05 ±1.17; p=0.001) and the other LIF group (12.51 ± 1.06; p=0.03) (16). Serum iron, an indicator of iron status, was not different among the groups at either time-point and actually dropped over time (16).

The authors speculate that different findings at three and six months may be due to changes in the sources of drinking water during the study associated with seasonal differences. For the first three months of this trial during the rainy season women relied on rainwater for cooking, but during the last three months of this trial, which was the dry season, water was collected from wells. In Cambodia, well water is known to be high in arsenic, which binds to iron preventing absorption (16). On the other hand, in Cambodia well water is often high in iron (39,40). However, the iron content of drinking water was not measured (16).

This study used serum iron as a biomarker of iron status. Serum iron is a transient form of iron only circulating in the blood for approximately forty-five minutes (8). Serum iron is affected by dietary intake, and these blood samples were non-fasted, and it is unclear whether the samples were taken at the same time of day which may lead to a systematic measurement error limiting the interpretation of the results (8). A recent study reported that Kandal Province has reported high levels of *Opistorchis viverrini* and *Strongyloides stercoralis* infection (77,101). Research indicates that parasitic infections are a major contributor to anemia of chronic disease and thus the serum iron measurements may have been confounded (44). Genetic hemoglobin disorders were not measured despite their known prevalence in Cambodia (41,51,55). This is important as

hemoglobin disorders confound biomarkers of iron status such as hemoglobin and serum iron (8). Finally, the primary outcome was not specified, and the power calculation is unclear, leading to the inability to draw conclusions from this trial.

Another study on the LIF has been published recently, showing potential success. This study was a three arm randomized controlled trial in Kandal province with non-pregnant women of reproductive age (17). A group received the LIF without follow-up (n=104), another group received the LIF with six follow-up sessions that included education on nutrition (n=104), and a control group did not receive anything other than follow-up (n=102) (17). This trial was stronger than the previous LIF trial as it was one year in duration, and it measured serum ferritin, CRP, and hemoglobin concentration determined using venous blood on the Hemocue which is known to be less variable than capillary blood (102). This clinical trial reported a significant improvement in hemoglobin concentration after 9 months of follow-up for the intervention group (123 (122,125) g/L) compared to the control group (118 (116,121) g/L) $p < 0.05$ (17). This remained consistent at 12 months as well, the intervention group had significantly higher hemoglobin (130 (128,132) than the control group (120 (127,123)), $p<0.05$ (17). The study reports a significant improvement $p<0.05$ in serum ferritin at 12 months for the intervention group (102 (89, 116)) compared to the control group (66 (57, 76)) (17). However, these results are limited by the low prevalence of iron deficiency (<16%) and IDA (<14%) at baseline (17). A summary of these results can be found in Table 4. This questions the efficacy of the LIF in Cambodia as it is designed to address iron deficiency, which appears to be quite low in this population (6,41).

However, conclusions about the efficacy of the LIF could not be drawn from this trial either as there are several methodological flaws. First, this study allowed more than one woman from each household to be included. In Cambodia it is culturally and traditionally common for those residing in the same household to eat from the same cooking pot (personal communication, 2015). Therefore, it is likely that a woman from the control group was sharing a pot with a woman in either intervention group.

Second, while it is a strength that CRP was measured, it is unclear why the authors did not correct serum ferritin for CRP values. Rather the authors excluded all women with CRP >10 mg/L which is not commonly utilized in practice. Instead generally established correction factors are used, or women with CRP>5 mg/L are excluded from analysis (21,103).

When analyzing and presenting the results the two intervention groups are pooled together, and it is difficult to determine if the education and follow-up visits had anything to do with the increase in hemoglobin concentration and serum ferritin for the LIF groups. It is unclear if the power calculation presented is for two or three groups, and what the primary outcome variable is. Additionally, it is unclear if having more women in the intervention group was an issue in their data analysis by narrowing the CI.

Therefore, before the LIF could be widely recommended, more research had to be done. The three-arm randomized control trial presented in this thesis addresses gaps to previous research. This trial was one year in duration, uses more robust biomarkers of iron status, and considers genetic hemoglobin disorders to determine the efficacy of the LIF in rural Cambodian women of reproductive age.

Table 4: Summary of the most recent LIF trial

Year	Sample Size and Design	Outcomes	Results
2015	Intervention n= 104 LIF follow- up n= 104 LIF no follow-up n= 102 control: nothing	Hemoglobin and serum ferritin at 3, 6, 9, and 12 months	

	Control (n=84)	Intervention (n=164)	p-value
Hb baseline	119 (116, 122)	117 (114, 119)	
Hb 9 months	118 (116, 121)	123 (122, 125)	p <0.05
Hb endline	120 (117, 123)	130 (128, 132)	p <0.05
SF baseline	54 (46, 61)	59 (51, 68)	NS
SF 9 months	61 (51, 71)	75 (63, 87)	NS
SF endline	66 (57, 76)	102 (89, 116)	p <0.05

Note: Hb = hemoglobin, SF = serum ferritin, NS = not significant Data from Charles *et al.* 2015 (17)

2.10 Objectives

The aim of this research was to determine the efficacy of an iron ingot (LIF) to improve hemoglobin and biomarkers of iron status among anemic rural Cambodian women. Partnered with the University of Guelph, Nutrifood Cambodia, and with support from the Ministry of Health in Cambodia and the National Institute of Public Health Laboratory (Laboratory (Phnom Penh, Cambodia), we conducted a one year randomized controlled trial to determine the efficacy of the LIF on hemoglobin concentration compared to a similar dose iron supplement and control.

Primary Research Question:

Is there a difference in hemoglobin concentration among rural Cambodian anemic women (18-49 y) cooking with the LIF compared to a group taking a daily iron supplement and or control after 6 months or one year?

Secondary Research Questions:

Is there a difference in anemia prevalence among rural Cambodian anemic women (18-49 y) cooking with the LIF compared to a group taking a daily iron supplement and or control after 6 months or one year?

Is there a difference in biomarkers of iron status (serum ferritin and/or sTfR) among rural Cambodian anemic women (18-49 y) cooking with the LIF compared to a group taking a daily iron supplement and or control after six months one year?

Is there a difference in iron deficiency and/or iron deficiency anemia prevalence among rural Cambodian anemic women (18-49) cooking with the LIF compared to a group taking a daily iron supplement and or control after six months or one year?

2.11 Hypotheses

Primary

Null Hypothesis (H_0): The use of the LIF and iron supplements over a 12-month period will not result in a significantly higher hemoglobin concentration compared to the control.

Research Hypothesis (H_A): The use of the LIF and iron supplements over a 12 month period will result in a significantly higher hemoglobin concentration compared to the control.

Secondary

Null Hypothesis (H_0): The use of the LIF and iron supplements over a 12-month period will not result in a significantly lower anemia prevalence compared to the control.

Research Hypothesis (H_A): The use of the LIF and iron supplements over a 12-month period will result in a significantly lower anemia prevalence compared to the control.

Null Hypothesis (H_0): The use of the LIF and iron supplements over a 12-month period will not result in a significant improvement of biomarkers of iron status (ferritin, sTfR) compared to the control.

Research Hypothesis (H_A): The use of the LIF and iron supplements over a 12-month period will result in a significant improvement of biomarkers of iron status (ferritin, sTfR) compared to the control.

Null Hypothesis (H_0): The use of the LIF and iron supplements over a 12-month period will not result in a significant improvement in iron deficiency and/or iron deficiency anemia compared to the control.

Research Hypothesis (H_A): The use of the LIF and iron supplements over a 12 month period will result in a significant improvement in iron deficiency and/or iron deficiency anemia compared to the control.

Chapter 3: Research design and methods

3.1 Purpose

The primary purpose of this research was to determine if cooking with the LIF results in higher hemoglobin concentration compared to iron supplements providing an equivalent amount of iron and a control group in anemic women of reproductive age living in Preah Vihear, Cambodia after one year. Outcomes from this clinical trial will help inform policy within the Ministry of Health, and the Ministry of Planning on the use of the LIF in Cambodia. A secondary objective of this trial was to determine if cooking with the LIF results in better iron status based on iron biomarkers (ferritin, sTfR) compared to the women consuming iron supplements, and control group after 12 months.

3.2 Overview of study design

This study was a randomized control trial in Preah Vihear Province Cambodia. Women were screened for anemia over ten days in April 2015 from 18 different villages in 8 different communes in Rovieng district, Preah Vihear Province. We aimed to recruit 330 women with mild or moderate anemia (hemoglobin <120 g/L) for this clinical trial.

After recruitment, women were randomized to receive LIF, 18 mg/d of elemental iron, or control. All three groups received nutrition education. Training on the use of the LIF, and on how to take iron supplements were provided to women randomized to those groups. Blood was collected at baseline, 6 months and 12 months. Women were followed up monthly to measure adherence and adverse effects.

3.3 Setting and rationale

We chose to conduct the research in Preah Vihear Province as its topography differs from Kandal, where past research on the LIF was conducted. Kandal's topography is planar and it is located in the southeast part of Cambodia. Two of Cambodia's largest rivers (the Bassac, and the Mekong) flow through this province. Preah Vihear is a plateau and mountainous region and is located in northern Cambodia boarding Thailand and Laos. Unlike Kandal, there are no rivers in Preah Vihear creating a different food environment. A list of all participating villages and communes can be found in (Appendix A).

A majority, 80%, of Cambodia's total population lives in rural areas, which is another reason Preah Vihear Province was chosen as a study location for this clinical trial (6). This province is one of the least developed provinces in Cambodia with approximately 47% of its inhabitants living in the lowest wealth quintile (6). Preah Vihear Province has a slightly higher anemia rate (54%) than the national average in Cambodia (45%) (6). Preah Vihear Province has one the highest rates of infant and under five mortality in the country at 70 deaths per 1000 live births and 79 deaths per 1000 live births, respectively (12). Preah Vihear has the highest rate of stunting and also has a high percentage of children under 5 that are wasted, 44% and 15%, respectively (6). Children are twice as likely to be wasted if their mothers are underweight, defined as having a BMI <18.5 kg/m^2 (6,104). Likewise, women in Preah Vihear Province, are more likely to be underweight (16.4%) than the national average for women living in rural areas (14.1%) (6).

3.4 Iron content of supplements and the LIF

A supplement dose of 18 mg per day of elemental iron (56.94 mg ferrous sulfate) was chosen for this study. This dose was chosen because previous research determined that women are obtaining approximately 8 – 15 mg of iron daily from the LIF depending on the number of uses, amount consumed, and content (i.e. pH) it was boiled with. Iron leached from the LIF has been tested and proved to be in its ferrous state and bioavailable with acidity and cooking time impacting the amount of iron leached (15,105).

In order to ensure that the LIF is safe to use in terms of its metal contaminants, and that it leaches comparable quantities of iron into the water during the boiling process as iron supplements I conducted a small study at the University of British Columbia. Water samples were analyzed at Agat laboratories (Burnaby, BC Canada). Data collection information and results from this study can be found in (Appendix B).

3.5 Ethics

Ethical approval to conduct this research was granted by the Cambodian National Ethics Committee for Health Research (NECHR) No: 0319 NECHR and UBC's Clinical Research Ethics Board, approval number H14-02551. Written informed consent was obtained from all women (Appendix C).

3.6 Recruitment

A convenience sample from 18 different villages in 8 different communes in Rovieng district was obtained.

3.7 Study participants

Inclusion criteria

To participate in this study women must have met the following criteria:

i. 18 to 49 y
ii. Be the female head of the household
iii. Have mild or moderate anemia defined by a hemoglobin concentration between 80 and 119 g/L at screening as assessed using a portable hemoglobinometer (Hemocue Hb 301)
iv. Willing to provide a finger prick and venous blood sample at baseline, 6 months, and 12 months
v. Not ill or taking medications
vi. Not pregnant (based on self-report)
vii. Not currently consuming or planning to consume iron supplements in the next 12 months
viii. Not planning to move in the next 12 months
ix. Not currently participating in any other nutrition intervention
x. Able to provide informed written consent

3.8 Procedures

Two days of training were held for the research team before each data collection time point. Ly Sokhoing from Helen Keller International led the training. During the first day of training, data collectors were given instructions on how to properly fill out the consent form, complete the questionnaire, and take anthropometric measurements. During the second day of training, a field pre-test was conducted using the consent forms and questionnaires at the Niroth Health Centre in Mean Chey, Phnom Penh. Following the pre-test a meeting was held to discuss inconsistencies. The questionnaire was then modified to fix errors in translation and prompts were added to ensure appropriate responses were recorded. At 6 and 12 months data collection,

training was identical but included instructions on how to correctly fill out the monitoring and evaluation forms used monthly in this study.

3.8.1 Screening and recruitment

Informed consent was obtained from all women. After obtaining consent, if the women met eligibility requirements a finger prick blood sample was obtained by a trained staff member from National Institute of Public Health Laboratory to determine hemoglobin concentration using the Hemocue Hb 301.

3.8.2 Randomization

Each participant was given a unique identification (ID) number (001 to 340). After recruitment, women were randomized into one of three groups using a random digit generator on Microsoft excel (Microsoft Corporation, Redmond, WA, 2010).

3.8.3 Data collection

At baseline, 6 months and 12 months a team travelled to 18 villages over 8 days. Depending on the village, we met the women at the village chief home, the health centre, or the Buddhist hall in the community between 6:00 am and 7:00 am for the morning, and around 1:00 pm in the afternoon. A sample schedule can be found in (Appendix D). At each time point (baseline, 6 months, and 12 months) capillary blood was used to determine hemoglobin concentration on the Hemocue Hb 301 and venous blood was collected, a questionnaire was administered, and anthropometry was measured. Adherence and adverse events were monitored monthly.

During baseline, training on the use of the LIF was provided to women randomized to this group by the Cambodian Operations Manager of the LIF. Women were instructed to use the LIF once per day, and to add an acidic food (i.e. lemon) when boiling the LIF. At the same time,

training on how to best take the iron supplements, and on how to open the pill bottles was provided to women in this group.

After baseline data collection, women received a sarong with a value of (~$2.50 USD). At 6 months and 12 months, women were given a sarong, a bag of laundry soap, a bottle of fish sauce, and two cans of juice for participating in this data collection period (~$4.00 USD).

3.8.4 Biochemical assessment

3.8.4.1 Capillary blood

At each time point, hemoglobin concentration was determined using capillary blood on the Hemocue Hb 301 machine (106). The Hemocue machines were cleaned daily and checked using quality control solutions (Hemotrol Level 1, 2, and 3 solutions; Eurotrol E.V.; Ede, The Netherlands).

3.8.4.2 Venous blood

A non-fasted venous blood sample was taken at baseline, 6 months, and 12 months, the collection procedure at 6 months differed from that at baseline and 12 months. Blood samples were transported to Siem Reap Provincial Hospital laboratory daily after collection where they were processed and stored at -20°C. Once a week, samples were transported on dry ice to the National Institute of Public Health Laboratory for storage at -80°C until shipment to the University of British Columbia. Information on blood processing at baseline and 12 months can be found in (Appendix E).

Venous blood samples were analyzed for:

a) Complete Blood Count ((CBC); including hemoglobin concentration))
b) Biomarkers of iron status: ferritin, sTfR, retinol binding protein (RBP), CRP and AGP (Erhardt Laboratory, Willstaett Germany).

c) At 6 months only, genetic hemoglobin disorders were measured using methods of hemoglobin electrophoresis (Sebia MINICAP analyzer).

At baseline and endline, 2 mL of venous blood were collected into an EDTA tube, and 6mL were collected into a trace element free vacutainer. Venous blood from the EDTA tube was used for the CBC analysis on a Sysmex automated hematology analyzer at the Siem Reap Provincial Hospital Laboratory. Serum from the trace element free vacutainer was aliquoted prior to storage for analysis of biomarkers of iron status.

At 6 months, 12 mL of venous blood sample were collected: 6mL was collected into an EDTA tube, and 6mL were collected into a trace element free vacutainer. The additional blood in the EDTA tube was used to collect RBCs for hemoglobin electrophoresis that was conducted at the National Pediatric Hospital in Phnom Penh by external consultant Robyn Devenish. Hemoglobin electrophoresis is a technique used to identify and measure the hemoglobin proteins (52). After processing the CBC at the Siem Reap Provincial Hospital, the EDTA samples were centrifuged at 3000 rpm for 10 minutes. The plasma and buffy coat were removed; only RBCs remained in the EDTA tube. The RBCs were stored at 4°C at the Siem Reap Provincial Hospital before transport on ice to NPH in Phnom Penh for hemoglobin electrophoresis on a SEBIA MINICAP analyzer, using the hemoglobin (E) program. The trace element free vacutainer procedure was consistent with the baseline and 12 month procedure. The visuals on the 6-month procedure can be found in (Appendix F).

If at any time point a woman's hemoglobin concentration fell below 80g/L she was referred to a health centre for treatment.

At 6 months, the laboratory at the provincial hospital in Siem Reap had a new Sysmex automated hematology analyzer and was not using their old machine any longer. Every effort

was made to ensure we had accurate measurements of hemoglobin concentration. First, 10 samples were run on the old machine as well as on the new machine. In addition, the Hemocue was utilized to measure hemoglobin concentration at all time periods. Further information on this can be found in (Appendix G).

3.8.5 Anthropometry

At all time periods, height and weight were recorded for body mass index (BMI) classification using standardized techniques (107) . Multiple measurements of height and weight were taken in alternating order. Two measurements for height were taken in alternating order with weight. If the difference in measurements for height between the first and second was greater than 2 cm, a third measurement was taken. Weight was measured using SECA mobile electronic patient weighing scale. The scale was calibrated daily. If the difference between the first and second measurement were greater than 0.1 kg and third measurement was taken.

3.8.6 Questionnaires

Questionnaires were translated from English to Khmer. Trained enumerators administered the questionnaires in Khmer to ensure the integrity of the results. After obtaining the results from the questionnaires they were translated back into English for analysis. The questionnaires were field tested outside Phnom Penh, at Niroth health centre in the Mean Chey district. The questionnaires were field tested on 20 participants. Based on this, confusing questions, errors in the skip function, and unclear questions were fixed. The final questionnaires including the following modules can be found in (Appendix H).

Baseline Questionnaire

- Module 1 – Inclusion Criteria
- Module 2 – Participant Information

- Module 3 – Food Intake
- Module 4 – Physical Activity Status
- Module 5 – Water
- Module 6 – Knowledge of iron deficiency and IDA
- Module 7 – Knowledge of Malaria
- Module 8 – Perceptions of Supplement Use
- Module 9 – Anthropometric Measurements

6-month questionnaire

- Module 1 – Participant Information
- Module 2 – Food Intake
- Module 3 – Knowledge of iron deficiency and IDA
- Module 4 – Water
- Module 5 – Physical Activity Status
- Module 6 – Perceptions of Supplement Use
- Module 7 – Smoking
- Module 8 – Anthropometric measurements

12-month questionnaire

- Module 1 – Participant Information
- Module 2 – Food Intake
- Module 3 – Knowledge of iron deficiency and IDA
- Module 4 – Water
- Module 5 – Physical Activity Status

- Module 6 – Anthropometry

The baseline questionnaire consisted of nine modules and differed slightly from the 6-month questionnaire, which had only 8 modules.

After baseline, the 2014 Cambodia Demographic and Health Survey results were released. In Preah Vihear Province, 14% of women smoke tobacco. Smoking cigarettes is associated with a significantly higher mean ± SE hemoglobin compared to women who do not smoke (108). Not only is this relationship significant, but a dose response relationship was found, with more cigarettes smoked a higher hemoglobin concentration was reported (108). As such, at 6 months a module was added related to tobacco use.

3.8.7 Nutrition education

Within the first month of baseline data collection, nutrition education was provided to all study participants. The education was based on principles outlined by the National Nutrition Program. Education was provided to village health support group members, and then delivered to the women enrolled in this study. Along with education, posters and handouts were provided to each village health support group member. Enough handouts were supplied for each woman enrolled in our study to receive one. A copy of this can be found in (Appendix I).

3.9 Monitoring

Data were collected monthly to monitor each participant for adverse outcomes and to measure adherence to the intervention. Simple questionnaires specific to their allocated treatment group were developed. The questionnaire remained the same throughout the study, with the exception of an extended questionnaire administered at 3, 6, and 12 months. The basic form of the questionnaire asked questions about adherence, and health outcomes. The extended version asked the same questions about adherence and health outcomes but also allowed additional

information to be collected on attitudes and practices of use of the LIF and iron supplements (Appendix J).

In order to ensure the consistency of data collected, three data collectors were chosen to follow-up with the women each month. Mr. Pen Bunroth was responsible for all women in the LIF group, Ms. Tren Borey was responsible for all women in the IS group, and Mr. Eth Narorng was responsible for all women in the control group. Each month, after monitoring the women received a small bag of laundry detergent as a gift of appreciation (~$0.65 USD).

3.10 Sample size

The sample size for this trial was calculated using an online tool and in consultation with a biostatistician. Sample size was determined on the basis that there is a 90% probability that the study will detect a difference at a two-sided alpha of 0.05 significance level, if the true difference in hemoglobin concentration between groups is 5 g/L. This is based on the assumption that the SD of the response variable (hemoglobin) is 11 g/L (29). As such, a sample size of n=90 women in each arm is required. To account for drop out and missing data, we increased the number of women in each arm by ~20%. Therefore the final sample size required was n=110 in each trial arm, for a total of 330 women.

3.11 Data management

Data in Khmer are stored securely in a locked cabinet at Nutrifood Cambodia in Phnom Penh. De-identified CBC test results, and data collected in English are stored at the University of British Columbia in a locked cabinet. Data collected in English (hemoglobin concentration, and CBC reports) were entered into Excel™ (Microsoft Corporation, Redmond, WA, 2010) on the

same day the data were collected. Data on hemoglobin concentration and CBC reports were entered twice and crosschecked to ensure precision.

Questionnaire data collected in Khmer were checked daily by the trained enumerator supervisor in the field on the day the data were collected. Each survey was double checked for completeness and errors.

3.12 Data analysis

Data were checked for normality to ensure the proper use of parametric tests. Generally, histograms were utilized. However, in the case where normality could not be determined, kurtosis was calculated. In order to ensure variation around the mean was equal between groups, as well as to check for outliers, boxplots were used. If data failed to meet one of these assumptions, they were transformed in order to meet these requirements prior to analysis.

For participant characteristics at baseline, mean ± SD are reported for continuous data. For categorical data, n (%) is reported.

Data were analyzed based on intent to treat (ITT) for all outcomes where the last recorded value was carried forward. An additional analysis was conducted for subjects who completed the study and had no missing data. In both cases, a general linear model was conducted and values were adjusted for baseline value, and the presence or absence of structural hemoglobin variant.

For the primary outcome variable, hemoglobin concentration, mean (95% CI) was compared between intervention groups and control at 6 months and at 12 months. Data were collected on the Hemocue 301 as well as the Sysmex, therefore, hemoglobin concentration was analyzed separately using a general linear model. All women were included in the model where hemoglobin concentration was measured on the Hemocue. Only 50% of women were anemic

according to the Sysmex at baseline, as such in order to capture the response to the treatment for those who had anemia, results are presented for all women, and for only women with anemia at baseline according to the Sysmex hematology analyzer. Hemoglobin concentration at baseline was adjusted for in the model as were variables with significant differences between groups at baseline.

For the secondary objective, serum ferritin and sTfR, data were corrected for inflammation based on established cutoffs using the biomarkers CRP and AGP (78). As ferritin and sTfR are not usually normally distributed variables, they were transformed using a natural logarithmic scale in order to conduct parametric tests. A generalized linear model was also utilized to determine differences between groups.

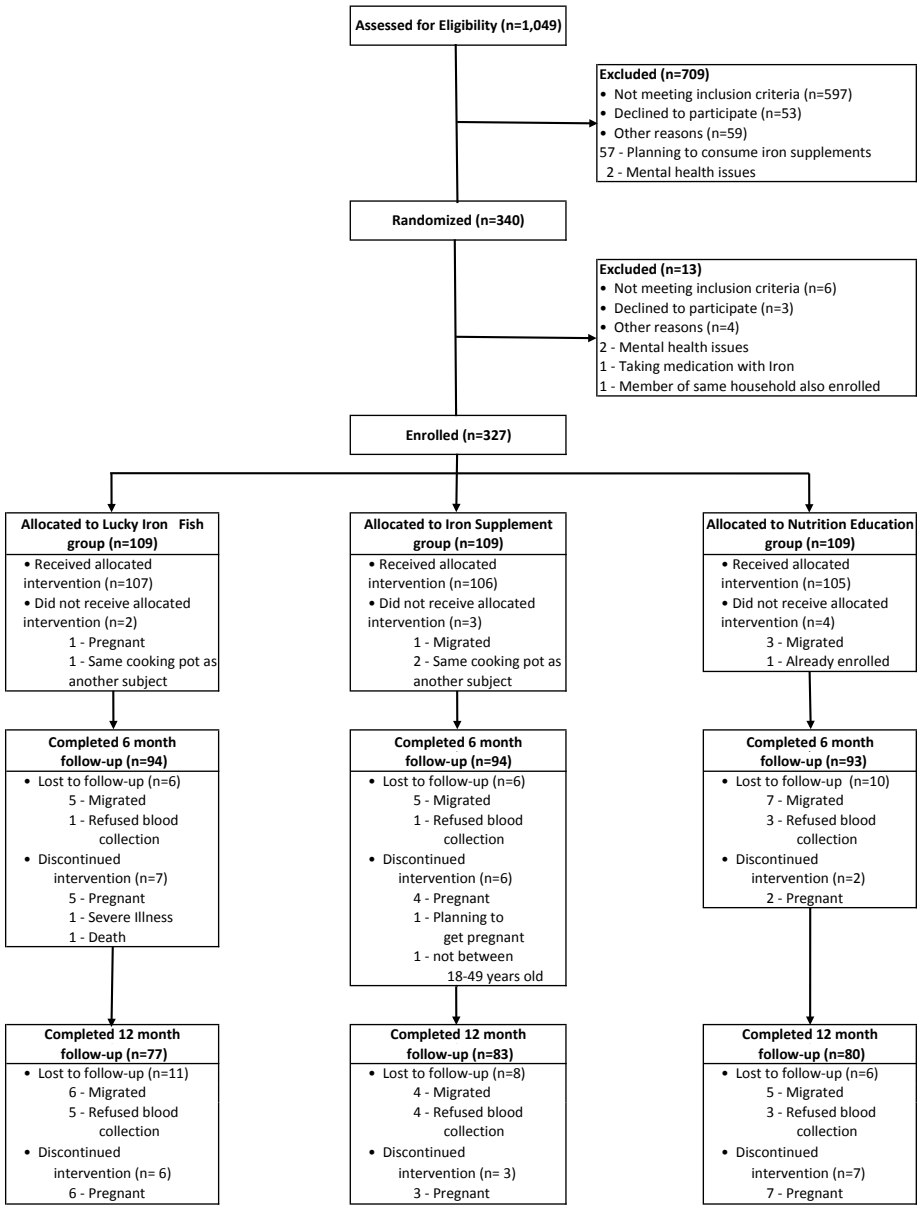
Tests were two tailed and a significance level of $p<0.05$ was set. Data were analyzed using STATA version SE 14.1 for Mac (Stata Corp, College Station, Texas).

Chapter 4: Results

4.1 Recruitment and follow-up

During recruitment, 1,049 women were screened for eligibility. A total of 760 women were screened for anemia, 419 women did not meet inclusion requirements because they had normal hemoglobin concentration (>120 g/L), 69 were not the female head of the household, and 22 were pregnant or planning to become pregnant. 340 women were enrolled in the study, and 327 (n=109 per group) remained at baseline. At 6 months, 281 women remained and attrition (14.1%) was similar across the groups. Loss to follow-up occurred primarily because of migration for work (n = 36), or women refusing the blood test (n = 17). Some women discontinued use of their intervention because of pregnancy (n=27). At 12 months, 240 women remained, and attrition was 26.6%; the reasons for loss of follow-up and discontinued intervention use were similar to those at 6 months. Participant flow and follow-up is provided in Figure 3.

Figure 3: Participation flow chart



4.2 Participant characteristics

Baseline participant characteristics are given in Table 5. The mean age was ~32 y and almost all women had completed some schooling. Around 11% of women received no schooling, and approximately 11% received some upper secondary school (equivalent to grades 10 thru 12). Most women had a normal BMI (18.5-24.9 kg/m^2); the control group was more likely to be classified as overweight and the LIF group had the greatest number of underweight women (16.5%).

The mean household size was similar across the three groups, with 5-6 people living in each home. Generally, most households earned less than $1800 USD in the previous 12 months, equivalent to less than $150 USD per month, however a small proportion of households earned between $1801 and $5000 USD.

Overall less than 5% of women smoked. Table 5 also includes information on structural hemoglobin variants analyzed at 6 months. A total of 194 (69%) of women had a structural hemoglobin variant. However, it is important to note that an additional 52 of the 87 women without one may have the α or β thalassemia trait but these cannot be ruled out or assessed using hemoglobin electrophoresis. As such, the prevalence of women having some sort of genetic hemoglobin disorder may be higher in this population. However, further analysis and genotyping is required to determine this. A total of 35 women (12.5%) had completely normal hemoglobin results based on the hemoglobin electrophoresis and CBC report at 6 months.

Table 5: Participant characteristics

Participant characteristics	LIF group (n= 109)	IS group (n=109)	Control group (n=108)
Women's age, *y*	31.5 ± 7.9	32.9 ± 8.4	32.6 ± 8.6
Number of people living in the household	5.4 ± 1.7	5.8 ± 1.9	5.3 ± 1.8
Parity	2.8 ± 1.9	2.9 ± 2.2	2.8 ± 2.4
Highest level of schooling completed			
No schooling	12 (11.0)	15 (13.8)	13 (12.0)
Some primary	62 (56.9)	60 (55.0)	66 (61.1)
Some lower secondary	21 (19.3)	22 (20.2)	19 (17.6)
Some upper secondary	14 (12.8)	12 (11.0)	10 (9.3)
BMI, kg/m^{2a}	21.3 ± 3.1	22.0 ± 3.3	22.1 ± 3.0
Underweight, *<18.5*	18 (16.5)	11(10.1)	8 (7.4)
Normal weight, *18.5-24.9*	78 (71.5)	81 (74.3)	78 (72.2)
Overweight, *25-29.9*	10 (9.2)	14 (12.8)	21 (19.4)
Obese, *≥30*	3 (2.8)	3 (2.8)	1 (0.9)
Income for the last 12 months[b]			
Lowest	81 (74.3)	86 (78.9)	86 (79.6)
Lower-Middle	24 (22.0)	18 (16.5)	20 (18.5)
Upper-Middle	3 (2.8)	4 (3.7)	0 (0)
Highest	1 (0.9)	1 (0.9)	2 (1.9)
Anemia Severity[c] Hemocue			
Normal, *≥ 120 g/L*	0 (0)	0 (0)	0 (0)
Mild anemia, *110-119 g/L*	69 (63.3)	63 (57.8)	69 (63.9)
Moderate anemia, *80-109 g/L*	40 (36.7)	46 (42.2)	39 (36.1)
Severe anemia, *<80 g/L*	0 (0)	0 (0)	0 (0)
Anemia Severity[c] CBC			
Normal, *≥ 120 g/L*	61 (56)	50 (45.9)	51(46.8)
Mild anemia, *110-119 g/L*	27 (24.8)	39 (35.8)	36 (33.0)
Moderate anemia, *80-109 g/L*	19 (17.4)	19 (17.4)	21 (19.3)
Severe anemia, *<80 g/L*	2 (1.8)	1 (0.9)	1 (0.9)
	(n= 94)	(n=94)	(n=93)
Smoking	5 (5.3)	3 (3.2)	2 (2.2)
Normal hemoglobin	29 (33)	28 (32)	30 (34)
Hemoglobin E trait	37 (36)	34 (33)	32 (31)
Hemoglobin EE	14 (15)	17 (18)	13 (14)
Hemoglobin CS (Constant Spring)	4 (4)	12 (13)	7 (7)
Other variants	10 (11)	3 (3)	9 (10)

Notes: For continuous data, mean ± (SD) are shown. For categorical data, n (%)
[a]Based on the WHO Cutoffs for women (104)
[b] Lowest: ≤1800 USD, Lower-Middle: 1801-5999 USD, Upper-Middle: 6000-11999 USD, Highest: 12000+ USD.
[c]Based on WHO Cutoffs for non-pregnant women older than 15 years (19)

4.3 Attrition differences

Table 6 compares participant characteristics for those who dropped out of the study, to those who remained at 12 months. Participants who dropped out tended to be older, had more children, and as such, had more people living in their household. Women who dropped out tended to be less educated, 20% received no schooling compared to only 9% in the non-dropout group. Slightly fewer women (n=5) in the non-dropout group earned more than $6000 USD compared to the group who dropped out. Women who dropped out had slightly lower hemoglobin concentration and serum ferritin than women who remained in the study. Women who remained in the study had lower sTfR (8.9 mg/L) than women who dropped out (9.7 mg/L). There were no major differences between having a hemoglobin variant for women who remained in the study (n=68%) compared to those who dropped out (n=76%).

Table 6: Characteristics of loss of follow-up participants to active participants

Participant characteristics	Dropout (n=86)	Non-dropouts (n=241)*
Women's age, *y*	29.9 ± 7.6	33.2 ± 8.4
Number of people living in the household	5.2 ± 1.8	5.6 ± 1.8
Parity	2.4 ± 1.9	3.0 ± 2.2
Highest level of schooling completed		
No schooling	17 (20)	22 (9)
Some primary	45 (52)	144 (60)
Some lower secondary	13 (15)	49 (20)
Some upper secondary	11 (13)	25 (10)
BMI, kg/m^{2a}	21.5 ± 3.0	21.9 ± 3.2
Income for the last 12 months[b]		
Lowest	70 (81)	183 (76)
Lower-Middle	16 (19)	46 (19)
Upper-Middle	0 (0)	7 (3)
Highest	0 (0)	4 (2)
Hemoglobin Concentration g/L, *Hemocue*	107 ± 9.0	110 ± 8.1
Hemoglobin Concentration g/L, *Sysmex*	116 ± 12	118 ± 11
Serum ferritin concentration µg/L	64 ± 50	74 ± 47
Serum ferritin concentration <15 µg/L	8 (9)	22 (9)
sTfR concentration mg/L	9.7 ± 7	8.9 ± 7
sTfR concentration <8.3 mg/L	32 (37)	65 (27)
6 month characteristics	(n= 41)	(n=240)
Normal hemoglobin	10 (24)	77 (32)
Abnormal hemoglobin	31 (76)	163 (68)

Notes: For continuous data, mean ± (SD) are shown. For categorical data, n (%)

[a]Based on the WHO Cutoffs for women (104)

[b] Lowest: ≤1800 USD, Lower-Middle: 1801-5999 USD, Upper-Middle: 6000-11999 USD, Highest: 12000+ USD.

*For participant characteristic values n=240 and n=241 for blood parameters

4.4 Hemoglobin concentration

In this clinical trial, hemoglobin concentration is reported on capillary blood collected by finger prick and measured by the Hemocue, as well as venous blood measured on automated hematology analyzers at each time point. Hemoglobin concentration is reported at 6 months and 12 months and is adjusted for baseline hemoglobin concentration. Data are reported as mean (95% CI). Two separate analyses were conducted, ITT where last measured values were carried forward and per protocol, where subjects with missing data were excluded from analysis. Mean hemoglobin concentration on the Hemocue Hb 301 was roughly 109 (107-111) g/L Table 7. There were no differences between groups in mean hemoglobin concentration measured on the automated hematology analyzer, mean (95% CI) hemoglobin was 117 g/L (115 – 121) g/L for all women Table 8.

Only 50% of women (n=165) had anemia at baseline based on measurements made on venous blood using a Sysmex automated hematology analyzer. For these women, mean (95% CI) hemoglobin concentration was 109 (105 – 113) g/L. There were no differences between groups. Table 9.

4.4.1 Hemocue

Using ITT, there were no significant differences in mean hemoglobin concentrations between the treatment groups either at 6 or 12 months. The mean hemoglobin concentration at 12 months for the control group (n=109) was 115 (114, 119) g/L, for the iron supplement group (n=109) was 115 (114, 119) g/L, and for the LIF group (n=109) was 116 (114, 119) g/L. There was a temporal change in hemoglobin concentration such that hemoglobin concentrations were 10 and 7 g/L higher than baseline at 6 months and 12 months, respectively, regardless of treatment Table 7. Using per protocol analysis did not change the findings markedly.

4.4.2 Sysmex

Based on ITT, similar to the findings on the Hemocue Hb 301, there was no difference in mean hemoglobin concentration between the three groups at 6 or 12 months Table 8. The mean hemoglobin concentration at 12 months for the control group (n=109) was 116 (115, 118) g/L, for the iron supplement group (n=109) was 116 (114, 118) g/L, and for the LIF group (n=109) was 117 (115, 118) g/L. The temporal changes in hemoglobin concentration observed with the Hemocue Hb 301 hemoglobin measurement were not observed with the Sysmex measurements Table 8. Using per protocol analysis did not change the findings.

When we restricted our analysis to only women with anemia (based on Sysmex at baseline) there was no significant difference between the groups Table 9. The mean hemoglobin concentration at 12 months for the control group (n=58) was 112 (109, 114) g/L, for the iron supplement group (n=59) was 113 (110, 116) g/L, and for the LIF group (n=48) was 111 (108, 114) g/L. Using per protocol analysis did not change the findings Table 9.

Table 7: Hemoglobin concentration: Hemocue[1]

	n	Baseline	n	Midline (6 months)[2]	n	Endline (12 months)[2]
Intent to treat[2]						
Control	109	109 (107, 110)[a]	109	120 (118, 123)[a]	109	117 (114, 119)[a]
Iron Supplement	109	109 (107, 111)[a]	109	119 (117, 122)[a]	109	116 (114, 119)[a]
LIF	109	109 (108, 111)[a]	109	120 (117, 122)[a]	109	116 (114, 119)[a]
Per protocol analysis[3]						
Control	109	109 (107, 110)[a]	93	120 (117, 122)[a]	79	115 (113, 118)[a]
Iron Supplement	109	109 (107, 111)[a]	94	118 (116, 121)[a]	83	115 (112, 117)[a]
LIF	109	109 (108, 111)[a]	94	120 (117, 122)[a]	77	116 (113, 118)[a]

Notes: Data are expressed as mean (95% CI)

[1] Generalized linear model was used to assess differences between groups. Adjustments for baseline hemoglobin concentration and the presence of a hemoglobin variant with a significance level of 0.05 for all analyses.

[2] Intent to treat analysis where last recorded values were carried forward

[3] Per protocol analysis where dropouts were excluded

[a-c] Values in the table with a different superscript are significantly different ($p < 0.05$)

Table 8: Hemoglobin concentration: All women Sysmex[1]

	n	Baseline	n	Midline (6 months)	n	Endline (12 months)
Intent to treat[2]						
Control	109	117 (115, 120)[a]	109	118 (115, 120)[a]	109	116 (115, 118)[a]
Iron Supplement	109	117 (115, 119)[a]	109	117 (115, 119)[a]	109	116 (115, 118)[a]
LIF	109	118 (116, 121)[a]	109	118 (116, 121)[a]	109	117 (115, 118)[a]
Per protocol analysis[3]						
Control	109	117 (115, 120)[a]	93	118 (115, 120)[a]	79	116 (114, 119)[a]
Iron Supplement	109	117 (115, 119)[a]	94	116 (114, 118)[a]	82	116 (115 118)[a]
LIF	109	118 (116, 121)[a]	94	117 (115, 119)[a]	77	115 (114, 117)[a]

Notes: Data are expressed as mean (95% CI)
[1] Generalized linear model was used to assess differences between groups. Adjustments for baseline hemoglobin concentration and the presence of a hemoglobin variant with a significance level of 0.05 for all analyses.
[2]Intent to treat analysis where last recorded values were carried forward
[3] Per protocol analysis where dropouts were excluded
[a-c] Values in the table with a different superscript are significantly different ($p < 0.05$)

Table 9: Hemoglobin concentration: Anemic women at baseline Sysmex[1]

	n	Baseline	n	Midline (6 months)	n	Endline (12 months)
Intent to treat[2]						
Control	58	110 (107, 112)[a]	58	113 (110, 116)[a]	58	112 (109, 114)[a]
Iron Supplement	59	110 (108, 113)[a]	59	113 (110, 116)[a]	59	113 (110, 116)[a]
LIF	48	108 (105, 112)[a]	48	112 (109, 116)[a]	48	111 (108, 114)[a]
Per protocol analysis[3]						
Control	58	110 (107, 112)[a]	47	110 (107, 114)[a]	39	110 (108, 113)[a]
Iron Supplement	59	110 (108, 113)[a]	52	110 (107, 113)[a]	46	111 (108, 113)[a]
LIF	48	108 (105, 112)[a]	42	113 (110, 116)[a]	30	109 (106, 112)[a]

Notes: Data are expressed as mean (95% CI)
[1] Generalized linear model was used to assess differences between groups. Adjustments for baseline hemoglobin concentration and the presence of a hemoglobin variant with a significance level of 0.05 for all analyses.
[2]Intent to treat analysis where last recorded values were carried forward
[3] Per protocol analysis where dropouts were excluded
[a-c] Values in the table with a different superscript are significantly different ($p < 0.05$)

4.5 Anemia prevalence

With one exception there was no difference in the prevalence of anemia using either Hemocue or Sysmex at 6 and 12 months. Using the Sysmex but not Hemocue, anemia prevalence was lower at 6 months in the LIF (47%) than the iron supplement (63%) or control (67%) group ($p<0.05$). Even though there were no differences between groups at baseline, this is not surprising as anemia prevalence was slightly lower for the LIF group at baseline. This difference was not present at 12 months Table 10.

Table 10: Anemia prevalence

	LIF group	IS group	Control group
Baseline	n=109	n=109	n=109
Anemia[1] *Hemocue*	109 (100)[a]	109 (100)[a]	109 (100)[a]
Anemia[1] *Sysmex*	48 (44)[a]	59 (54)[a]	58 (53)[a]
Midline (6 months)	n=94	n=94	n=93
Anemia[1] *Hemocue*	43 (46)[a]	50 (53)[a]	46 (49)[a]
Anemia[1] *Sysmex*	44 (47)[a]	59 (63)[b]	62 (67)[b]
Endline (12 months)	n=77	n=83	n=79
Anemia[1] *Hemocue*	47 (61)[a]	56 (67)[a]	49 (62)[a]
Anemia[1] *Sysmex*	46 (60)[a]	56 (67)[a]	48 (61)[a]

Notes: Data are expressed as n (%)

[1]Based on WHO Cutoffs for non-pregnant women older than 15 years (19).

[a-c] Values in the table with a different superscript are significantly different ($p < 0.05$).

4.6 Iron status biomarkers

Table 11 presents data on iron biomarkers. The prevalence of iron deficiency in this population differed by biomarker. Biomarkers of iron status are reported at 6 and 12 months and are adjusted for baseline values. Data are reported as mean (95% CI). Two separate analyses were conducted, ITT where last measured values were carried forward and per protocol, where subjects with missing data were excluded from analysis. There was no interaction between treatment and the presence of a hemoglobin variant.

4.6.1 Serum ferritin

Using ITT, there was a significant improvement in mean serum ferritin concentration in the iron supplement group at both 6 and 12 months compared to the LIF and control groups. The mean serum ferritin concentration at 12 months for the control group (n=109) was 76 (71, 85) μg/L, for the iron supplement group (n=109) was 94 (86, 102) μg/L, and for the LIF group (n=109) was 78 (69, 83) μg/L Table 11. Using per protocol analysis did not change the findings.

4.6.2 sTfR

Using ITT, there was a significant improvement in mean sTfR concentration for the iron supplement group at both 6 and 12 months compared to the LIF and control groups. The mean sTfR concentration at 12 months for the control group (n=109) was 7.0 (6.3, 7.7) mg/L, for the iron supplement group (n=109) was 6.0 (5.3, 6.7) mg/L, and for the LIF group (n=109) was 8.1 (7.3, 8.8) mg/L Table 11. Results from the per protocol analysis did not differ from the ITT.

Table 11: Biomarkers of iron status[1]

Serum Ferritin (μg/L)						
	n	Baseline	n	Midline (6 months)[2]	n	Endline (12 months)[2]
Intent to treat[2]						
Control	109	71 (62, 80)[a]	109	68 (63, 73)[a]	109	76 (71, 85)[a]
Iron Supplement	109	61 (52, 70)[a]	109	85 (79, 91)[b]	109	94 (86, 102)[b]
LIF	109	68 (59, 78)[a]	109	69 (64, 75)[a]	109	78 (69, 83)[a]
Per protocol analysis[3]						
Control	109	71 (62, 80)[a]	93	68 (63, 73)[a]	79	83 (74, 91)[a]
Iron Supplement	109	61 (52, 70)[a]	94	85 (79, 91)[b]	83	97 (88, 105)[b]
LIF	109	68 (59, 78)[a]	94	69 (64, 75)[a]	77	80 (72, 88)[a]
sTfR (mg/L)						
	n	Baseline	n	Midline (6 months)[2]	n	Endline (12 months)[2]
Intent to treat[2]						
Control	109	8.1 (6.7, 9.5)[a]	109	7.4 (6.6, 8.2)[a]	109	7.0 (6.3, 7.7)[b]
Iron Supplement	109	8.7 (7.4, 10.1)[a]	109	6.0 (5.2, 6.8)[b]	109	6.0 (5.3, 6.7)[b]
LIF	109	8.5 (7.2, 9.9)[a]	109	8.3 (7.4, 9.1)[a]	109	8.1 (7.3, 8.8)[a]
Per protocol analysis[3]						
Control	109	8.1 (6.7, 9.5)[a]	93	7.4 (6.6, 8.2)[a]	79	7.1 (6.5, 7.8)[a]
Iron Supplement	109	8.7 (7.4, 10.1)[a]	94	6.0 (5.2, 6.8)[b]	83	5.9 (5.2, 6.6)[b]
LIF	109	8.5 (7.2, 9.9)[a]	94	8.3 (7.3, 9.2)[a]	77	7.5 (6.8, 8.2)[a]

Inflammation						
Acute[4], *CRP >5 mg/L*	327	25 (7.6)	281	10 (3.6)	239	9 (3.8)
Chronic[4], *AGP >1 g/L*	327	30 (9.2)	281	5 (1.8)	239	9 (3.8)

Notes: Data are expressed as mean (95% CI)

[1] Generalized linear model was used to assess differences between groups. Adjustments for baseline serum ferritin and sTfR and the presence of a hemoglobin variant with a significance level of 0.05 for all analyses.

[2]Intent to treat analysis where last recorded values were carried forward

[3] Per protocol analysis where dropouts were excluded.

[4]Inflammation biomarkers were used to correct serum ferritin for inflammation stage, incubation, early convalescence or late convalescence

[a-c] Values in the table with a different superscript are significantly different ($p < 0.05$).

4.7 Iron deficiency and IDA prevalence

The prevalence of iron deficiency in this population differed by biomarker. Approximately 9% were iron deficient using the ferritin biomarker corrected for inflammation, and 30% based on the sTfR biomarker.

At 6 months, the prevalence of iron deficiency (serum ferritin <15 *μg/L*) and IDA was lower in the iron supplement group (hemoglobin <120 g/L and serum ferritin <15 *μg/L*) than the LIF and control groups. This difference was similar using the Hemocue and Sysmex but this difference was not present at 12 months Table 12.

Table 12: Iron deficiency and IDA prevalence

	LIF group	IS group	Control group
Baseline	n=109	n=109	n=109
iron deficiency [3], *serum ferritin*	12 (11)[a]	9 (8)[a]	9 (8)[a]
iron deficiency, *sTfR*	33 (30)[a]	35 (32)[a]	29 (27)[a]
IDA[2] *Hemocue*	12 (11)[a]	9 (8)[a]	9 (8)[a]
IDA[2] *Sysmex*	10 (9.2)[a]	8 (7.4)[a]	8 (7.3)[a]
Midline (6 months)	n=94	n=94	n=93
iron deficiency [3], *serum ferritin*	15 (16)[a]	1 (1)[b]	9 (10)[a]
iron deficiency, *sTfR*	18 (19)[a]	19 (20)[a]	21 (23)[a]
IDA[2, 3] *Hemocue*	11 (10)[a]	1 (1)[b]	6 (6)[a]
IDA[2, 3] *Sysmex*	9 (10)[a]	1 (1)[b]	7 (8)[a]
Endline (12 months)	n=77	n=83	n=79
iron deficiency [3], *serum ferritin*	7 (9)[a]	9 (11)[a]	7 (11)[a]
iron deficiency, *sTfR*	11 (14)[a]	13 (16)[a]	19 (24)[a]
IDA[2, 3] *Hemocue*	5 (7)[a]	7 (8)[a]	4 (5)[a]
IDA[2, 3] *Sysmex*	6 (6)[a]	6 (6)[a]	5 (5)[a]

Notes: Data are expressed as n (%)

[2]Ferritin <15 *μg/L*, Hemoglobin<120 g/L

[3]Values were corrected for inflammation based on established cutoffs for biomarkers CRP and AGP (78).

[a-c] Values in the table with a different superscript are significantly different ($p < 0.05$)

4.8 Adherence to treatment

Monitoring was conducted monthly to measure adherence. For the iron supplement group, women were considered adherent if they took more than 80% of pills in their supplement bottle for the month. Women were met with monthly and remaining pills in the bottle were counted. For the first six months 82% of women were adherent. For the second six months of the study, adherence to iron supplement treatment was similar, at 83% Table 13.

For the LIF group, adherence was calculated based on whether or not the LIF was used in the previous day, and if this pattern was of typical routine for the woman. During the first six months, 83% of women reported using the fish yesterday, and in the second six months 90% of women reported cooking with the LIF in the previous day Table 13.

Table 13: Adherence to treatment by group

	LIF	Total n	Iron Supplement	Total n
Adherence (month 1 – 6)	499 (83%)	611	522 (86%)	608
Adherence (month 7 – 12)	497 (90%)	551	454 (83%)	547

Notes: Data is presented as n (%)

Chapter 5: Discussion

Based on WHO criteria, anemia is classified a severe public health problem in Cambodia, affecting 45% of women of childbearing age (5,6). Generally, iron deficiency is thought to be the primary cause of anemia and, as such, the WHO recommends intermittent IFA supplementation when the anemia prevalence is above 20%, and daily blanket IFA supplementation when the anemia prevalence is above 40% (10,82). Therefore, the Ministry of Health in Cambodia has policies to address anemia, mainly through iron supplementation (11). However, there are issues with cost, distribution and compliance (14,109). As such, the LIF was developed to address anemia in Cambodia as it is expected to have better adherence than iron supplements. Results from this RCT do not support the use of the LIF in Cambodia. There was no difference in hemoglobin concentration across the groups in this study after six months or one year measured on either the Hemocue Hb 301 or an automated hematology analyzer. The lack of effect of either the LIF or the iron supplement on anemia may be in part due to the very low prevalence of iron deficiency in this population. The finding of a low prevalence of iron deficiency despite high anemia is consistent with the findings of other researchers in Cambodia (6,41). However, serum ferritin was higher in the iron supplement group but not in the LIF group compared with the control group. Here, I will discuss the anemia paradox in Cambodia, the low prevalence of iron deficiency in this population, as well as interpret the results in the context of current and past literature. Strengths and limitations of this randomized controlled trial, and future directions of further research will be discussed as well.

5.1 Participant characteristics and anemia prevalence

The characteristics of the participants in this trial were consistent with national survey data in Cambodia. Mean BMI in this study was 21.8 kg/m^2, which was very similar to the mean BMI for women living in rural areas in Cambodia, 21.9 kg/m^2 (6). The average household size in the 2014 Cambodia Demographic and Health survey was 4.5 and 5.0 people for rural and urban areas, respectively, compared with 5.5 people in our study (6).

In our study, 11% of women received no school, 58% completed some primary school, 19% received some lower secondary and finally, 11% received some upper secondary. This is similar to the 2014 CDHS results, where 12% did not receive any schooling, 53% received some primary school, and 27% received some secondary school (6).

The overall prevalence of anemia was 45%, around 10% lower than reported in a representative survey of women in Preah Vihear. (6). However, it is the same as the 2014 CDHS national anemia prevalence, 45%, and thus remains classified a severe public health problem according to WHO guidelines (5,6). All of the women in this study had mild or moderate anemia, as those with severe anemia were excluded and referred to the health centre for treatment. During screening we found that less than 1% of women had severe anemia, which again was consistent with the 2014 Cambodia Demographic and Health Survey results (6).

This study only assessed the prevalence of structural hemoglobin variants not thalassemias. The frequency of the variants found in this trial was slightly higher than reported in previous studies from Cambodia (41,55,57). We found that 31% of women had normal hemoglobin, 37% had the hemoglobin EA variant, 17% of women were EE homozygous, and 9% had Constant Spring. Unfortunately due to funding and time constraints, PCR analysis to determine the presence of α and β thalassemia was not conducted. The types of structural

hemoglobin variants found in our trial were similar to previous research in Cambodia (41,55,57). A recent study conducted in Battambang, Preah Vihear, and Phnom Penh reported that 44% of women had normal hemoglobin, 38% had the hemoglobin EA variant, 10% of women were EE homozygous, and 8% had Constant Spring (57). Similarly, a recent trial from Prey Veng Cambodia reported that 46% of women had normal hemoglobin, 31% had hemoglobin EA, 7% had hemoglobin EE, and 4% had Constant Spring (41). More women in this study had normal hemoglobin than did in Preah Vihear (41). Another study in Cambodian children in Siem Reap Province also found more children had normal hemoglobin (49%) than this study, 29% had hemoglobin EA, 4% had hemoglobin EE and 5% had Constant Spring (55). Our findings compare similarly to the findings in Preah Vihear, where a higher proportion of women had hemoglobin EE and hemoglobin Constant Spring. More research, such as PCR analysis to detect α and β thalassemias is needed to gain a full understanding of the genetic hemoglobin disorders in Preah Vihear, Cambodia.

5.2 Differences in mean hemoglobin concentration and anemia

At 6 and 12 months, there were no significant differences in mean hemoglobin concentration between groups regardless of method used to measure hemoglobin or just including women who were classified as anemic at baseline on the Sysmex. These findings are not consistent with previous research conducted on the LIF. In the first study of the LIF conducted in Kandal Province, serum iron, CRP, and hemoglobin concentration were measured at 3, and 6 months (16). There were 3 groups in this study, a LIF group that received follow up (n=60), a LIF group without follow up (n=60) and a control group (n=60). Hemoglobin concentration for the LIF group who received follow-up was 130 ± 10 g/L compared to the LIF group without follow-up 125 ± 11 g/L and the control group 121 ± 12 g/L p=0.001. This

observed difference in hemoglobin concentration was not consistent at 6 months (16). At 6 months, the mean ± SD hemoglobin concentration for the LIF group who received follow-up was 129 ± 10 g/L compared to the LIF group without follow-up 126 ± 13 g/L and the control group 129 ± 13 g/L p=0.51 (16). The authors did not report anemia prevalence and did not include robust biomarkers of iron status such as ferritin or sTfR. However, they did include serum iron, which is not considered a reliable marker of iron status; however there were no differences between groups at either time point. Finally, the study was small with only 60 subjects per group and faced a 30% attrition rate.

It is interesting that use the LIF resulted in higher hemoglobin at some points but not others. The authors suggest that this is due to the different availability of iron rich foods in the wet and dry season. They go on to suggest that the LIF works only when iron in the food supply is low. We did not see any difference between treatment groups at either 6 months or 12 months, corresponding to the dry and wet seasons, suggesting this was not the case.

In another study in Kandal Province, with two LIF groups (n=164) and a control group (n=84) the same authors reported a significantly higher hemoglobin concentration in the groups receiving the LIF compared to the control group at 9 and 12 months. The mean (95% CI) for the intervention group was 123 (122, 125) and 130 (128, 132) compared to the control group 118 (116, 121) and 120 (117, 123), at 9 and 12 months respectively for both groups. Similar to the trial described in this thesis, the authors report a low prevalence of iron deficiency in this population, 11% (17). However, unlike findings from this trial, previous research on the LIF reported a significant reduction in anemia prevalence for the intervention group (n=164) at 9 and 12 months. Remarkably, anemia was reduced by 34% and 46% at 9 and 12 months, respectively despite the low prevalence of iron deficiency at baseline.

In our study there were temporal changes in hemoglobin concentration, based on Hemocue Hb 301 measurement but not on the automated hematology analyzer. There was a ~10 g/L improvement in hemoglobin concentration from baseline to 6 months, irrespective of treatment. At 12 months, hemoglobin concentration measured on the Hemocue dropped by approximately 4 g/L across all treatment groups from 6 months. The overall improvement in hemoglobin concentration from baseline to 12 months was 7 g/L from baseline across all treatment groups assessed with the Hemocue. This temporal change was not observed when Hb concentration was measured on venous blood on an automated hematology analyzer suggesting that regression to the mean accounts for much of the temporal change seen with the Hemocue. However, we cannot discount other factors such as seasonal differences.

5.3 Prevalence of iron deficiency and iron deficiency anemia

The aim of this clinical trial was to address anemia caused by iron deficiency. A finding of this study was that although iron deficiency was low it varied considerably by biomarker. Approximately 9% were iron deficient using the ferritin biomarker corrected for inflammation by methods suggested by Thurnham *et al.* (97), and 30% based on the sTfR biomarker (97). This is consistent with previous recent findings on similar biomarkers of iron status in Cambodia, and globally (21,41,80). A recent study conducted in Prey Veng (n=420) reported similar results in non-pregnant women; anemia was common (30 %), and iron deficiency rates were low, and differed by biomarker, 2% based on serum ferritin and 20% with sTfR (39). Similarly, in the 2014 Cambodia Demographic and Health Survey iron deficiency in women of reproductive age was 3% and 34% based on serum ferritin and sTfR, respectively (6). The biomarker, sTfR is known to be falsely elevated in people with genetic hemoglobin disorders (80). Findings from

this trial support this: mean ± SD sTfR was higher at baseline in those who had a hemoglobin disorder compared to those who did not (9.4 ± 0.5 vs. 7.6 ± 0.6; p<0.02) (80).

Over the last ten years in Cambodia, there has been a push to increase the dietary intake of iron as well as to increase the availability of iron supplements. Iron fortified fish and soy sauce provide a daily source of bioavailable iron (NaFeEDTA). There have also been efforts to increase dietary intake of iron in recent years leading to high iron status of Cambodian women (41,42,110). It is not clear whether these interventions are responsible for the low rate of iron deficiency or whether there never was iron deficiency in Cambodia. Earlier research in Cambodia relied on anemia as a proxy of iron deficiency and did not include biomarkers of iron status. Also well water has been show to be a source of iron in some areas (39,40) Given the lack of apparent iron deficiency, it is not surprising that hemoglobin did not respond to either the LIF or iron supplementation (41).

In our study, at both time-points, mean serum ferritin was higher in the group receiving the iron supplement compared to the LIF and control groups. At 12 months, ferritin was 33 ± 8 *μg/L* higher in the iron supplement group compared to control (p=0.002). This suggests that ferritin can be improved with iron supplementation in this population. Although the prevalence of iron deficiency (ferritin < 15 μg/L), was reduced in the iron supplement group compared to control at 6 months only, the number of participants with iron deficiency was very low (n=25; 9%) so this should be interpreted with caution. Curiously, sTfR was lower at 12 months but not 6 months in the iron-supplemented group, suggesting less tissue iron deficiency, than the LIF but not the control. We are unsure of what to make of this finding.

Another study of the LIF measured serum ferritin as a biomarkers of iron status as was done in this thesis research. This previous study found a significant improvement in mean (95%

CI) serum ferritin for the intervention group 102 (89, 116) after one year compared to the control group 66 (57, 76) in contrast to the results presented in this thesis where no significant improvement in biomarkers of iron status for the LIF were found after one year (17). Contrary, the present study found that serum ferritin can be significantly improved and sTfR significantly lower with ferrous sulfate supplementation while the LIF did not show any changes in these biomarkers. This suggests that the iron provided by the LIF is not bioavailable or other metal contaminants leached from the LIF compete with iron for absorption. However, more research is needed to confirm this hypothesis.

The findings in this trial are in line with those reported for cast iron cookware. Zlotkin *et al.* conducted a RCT in Benin, comparing groups of women and children who consumed food from one of two different types of iron cookware to a control group who received iron supplements or MNP in a population with 50% anemia (97). After one year, there was no significant difference in hemoglobin concentration among the three groups (97). However, the iron supplement and MNP group had significantly higher serum ferritin than cast iron cookware groups (97). Cast iron cookware has a larger surface area compared to the LIF. In addition, the recommended cooking time for the LIF is only 10 minutes, while cast iron cookware is utilized through the entire cooking process. Therefore, if cast iron cookware cannot improve biomarkers of iron status, it is no surprise that the LIF did not improve these biomarkers either, questioning the biological plausibility of this mechanism of iron transfer.

5.4 Adherence to treatment

The authors of a previous study in 2011 in non-pregnant Cambodian women reported 47% adherence to IFA supplements (14). Previous research in Cambodia also determined that follow-up visits, number of tablets received, education, and knowledge of anemia influence

adherence to IFA supplements (14). During the first six months of this study there was 83% adherence to the LIF and 86% adherence to iron supplements. For the latter six months of this study there was 90% adherence to the LIF and 83% adherence to iron supplements. As this was an efficacy study and monitoring was conducted to measure adherence the high adherence is expected.

5.5 Limitations

Our study had several limitations. First the low rate of iron deficiency would make it unlikely that additional iron either from the supplement or LIF would impact anemia. If the LIF is to be trialed again it should be done in an area with proven iron deficiency based on biomarkers of iron status. Second, anemia in Cambodia and elsewhere is generally treated with an iron supplement containing 60 mg of iron and 400 μg of folic acid (11,13). However, the dose of 18 mg of iron per day was chosen in this study to parallel the dose of iron received by the women cooking with the LIF. It is possible that a higher dose of iron may have had an effect on hemoglobin, but given the low rate of iron deficiency this is unlikely. Third, the prevalence of genetic hemoglobin disorders was very high in this population. Genetic hemoglobin disorders are known to increase sTfR and thought to increase ferritin (80). It is possible that those with genetic hemoglobin disorders respond differently to iron supplementation. However, we found no interaction between treatment and the presence of a hemoglobin disorder for any outcome, suggesting this was not an issue. Fourth, we did not distribute deworming medications to women nor did we test for the presence of malaria. Malaria prevalence is thought to be low (12.3%) in Preah Vihear, Cambodia (47). However, past research in Cambodia, specifically in Rovieng district, Preah Vihear Province where this trial took place suggests a high prevalence of schistosomiasis and worm infection (32). Because randomization was done at a sample level, it

can be assumed that there is an equal distribution of parasitic infection across treatment groups. However, a high prevalence of infection would limit the efficacy of iron in any form to reduce anemia

Finally, attrition was high because women frequently migrate for work, and become pregnant. Surprisingly, women who dropped out were worse off than women who remained in the study. However, as this was a randomized trial, this does not impact the results. In addition, differences between dropouts and non-dropouts are not large enough to impact the observed results.

We faced challenges associated with the blood collection in Cambodia. It is not common practice in the country to have blood taken. Many women were scared of the blood test, and family members forbid women to have their blood taken. It is common belief that blood tests cause women to feel fatigued, sleep excessively, have a headache, nausea, weakness, and inability to perform work or household chores. Some women refused having their blood taken because they believed that their blood would be taken and sold on the black market. In addition, in 2014 there was an HIV outbreak in Battambang Province. Many women believed that by having their blood tested for hemoglobin concentration that they would become HIV positive. This led to higher attrition than we accounted for in our original sample size calculation.

5.6 Future research

This clinical trial does not support future research with the LIF as a means to address IDA, or iron deficiency in Cambodia. First, we found a low prevalence of IDA and iron deficiency consistent with previous research from Cambodia (6,41). If the LIF was to be trialed in Cambodia it should be in women with proven IDA. However, with only 10% of women with anemia having iron deficiency a lot of women would need to be screened and then it would be

simpler to treat with an iron supplement. While the LIF is unsuited for use in Cambodia, it is not clear whether or not the LIF has the potential to be a solution to address iron deficiency and IDA in a setting with a high burden of iron deficiency.

Chapter 6: Conclusion

The primary objective of this study was to determine if the LIF and iron supplements could improve the hemoglobin concentration in non-pregnant women of reproductive age in rural Cambodia. After one year the hemoglobin concentration did not significantly differ between control and either the LIF or the iron supplement groups. Similarly, the use of the LIF and iron supplements over 12 months did not result in significant differences in anemia prevalence compared to the control.

An additional objective of this research was to determine the impact of the LIF and iron supplements on biomarkers of iron status. Despite the low prevalence of iron deficiency, results found a significant improvement in serum ferritin concentration for women consuming iron supplements after 12 months. This suggests that in women of reproductive age in Cambodia, biomarkers of iron status can be addressed with a low dose (18 mg/day) of ferrous sulfate with monthly visits to monitor adherence. The same trend was not seen in the LIF group suggesting it does not have the same impact on women of reproductive age as iron supplements do. Perhaps this is attributed to the bioavailability of the iron provided in the LIF, metal contaminants competing with iron for absorption, the biological plausibility of iron cookware in general, or poor adherence to the LIF.

Another objective of this research was to determine the impact of the LIF and iron supplements on the prevalence of iron deficiency and IDA. While the iron supplement group found a significant reduction of IDA at midline compared to the other groups, these findings were not consistent at 12 months.

A take away message from this study is that interventions should not be done without knowing the true cause of the anemia. As seen in this study, iron deficiency prevalence in this population is low and does not contribute to the high burden of anemia in Cambodia.

References

1. United Nations Statistics Division. World Statistics Pocketbook 2015 edition. New York, NY; 2015.
2. CELADE, ECLAC, National Institute of Statistics. Cambodia General Population Census [Internet]. 2008 [cited 2016 Jun 3]. Available from: http://celade.cepal.org/redkhm/census/khm2008/
3. Ministry of Planning, National Institute of Statistics. Cambodia Inter-Censal Population Survey. Phnom Penh, Cambodia; 2013.
4. FAO. Cambodia - aquastat survey. 2011.
5. De Benoist B, McLean E, Egli I, Mary C, Editors, editors. Worldwide prevalence of anaemia 1993-2005: WHO global database on anaemia [Internet]. Geneva: WHO; 2008 [cited 2016 Jan 13]. Available from: http://www.who.int/vmnis/publications/anaemia_prevalence/en/
6. National Institute of Statistics, Directorate General for Health, ICF International. Cambodia Demographic and Health Survey 2014. Phnom Penh, Camboda and Rockville, Maryland, USA; 2015.
7. Balarajan Y, Ramakrishnan U, Özaltin E, Shankar AH, Subramanian S V. Anaemia in low-income and middle-income countries. Lancet. Elsevier Ltd; 2011;378(9809):2123–35.
8. World Health Organization; Centre for Disease Control and Prevention. Assessing the iron status of populations. 2nd ed [Internet]. Geneva; 2007 [cited 2016 Jan 13]. Available from: http://www.who.int/nutrition/publications/micronutrients/anaemia_iron_deficiency/9789241596107/en/

9. Say L, Chou D, Gemmill A, Tuncalp O, Moller AB, Daniels J, et al. Global causes of maternal death: A WHO systematic analysis. Lancet Glob Heal. 2014;2:323–33.
10. World Health Organization. Guideline: Intermittent iron and folic acid supplementation in menstruating women. Geneva, Switzerland; 2011.
11. Huot E. National Guidelines for the Use of Iron Folate Supplementation to Prevent and Treat Anemia in Pregnant and Post-Partum Women. Phnom Penh, Cambodia; 2007.
12. National Nutrition Programme. National Policy and Guidelines for Micronutrient Supplementation to Prevent and Control Deficiencies in Cambodia. Phnom Penh, Cambodia; 2012.
13. Vir SC, Sforza-Cavalli LT. Weekly iron and folic acid supplementation programmes for women of reproductive age: An analysis of best programme practices. Geneva, Switzerland; 2011.
14. Lacerte P, Pradipasen M, Temcharoen P, Imamee N, Vorapongsathorn T. Determinants of adherence to iron/folate supplementation during pregnancy in two provinces in Cambodia. Asia-Pacific J public Heal. 2011 May;23(3):315–23.
15. Charles C V, Summerlee AJS, Dewey CE. Iron content of Cambodian foods when prepared in cooking pots containing an iron ingot. Trop Med Int Heal. 2011;16(12):1518–24.
16. Charles C V, Dewey CE, Daniell WE, Summerlee AJS. Iron-deficiency anaemia in rural Cambodia: community trial of a novel iron supplementation technique. Eur J Public Health. 2011;21(1):43–8.
17. Charles C V, Dewey CE, Hall A, Hak C, Channary S, Summerlee AJS. A Randomized Control Trial Using a Fish-Shaped Iron Ingot for the Amelioration of Iron Deficiency

Anemia in Rural Cambodian Women. Trop Med Surg. 2015;3(3).

18. National Institute of Statistics, Directorate General for Health, Macro I. Cambodia Demographic and Health Survey 2010. Phnom Penh, Camboda and Calverton, Maryland, USA; 2011.

19. World Health Organization. Haemoglobin concentrations for the diagnosis of anaemia and assessment of severity [Internet]. Geneva; 2011 [cited 2016 Jan 13]. p. Vitamin and Mineral Nutrition Information System. Available from: http://www.who.int/vmnis/indicators/haemoglobin/en/

20. Sherwood L, Kell R. Human Physiology: From Cells to Systems. First Cana. Veitch E, Williams A, Fam P, Ryoji R, Grzeskowiak M, editors. Toronto, Ontario: Nelson Education Limited; 2010. 401-424 p.

21. Lynch S. Case studies: iron. Am J Clin Nutr. 2011;94(2):673S – 678S.

22. De Bonist B. Focusing on anaemia [Internet]. Geneva, Switzerland; 2004 [cited 2016 Apr 4]. p. World Health Organization and the United Nations C. Available from: http://www.who.int/nutrition/publications/micronutrients/WHOandUNICEF_statement_anaemia_en.pdf?ua=1

23. Gorstein J, Sullivan KM, Parvanta I, Begin F. Indicators and methods for cross-sectional surveys of vitamin and mineral status of populations. The Micronutrient Initative (Ottawa) and the Centers for Disease Control and Prevention (Atlanta); 2007.

24. Knowles J, Thurnham DI, Phengdy B, Houamboun K, Philavong K, Keomoungkhone I, et al. Impact of inflammation on the biomarkers of iron status in a cross-sectional survey of Lao women and children. Br J Nutr. 2013;110(12):2285–97.

25. Ezzati, M., Lopez, A., Rodgers, A., & Murrary C. Comparative Quantification of Health

Risks Global and Regional Burden of Disease Attributable to Selected Major Risk Factors. World Health Organization. Geneva, Switzerland; 2004.

26. Haas JD, Brownlie TI. Iron deficiency and reduced work capacity: a critical review of the research to determine a causal relationship. J Nutr. 2001;131(2s):676S – 690S.

27. Horton S, Ross J. The economics of iron deficiency. Food Policy. 2003;28(1):51–75.

28. United Nations Children's Fund, United Nations University, World Health Organization. Iron deficiency anaemia: assessment, prevention, and control, a guide for programme managers. Geneva, Switzerland; 2001.

29. Gibson R. Principles of nutrition assessment. 2nd ed. New York: Oxford; 2005.

30. Gropper SS, Smith JL, Groff JL. Advanced Nutrition and Human Metabolism. Fifth Edit. Adams P, Lustig A, Feldman E, Downs E, editors. Belmont, California: Wadsworth Cengage Learning; 2009.

31. Khush GS. Origin, dispersal, cultivation and variation of rice. Plant Mol Biol. 1997;35(1-2):25–34.

32. Inpankaew T, Schär F, Dalsgaard A, Khieu V, Chimnoi W, Chhoun C, et al. High Prevalence of Ancylostoma ceylanicum Hookworm Infections. Emerg Infect Dis. 2014;20(6):976–82.

33. Beard JL, Finch CA. Iron Deficiency. In: Clydesdale FM, Wiemer K, editors. Iron Fortification of Foods. Orlando, Florida: ACADEMIC PRESS, INC.; 1985. p. 3–16.

34. Tee E, Florentino R. Recommended Dietary Allowances: Harmonization in Southeast Asia. Singapore; 2005.

35. Institute of Medicine. Dietary Reference Intakes for Vitamin A, Vitamin K, Arsenic, Boron, Chromium, Copper, Iodine, Iron, Maganese, Molybdenum, Nickel, Silicon,

Vanadium, and Zinc. Washington, D.C.; 2001.

36. Verbowski VC. The effect of plant-based homestead food production with and without small-scale aquaculture on dietary intake of women farmers and their children in Prey Veng, Cambodia. University of British Columbia; 2015.

37. Wallace LJ, Summerlee AJS, Dewey CE, Hak C, Hall A, Charles C V. Women's nutrient intakes and food-related knowledge in rural Kandal province, Cambodia. Asia Pac J Clin Nutr. 2014;23(2):263–71.

38. National Nutrient Database for Standard Reference: Release 27. Basic Report: 11504, Swamp cabbage, cooked, boiled, drained, without salt [Internet]. United States Department of Agriculture: Agricultural Research Service. Washington, D.C.; 2005 [cited 2015 Mar 19]. Available from: http://ndb.nal.usda.gov/ndb/foods/show/3239?fgcd=&manu=&lfacet=&format=&count=&max=35&offset=&sort=&qlookup=cabbage

39. Karakochuk CD, Murphy HM, Whitfield KC, Barr SI, Vercauteren SM, Talukder A, et al. Elevated levels of iron in groundwater in Prey Veng province in Cambodia: a possible factor contributing to high iron stores in women. J Water Health. 2015;13(2):575–86.

40. Feldman PR, Rosenboom J-W, Saray M, Navuth P, Samnang C, Iddings S. Assessment of the chemical quality of drinking water in Cambodia. J Water Health. 2007;5(1):101–16.

41. Karakochuk CD, Whitfield KC, Barr SI, Lamers Y, Devlin AM, Vercauteren SM, et al. Genetic hemoglobin disorders rather than iron deficiency are a major predictor of hemoglobin concentration in women of reproductive age in rural Prey Veng, Cambodia. J Nutr. 2015;145(1):134–42.

42. Wieringa F, Sophonneary P, Whitney S, Mao B, Berger J, Conkle J, et al. Low Prevalence

of Iron and Vitamin A Deficiency among Cambodian Women of Reproductive Age. Nutrients. 2016;8(197).

43. Steinbicker AU. A novel treatment of anemia of inflammation. Blood. 2014;124(17):2618–9.

44. Weiss G, Goodnough LT. Anemia of chronic disease. N Engl J Med. 2005;352(10):1011–23.

45. Weiss G. Iron metabolism in the anemia of chronic disease. Biochim Biophys Acta. Elsevier B.V.; 2009;1790(7):682–93.

46. Nemeth E, Ganz T. Regulation of iron metabolism by hepcidin. Annu Rev Nutr. 2006;26:323–42.

47. Bosman P, Stassijns J, Nackers F, Canier L, Kim N, Khim S, et al. Plasmodium prevalence and artemisinin-resistant falciparum malaria in Preah Vihear Province, Cambodia: a cross-sectional population-based study. Malar J. 2014;13(394).

48. Incardona S, Vong S, Chiv L, Lim P, Nhem S, Sem R, et al. Large-scale malaria survey in Cambodia: novel insights on species distribution and risk factors. Malar J. 2007;6(37).

49. Centers for Disease Control and Prevention. Malaria Information and Prophylaxis, by Country: Cambodia [Internet]. Global Health - Division of Parastic Diseases and Malaria. 2014 [cited 2015 Jan 7]. Available from: https://www.cdc.gov/malaria/travelers/country_table/a.html

50. Weatherall D, Akinyanju O, Fucharoen S, Olivieri N, Musgrove P. Disease Control Priorities in Developing Countries: Chapter 34 - Inherited Disorders of Hemoglobin. 2nd Editio. Jamison D, Breman J, Measham A, Alleyne G, Claeson M, Evans DB, et al., editors. Disease Control Priorities in Developing Countries. New York: Oxford University

Press; 2006. 663-680 p.

51. Weatherall DJ, Clegg JB. Inherited haemoglobin disorders: an increasing global health problem. Bull World Health Organ. 2001;79(8):704–12.

52. Angastiniotis M, Eleftheriou A, Galanello R, Harteveld CL, Petrou M, Traeger-Synodinos J. Prevention of Thalassaemias and Other Haemoglobin Disorders: Volume 2. 2nd ed. Old J, editor. Nicosia, Cyprus: Thalassaemia International Federation; 2012.

53. Old JM. Screening and genetic diagnosis of haemoglobin disorders. Blood Rev. 2003;17:43–53.

54. Fucharoen S, Weatherall DJ. The Hemoglobin E Thalassemias. Cold Spring Harb Perspect Med. 2012;2(8).

55. Carnley BP, Prior JF, Gilbert A, Lim E, Devenish R, Sing H, et al. The prevalence and molecular basis of hemoglobinopathies in Cambodia. Hemoglobin. 2006;30(4):463–70.

56. George J, Yiannakis M, Main B, Devenish R, Anderson C, An US, et al. Genetic Hemoglobin Disorders, Infection, and Deficiencies of Iron and Vitamin A Determine Anemia in Young Cambodian Children. J Nutr. 2012;142(4):781–7.

57. Munkongdee T, Tanakulmas J, Butthep P, Winichagoon P, Main B, Yiannakis M, et al. Molecular Epidemiology of Hemoglobinopathies in Cambodia. Hemoglobin. 2016;40(3):163–7.

58. Zwart A, van Assendelft OW, Bull BS, England JM, Lewis SM, Zijlstra WG. Recommendations for reference method for haemoglobinometry in human blood (ICSH standard 1995) and specifications for international haemiglobinocyanide standard (4th edition). J Clin Pathol. 1996;49(4):271–4.

59. International Committee for Standardization in Haematology. Recommendations for

reference method for haemoglobinometry in human blood (ICSH standard EP 6/2: 1977) and specifications for international haemiglobincyanide reference preparation (ICSH standard EP 6/3: 1977). J Clin Pathol. 1978;31(2):139–43.

60. Sari M, De Pee S, Martini E, Herman S, Sugiatmi, Bloem MW, et al. Estimating the prevalence of anaemia: a comparison of three methods. Bull World Health Organ. 2001;79(6):506–11.

61. Srivastava T, Negandhi H, Neogi SB, Sharma J, Saxena R. Methods for hemoglobin estimation: a review of "what works." J Hematol Transfus. 2014;2(3).

62. Sysmex. Functional description. Sysmex XE-2100 Operator's Manual. Mundelein, IL; 2007.

63. Gulati GL, Hyun BH. Quality control in hematology. Clin Lab Med. 1986 Dec;6(4):675–88.

64. Karakochuk CD, Janmohamed A, Whitfield KC, Barr SI, Vercauteren SM, Kroeun H, et al. Evaluation of two methods to measure hemoglobin concentration among women with genetic hemoglobin disorders in Cambodia: A method-comparison study. Clin Chim Acta. 2015;441:148–55.

65. Moharram NMM, Aouad RE, Busaidy SA, Fabricius A, Heller S, Wood WG, et al. International collaborative assessment study of the AHD 575 method for the measurement of blood haemoglobin. East Mediterr Heal J. 2006;12(6):722–34.

66. Chakravarthy K V, Chandra ND, Prasanna SB, Rao JMT, Rao RD. Haemoglobin estimation by non-cyanide methods. J Clin Diagnostic Res. 2012;6(6):955–8.

67. Lafferty JD, Crowther MA, Ali MA, Levine M. The evaluation of various mathematical RBC indices and their efficacy in discriminating between thalassemic and non-thalassemic

microcytosis. Am J Clin Pathol. 1996;106(2):201–5.

68. HemoCue. HemoCue Hb 201+ operating manual. Angelholm; 1995.

69. Polin V, Coriat R, Perkins G, Dhooge M, Abitbol V, Leblanc S, et al. Iron deficiency: from diagnosis to treatment. Dig Liver Dis. 2013;45(10):803–9.

70. Zimmermann MB, Hurrell RF. Nutritional iron deficiency. Lancet. 2007;370(9586):511–20.

71. Garrett D a., Sangha JK, Kothari MT, Boyle D. Field-friendly techniques for assessment of biomarkers of nutrition for development. Am J Clin Nutr. 2011;94(2):685–90.

72. Beard JL. Iron Biology in Immune Function, Muscle Metabolism and Neuronal Functioning. J Nutr. 2001;131(2S-2):568S – 580S.

73. World Health Organization. Serum ferritin concentrations for the assessment of iron status and iron deficiency in populations. Geneva; 2011.

74. Wieringa FT, Dijkhuizen MA, West CE, Northrop-Clewes CA, Muhilal. Estimation of the effect of the acute phase response on indicators of micronutrient status in Indonesian infants. J Nutr. 2002;132(10):3061–6.

75. Thurnham DI, Mccabe GP. Influence of infection and inflammation on biomarkers of nutritional status with an emphasis on vitamin A and iron [Internet]. WHO Report: Priorities in the assessment of vitamin A and iron status in populations. Geneva; 2012 [cited 2014 Nov 14]. p. 63–80. Available from: http://www.who.int/nutrition/publications/micronutrients/background_paper4_report_assessment_vitAandIron_status.pdf

76. Engle-Stone R, Nankap M, Ndjebayi AO, Erhardt JG, Brown KH. Plasma ferritin and soluble transferrin receptor concentrations and body iron stores identify similar risk

factors for iron deficiency but result in different estimates of the national prevalence of iron deficiency and iron-deficiency anemia among women a. J Nutr. 2013 Mar;143(3):369–77.

77. Yong T, Chai J, Sohn W, Eom KS, Jeoung H, Hoang E. Prevalence of Intestinal Helminths among Inhabitants of Cambodia (2006-2011). Korean J Parasitol. 2014;52(6):661–6.

78. Thurnham DI, McCabe LD, Haldar S, Wieringa FT, Northrop-Clewes CA, McCabe GP. Adjusting plasma ferritin concentrations to remove the effects of subclinical inflammation in the assessment of iron deficiency: a meta-analysis. Am J Clin Nutr. 2010 Sep;92(3):546–55.

79. Skikne BS, Flowers CH, Cook JD. Serum transferrin receptor: a quantitative measure of tissue iron deficiency. Blood. 1990;75(9):1870–6.

80. Karakochuk CD, Whitfield KC, Rappaport AI, Barr SI, Vercauteren SM, Mclean J, et al. The homozygous hemoglobin EE genotype and chronic inflammation are associated with high serum ferritin and soluble transferrin receptor concentrations among women in rural Cambodia. J Nutr. 2015;2765–73.

81. Ministry of Planning. For production and consumption of Iron Fortified Fish Sauce and Soy Sauce Senior. Phnom Penh, Cambodia; 2012.

82. Stoltzfus RJ, Dreyfuss ML. Guidelines for the Use of Iron Supplements to Prevent and Treat Iron Deficiency Anemia. Washington, D.C.; 1998.

83. Gillespie S. Major Issues in the Control of Iron Deficiency. Ottawa, Ontario; 1998.

84. Theary C, Panagides D, Laillou A, Vonthanak S, Kanarath C, Chhorvann C, et al. Fish sauce, soy sauce, and vegetable oil fortification in Cambodia: Where do we stand to date?

Food Nutr Bull. 2013;34(2):S62–71.

85. Hurrell RF. Preventing Iron Deficiency Through Food Fortification. Nutr Rev. 1997;55(6):210–22.

86. Laillou A, Pfanner S, Chan T, Chea C, Mam B, Sambath P, et al. Beyond effectiveness - the adversities of implementing a fortification program. A case study on the quality of iron fortification of fish and soy sauce in Cambodia. Nutrients. 2016;8(94):1–8.

87. Hallberg L. Factors Influencing the Efficacy of Iron Fortification and the Selection of Fortification Vehicles. In: Clydesdale F, Wiemer K, editors. Iron Fortification of Foods. Orlando: Academic Press, INC.; 1985. p. 17–28.

88. Brittin HC, Kollipara UK. Increased iron content of some Indian foods due to cookware. J Am Diet Assoc. 1996;96(5):508.

89. Burroughs, P. H., Chan JJ. Iron Content of Some Mexican-American Foods.pdf. J Am Diet Assoc. 1972;60(2):123.

90. Brittin HC, Zhou Y-D. Increased iron content of some Chinese foods due to cooking in steel woks. J Am Diet Assoc. 1994;94(10):1153.

91. Geerligs PPD, Brabin BJ, Hart DJ, Fairweather-Tait SJ. Iron Contents of Malawian Foods when Prepared in Iron Cooking Pots. Int J Vtamin Nutr Res. 2004;74(1):21–6.

92. Cheng YJ, Brittin HC. Iron in Food: Effect of Continued Use of Iron Cookware. J Food Sci. 1991;56(2):584–5.

93. Rosanoff A, Kennedy BM. Bioavailability of Iron Produced by the Corrosion of Steel in Apples. J Food Sci. 1982;47(2):609–13.

94. Martinez FE, Vannucchi H. Bioavailability of Iron Added to the Diet by Cooking Food in an Iron Pot. Nutr Res. 1986;6(4):421–8.

95. Adish AA, Esrey SA, Gyorkos TW, Jean-Baptiste J, Rojhani A. Effect of consumption of food cooked in iron pots on iron status and growth of young children: a randomised trial. Lancet. 1999;353:712–6.

96. Geerligs PP, Brabin B, Mkumbwa A, Broadhead R, Cuevas LE. The effect on haemoglobin of the use of iron cooking pots in rural Malawian households in an area with high malaria prevalence: a randomized trial. Trop Med Int Heal. 2003;8(4):310–5.

97. Sharieff W, Dofonsou J, Zlotkin S. Is cooking food in iron pots an appropriate solution for the control of anaemia in developing countries? A randomised clinical trial in Benin. Public Health Nutr. 2008;11(9):971–7.

98. Geerligs PP, Brabin B, Mkumbwa A, Broadhead R, Cuevas LE. Acceptability of the use of iron cooking pots to reduce anaemia in developing countries. Public Health Nutr. 2002;5(5):619–24.

99. Tripp K, Mackeith N, Woodruff BA, Talley L, Mselle L, Mirghani Z, et al. Acceptability and use of iron and iron-alloy cooking pots: implications for anaemia control programmes. Public Health Nutr. England; 2010;13(1):123–30.

100. World Health Organization. WHO guidelines for drinking water quality. Geneva, Switzerland; 2011.

101. Dysoley L, Rithea L, Bunkea T, Babu S, Sim K, Chea N, et al. Cambodia Malaria Survey. Phnom Penh, Cambodia; 2010.

102. Morris SS, Ruel MT, Cohen RJ, Dewey KG, de la Brière B, Hassan MN. Precision, accuracy, and reliability of hemoglobin assessment with use of capillary blood. Am J Clin Nutr. 1999;69(6):1243–8.

103. Thurnham DI, Northrop-clewes CA, Knowles J. The Use of Adjustment Factors to

Address the Impact of Inflammation on Vitamin A and Iron Status in Humans. J Nutr. 2015;145(5):1137s – 1143s.

104. World Health Organization. BMI Classification [Internet]. 2016 [cited 2016 Jun 9]. Available from: http://apps.who.int/bmi/index.jsp?introPage=intro_3.html

105. Armstrong, R G, Dewey E C, Summerlee AJS. Iron release from the Lucky Iron Fish: Safety considerations. 2015;1–23.

106. HemoCue. HemoCue Hb 301 operating manual. Angelholm;

107. National Health and Nutrition Examination Survey (NHANES), Centre for Disease Control (CDC). Anthropometry Procedures Manual [Internet]. 2007. Available from: http://www.cdc.gov/nchs/data/nhanes/bm.pdf

108. Nordenberg D, Yip R, Binkin NJ. The effect of cigarette smoking on hemoglobin levels and anemia screening. JAMA. 1990;264(12):1556–9.

109. National Nutrition Programme. National Policy and Guidelines for Micronutrient Supplementation to Prevent and Control Deficiencies in Cambodia. 2011.

110. Wieringa F, Dahl M, Chamnan C, Poirot E, Kuong K, Sophonneary P, et al. The High Prevalence of Anemia in Cambodian Children and Women Cannot Be Satisfactorily Explained by Nutritional Deficiencies or Hemoglobin Disorders. Nutrients [Internet]. 2016;8(6):348. Available from: http://www.mdpi.com/2072-6643/8/6/348

111. Bland JM, Altman DG. Statistical methods for assessing agreement between two methods of clinical measurement. Lancet. 1986;8:307–10.

112. Altman DG, Bland JM. Measurement in medicine: the analysis of method comparison studies. J R Stat Soc. 1983;32:307–17.

Appendices

Appendix A: Village and commune list

Appendix B: Iron leached from the LIF

Appendix C: Original consent form

Appendix D: Schedule for 6-month data collection

Appendix E: Blood sampling procedure at baseline and 12 months

Appendix F: Blood sampling procedure at 6 months

Appendix G: Comparing two Sysmex machines at 6 months

Appendix H: Questionnaires utilized in data collection

Appendix I: National Nutrition Program education

Appendix J: Monitoring and evaluation forms

Appendix A: Village and commune list

Province	District	Commune	Village
Preah Vihear	Rovieng	Robieb	Tnaot Mlu
Preah Vihear	Rovieng	Robieb	Rovieng Cheung
Preah Vihear	Rovieng	Rung Raeung	Boh Pey
Preah Vihear	Rovieng	Rung Raeung	Thnal Kaeng
Preah Vihear	Rovieng	Rung Raeung	Srae Thum
Preah Vihear	Rovieng	Rik Reay	Boh
Preah Vihear	Rovieng	Rik Reay	Doung
Preah Vihear	Rovieng	Rik Reay	Pal Hal
Preah Vihear	Rovieng	Rumdaoh	Kouk Ampil
Preah Vihear	Rovieng	Rumdaoh	Thnal Kaong
Preah Vihear	Rovieng	Romtum	Tuol Rovieng
Preah Vihear	Rovieng	Romtum	Ou Talaok
Preah Vihear	Rovieng	Romoneiy	Ou Pou
Preah Vihear	Rovieng	Romoneiy	Rumchek
Preah Vihear	Rovieng	Rohas	Anlong Svay
Preah Vihear	Rovieng	Rohas	Sangkae Roung
Preah Vihear	Rovieng	Rohas	Chamlang
Preah Vihear	Rovieng	Reaksmei	Chambak Ph’aem

Appendix B: Iron Leached from the LIF

A LIF from the same batch as the ones given out in this clinical trial was utilized in this water content study. I cleaned all the equipment and used a large glass beaker to prepare 2L of deionized water with a squeeze of juice from one fresh lime (~24mL) as recommended by the LIF Company. Then I tested the pH of the water to ensure it was acidic (pH = 2.9). I separated the 2 L of water into two smaller glass beakers and boiled one of the beakers with the LIF on a hotplate for 10 minutes, and the other glass beaker with the LIF for 60 minutes. I measured the pH of each beaker of water to ensure they were equal to one another. I allowed the water to cool before placing it into the containers with preservatives provided by Agat laboratory and stored the samples in a cooler on ice.

Table 14 is a summary, and not all metal contaminants are listed in this table. Metal contaminants that were omitted fall well below the WHO drinking water standards, or have no established WHO Drinking water standard level. Most metal contaminants in the water fell below the WHO drinking water standards. Aluminum, Chromium, and Iron all fell above these standards. Iron was almost 1000 times greater than the WHO drinking water standard. Aluminum and Chromium only fell above the standard when boiled for 60 minutes, rather than the recommended 10.

Table 14: Metal contaminants and iron content of water boiled with the LIF

Parameter	Unit	Boiled 10 minutes	Boiled 60 minutes	WHO drinking water standard (100)
Aluminum	µg/L	59	175	100 µg/L
Antimony	µg/L	<0.5	<0.5	20 µg/L
Arsenic	µg/L	2.1	2.5	10 µg/L
Barium	µg/L	7.5	11.7	700 µg/L
Chromium	µg/L	45.5	103	50 µg/L
Copper	µg/L	12.1	14.9	2000 µg/L
Iron	**µg/L**	**121,000**	**299,000**	**300 µg/L**
Lead	µg/L	0.31	0.41	10 µg/L
Manganese	µg/L	593	1030	400 µg/L
Mercury	µg/L	0.03	0.05	6 µg/L
Nickel	µg/L	12.4	24.2	70 µg/L

The recommended cooking time for the LIF is 10 minutes. However, many women leave the fish in the pot throughout the entire cooking process, leading to excess metals being leached in the cooking pot. Aluminum, Chromium, and Iron are all above the WHO drinking water standards when boiled for 60 minutes. Iron is above these standards even when boiled for 10 minutes, however, this is expected as the goal of the LIF is to increase dietary intake of iron by leaching iron into the cooking pot.

The WHO recommends aluminum concentrations in water to be less than 100 µg/L, when boiled for 60 minutes, the LIF leaches 175 µg/L of aluminum into the water (100). Despite aluminum's abundance in the earth, there is some evidence indicating the orally ingested aluminum is acutely toxic to humans and that it may be a risk factor for the development and/or the acceleration of Alzheimer's disease (100). Past research in Cambodia has found high levels of aluminum in the water contributing to excess aluminum consumption (39,40). As such, if consumed daily in excess from the LIF and from the groundwater for an extended period of time, it may cause health risks to the population.

The LIF when boiled for 60 minutes leaches Chromium concentrations (103 μg/L) above the WHO drinking water standards (50 μg/L). In excess amounts, Chromium is of concern to human health, as chromium (VI) is considered a carcinogen to humans (100). This 50 μg/L guideline was set as chromium occurs in water naturally, but is considered to be of health significance in drinking water when found in excess (100). Over time Chromium may cause negative health issues for users of the LIF.

The presence of iron in drinking water at high concentrations may affect the acceptability of the water due to colour and taste changes (100). As such, the WHO recommends iron content to be below 300 μg/L (100). However the LIF leaches iron well above this amount: when boiled for 60 minutes, it leaches almost 1000 times more iron than the WHO drinking water guidelines (100). At first, it may seem like 121,000 μg/L (121 mg/L) and 299,000 μg/L (299 mg/L) is a lot and that it may pose toxicity issues. However, first it is important to think of the quantity of consumption. In an ideal setting, if a woman consumes about 200 mL of soup or water in which the fish was boiled for 10 minutes it is roughly equivalent of 24 mg of iron. If the LIF was left in the cooking pot for an hour, and 200 mL of soup is consumed it is equivalent of about 60 mg of iron, again in an ideal setting. It is important to consider the bioavailability of the iron provided. A recent paper published on the safety of the LIF, suggests that the iron is in its reduced form, $Fe^{2}+$, the form of iron generally used in food fortification and supplements as it has greater bioavailability (85). Therefore, if this is the case and the whole family is eating from the same cooking pot, the iron levels released from the LIF may pose toxicity issues for the non-anemic and/or non-iron deficient family members with lower iron requirements. However, based on the aforementioned results, it doesn't appear that cooking with the LIF improves iron status among women of reproductive age. Perhaps the iron from the LIF is not bioavailable.

Appendix C: Original consent form

A randomized control trial of the Lucky Iron Fish™ to improve hemoglobin concentration in women in Preah Vihear, Cambodia
Subject Information and Consent Form

Introduction

Anemia is a severe public health issue in Cambodia. The most common form of anemia is iron deficiency anemia (IDA), which is caused by a lack of iron in the diet, poor absorption of iron, and/or a loss of iron from the body. Anemia can lead to disease, feeling tired leading to an inability to work, and an increased risk of negative pregnancy outcomes, including maternal and child death.

In Cambodia, anemia is common, and half of all cases are believed to be caused by iron deficiency. Cambodia does not have the resources to screen or treat anemia. In addition, non-governmental organizations (NGOs) have had little success with iron supplements as a means to address anemia due to high cost, distribution issues, poor adherence, and sustainability. Therefore, a novel strategy to address anemia is needed. One promising option is the Lucky Iron Fish™ (LIF™), a piece of iron shaped like a fish that is placed in the pot during cooking. Iron leeches from the LIF™ into the food when cooked with lemon for 10 minutes. The goal of the LIF™ is to increase dietary intake of iron with a small, lightweight, and easy to clean system.

This study will take place in Preah Vihear province, Cambodia where 330 women will be invited to participate in this study. Women will be randomized (like flipping a coin) to one of three groups: LIF™ group (women receive a LIF™ for use during cooking in the common pot throughout the study), iron supplement group (women consume one tablet of 18mg elemental iron daily), or control group (women receive education on methods to increase dietary intake of iron). The aim of this study is to determine if the LIF™ will improve iron status of women of reproductive age (18-49y) compared to women consuming daily iron supplements (18mg elemental iron) or the control group, after 6 and 12 months.

Participation

Your participation in this study is entirely voluntary, so it is up to you to decide whether you would like to take part. Before you decide it is important for you to understand what the research involves. This consent form will tell you about the study, why the research is being done, what participation in the study will look like, and the possible benefits, risks, and discomforts.
If you wish to take part in the study, you will be asked to provide verbal consent to the trained interviewer and sign/thumb print this consent form. If you do decide to take part in this study, you are still free to withdraw at any time and without giving any reasons for your decision. If you do not wish to take part, you do not have to provide any reason for your decision not to participate.
Please take time to read the following information carefully and to discuss it with your family, friends, and Village Chief before you decide.

Who is conducting the study?

Researchers from Food, Nutrition, and Health in the Faculty of Land and Food Systems at the University of British Columbia in Canada are conducting this study with NutriFood Cambodia. Co-investigators of this study are [redacted], and [redacted]. You are entitled to request further details from the investigators.

Who Can Participate?

To participate in this study, individuals must:
- be a woman aged 18-49 years,
- have a hemoglobin concentration between 80-119 g/L at screening, indicating that the woman has mild or moderate anemia (women with severe anemia (hemoglobin less than (<) 80g/L) or normal hemoglobin (hemoglobin greater than or equal to (≥) 120g/L) at baseline screening will be excluded; those with severe anemia will be referred to the nearest health centre for physician consultation (transport fees will be covered for this initial visit),
- be willing to provide a finger prick blood sample at baseline, and venous blood samples at baseline, 6 months, and endline,
- live in Preah Vihear province and not be planning to move in the next 12 months,
- not be participating in/receiving another nutritional intervention,
- not be currently consuming, or planning to consume iron supplements over the next 12 months,
- not be ill or on medication,
- not be pregnant (based on self-report), and
- be able to provide written, informed consent.

Who Should Not Participate in This Study?

You should not particpcate in this study if you do not meet the criteria above, or if you are unable to provide informed consent.

Study Procedures

If you agree to participate, your hemoglobin level will be determined from a finger prick blood sample to ensure your eligibility to participate in this study. This screening test will take place in your home before baseline questionnaire administration. Following the finger prick blood test, you will be asked to complete a baseline questionnaire, then, you will be randomized (like flipping a coin) into one of the three groups (control, daily dose of 18 mg elemental iron, and Lucky Iron Fish^{TM}). Depending on your group, we will teach you how to use the LIF or how to consume the iron tablets.

Within the first month of the study, you will be invited to participate in an educational workshop with training regarding dietary sources of iron, iron deficiency anemia, and malaria.

Questionnaire (time: 1 hour)

1. You will review the study procedures and provide consent to a Khmer-speaking interviewer.
2. You will be asked to complete a questionnaire that includes modules on:
    a. yourself, such as your age and education level, and

b. consumption habits, typical food intakes, water treatment practices
c. usual physical activity status
d. current knowledge on anemia, iron deficiency anemia, and malaria
e. the length of time spent sleeping, and working,
f. demographic and socio-economic information such as: household members, housing characteristics and household possessions

3. You do not need to answer questions that make you uncomfortable.
4. Trained experts will measure your height and weight.

Blood Draw (time: 1 hour, including travel time)

There will be two components to the blood sample collection

1. **Hemocue test:** This test requires a finger prick blood sample to ensure you have mild or moderate anemia (hemoglobin levels between 80 – 120 g/L). If you have severe anemia (hemoglobin circulating level less than 80g/L) you will be referred for immediate medical attention and will not be eligible to participate in this study.
2. **Venous blood sample:** A 10mL venous blood sample will be collected now, at six, and at twelve months at the local health centre. This blood will be used for a Complete Blood Count (CBC), and will be analyzed for biomarkers of iron status.

Risks

The blood collection procedure may cause some discomfort and slight bruising or, very rarely, an infection at the site of the needle poke. After the blood draw you will immediately be given a bandage to cover the spot where the blood was taken.

Side effects of iron supplementation are mild, including: constipation, cramping, nausea, increased risk for bacterial infection, zinc deficiency from oral iron supplementation. or other discomfort that are associated with high-doses of iron. Women will be informed of these side effects and provided with information about how to reduce and/or avoid these symptoms.

Benefits

If you agree to take part in this study, there may or may not be a direct benefit to you. If you are in the iron supplement or the LIF™ group, you may see health benefits from increased dietary iron intake. Although you may not be receiving iron, nutrition education will be provided by trained local research staff. At the end of the study we will return to your village to inform you of your iron status.

We understand this survey will take some time away from your work and family. Now, and six months and a year from you will travel to the local health centre for a venous blood draw. As remuneration for your time, you will receive one sarong and will be provided with US$2.00 to reimburse travel expenses to and from your local health centre.

Confidentiality

Your confidentiality will be respected. However, research records and health or other source records identifying you may be inspected in the presence of the Investigator or his or her

designate by representatives of UBC Clinical Research Ethics Board, and Cambodian National Ethics Committee for Health Research for the purpose of monitoring the research. No information or records that disclose your identity will be published without your consent, nor will any information or records that disclose your identity be removed or released without your consent unless required by law. You will be assigned a unique study number as a subject in this study. Only this number will be used on any research-related information collected about you during the course of this study, so that your identity (i.e. your name or any other information that could identify you) as a subject in this study will be kept confidential. Information that contains your identity will remain only with the Principal Investigator and/or designate. No records that identify you by name or initials will be allowed to leave the Investigators' offices. De-identified information such as blood samples and other data will be sent to the researchers at the University of British Columbia. The list that matches your name to the unique study number that is used on your research-related information will not be removed or released without your consent unless required by law.

Consent

This study has been explained to you and you have been given the chance to ask questions about taking part in this study. If you have questions you can ask the interviewer, or contact [redacted], or [redacted], a Khmer speaking contact [redacted].

Participation and Withdrawal from this Study

Taking part in this study is voluntary. You may withdraw from this study at any time without giving reasons. If you choose to enter the study and then decide to withdraw at a later time, you have the right to request the withdrawal of your information and samples collected during the study. This request will be respected to the extent possible. Please note however that there may be exceptions where the data and samples will not be able to be withdrawn for example where the data and/or sample is no longer identifiable (meaning it cannot be linked in any way back to your identity) or where the data has been merged with other data. If you would like to request the withdrawal of your data [and/or samples], please let your study doctor know.

What if Something Goes Wrong?

Signing this consent form in no way limits your legal rights against the sponsor, investigators, or anyone else, and you do not release the study doctors or participating institutions from their legal and professional responsibilities.

Consent Form

- I have listened to or read and understood the subject information and consent form.
- I have had sufficient time to consider the information provided and to ask for advice.
- I have had the opportunity to ask questions and have had satisfactory response to my questions.
- I understand that all of the information collected will be kept confidential and that the results of this study will only be used for scientific objectives.

- I understand that participation in this study is voluntary and that I am completely free to refuse to participate or to withdraw from this study at any time without changing in any way the quality of care that I receive.
- I understand that I am not waiving any of my legal rights as a result of signing this consent form.
- I have read this form and I freely consent to participate in this study.
- I have been told that I will receive a dated and signed copy of this form.

I consent to participating in this study.

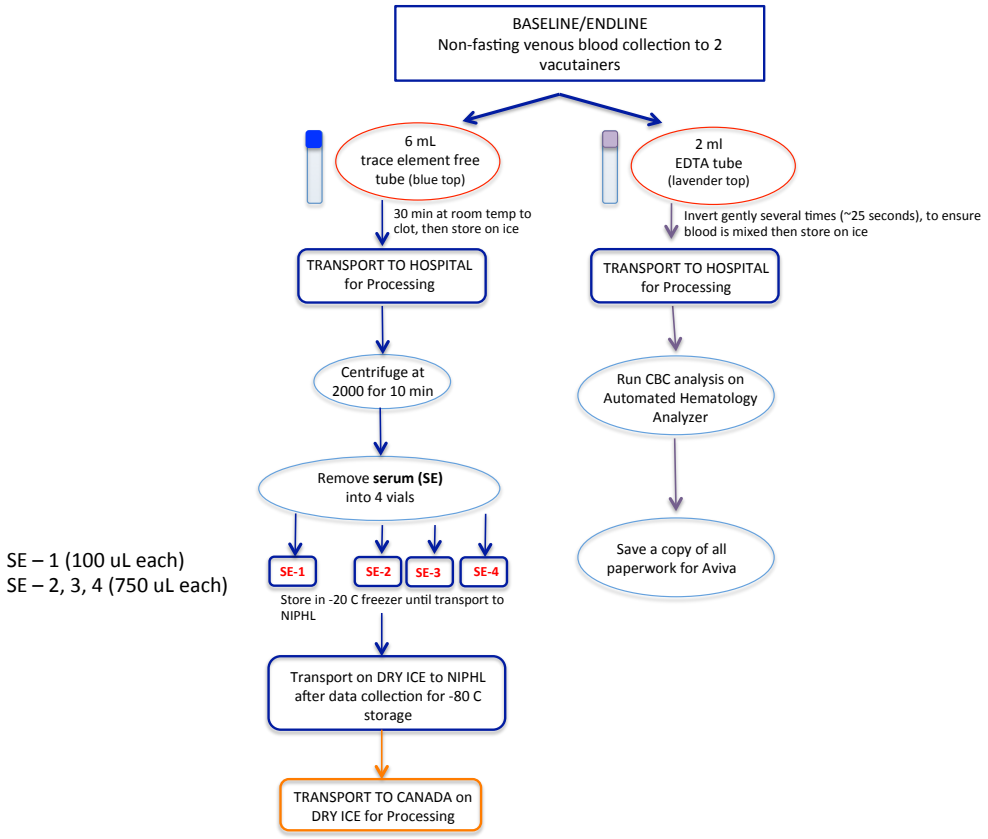
Printed Name of Subject	**Signature/Thumb Print**	**Date**

Printed Name of Person Obtaining Consent	**Study Role**	**Signature**	**Date**

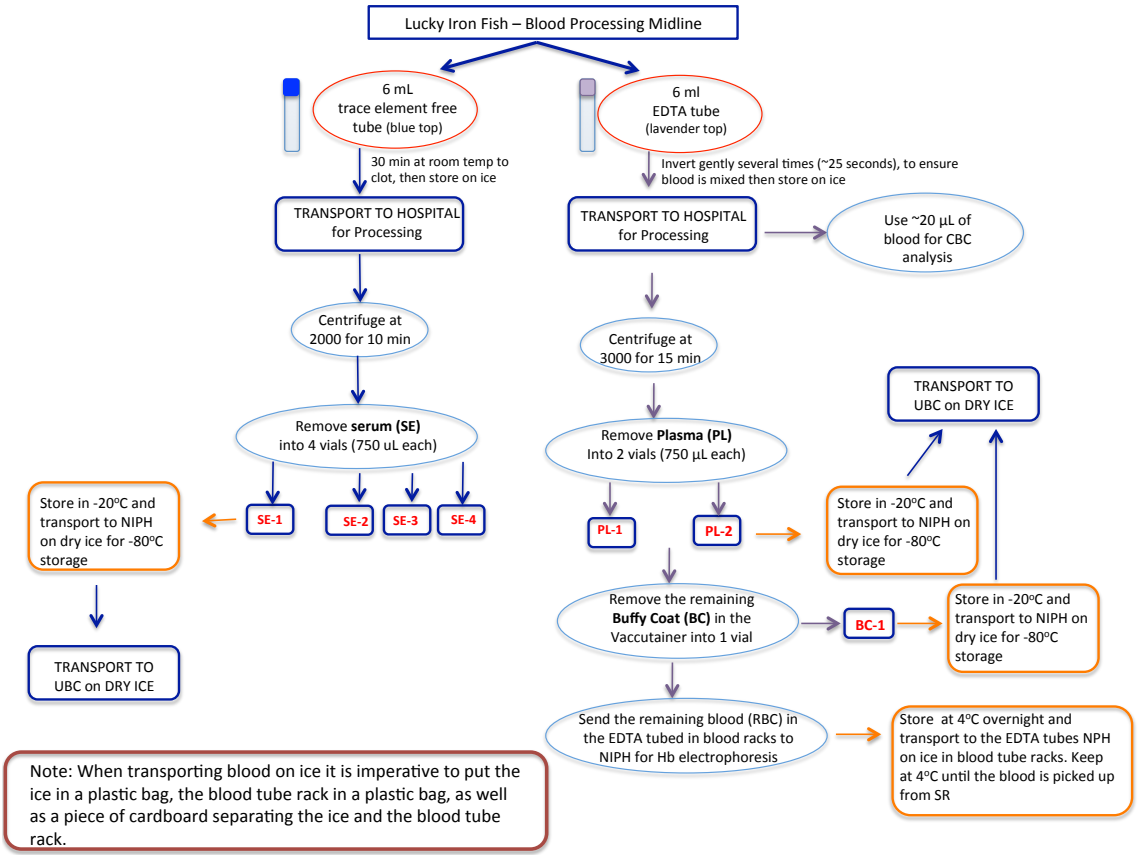
Appendix D: Schedule for ML data collection

	May 6th	May 7th	May 8th	May 9th	May 10th	May 11th	May 12th	May 13th
Village Morning	Rumchek Ou Pou	Doung 1 & Doung 2	Thnal Kaong & Kouk Ampil	Ou Talaok & Tuol Rovieng	Boh Pey & Srae Thum	Sangkae Roung & Chambak Ph'aem	Anlong Svay Chamlang	Pal Hal Boh
Village Afternoon	----------	----------	----------	Thnal Kaeng	Tnalot Mlu & Rovieng Cheung	----------	----------	----------
Team Van Morning	Drive team 1 to Rumchek then team 2 to Ou Pou	Drives to Doung 1, then gets women from Doung 2 to Doung 1	Drives team 2 Kouk Ampil then drives team 1 to Thnal Kaong	Drives team 2 to Toul Rovieng Then team 1 to Ou Talaok	Drives team 2 to Srae Thum, then Team 1 to Boh Pey	Drives team 1 to Sangkae Roung and team 2 to Chambak Ph'aem	Drives team 2 to Anlong Svay and then drops team 1 in Chamlang	Drives team 1 to Pal Hal then drops team 2 in Boh
Team Van Afternoon	Drives everyone to lunch, then to guesthouse	Drives everyone to lunch, then to guesthouse	Drives everyone to lunch, then to guesthouse	Drives everyone to lunch, then everyone to Thnal Kaeng	Drives everyone to lunch, then team 1 to Tnaot Mlu and Team 2 to Rovieng Cheung	Drives everyone to lunch, then to guesthouse	Drives everyone to lunch, then to guesthouse	Enumerator team goes home
White Car	Aviva will go with blood to Siem Reap and back to PV in the same day	Drive with Lin and the blood to Siem Reap and will come back to PV in the same day	Drive with blood to Siem Reap and bring paperwork to Aviva the following day	Pick up blood from Thnal Kaeng, drive it to SR. Bring paperwork to Aviva the following day	Pick up blood from Tnaot Mlu and Toul Rovieng, drive it to SR. Bring paperwork to Aviva the following day	Pick up the blood and drive it to SR. Bring paperwork to Aviva	Pick up blood then drive to SR. When blood is finished, drive back to PV, give Aviva paperwork	Take blood with Aviva and Lin to SR.
Seang's Car	Help Mesa arrange and coordinate with VHSG for future days	Help Mesa arrange and coordinate with VHSG for future days	Help Mesa arrange and coordinate with VHSG for future days	Drives blood from morning Ou Talaok and Toul Rovieng with Lin to hospital, and back to PV	Drives blood from Boh Pey and Srae Thum to hospital, and back to PV. Bring Aviva the paperwork	Help Mesa arrange and coordinate with VHSG for future days	Help Mesa arrange and coordinate with VHSG for future days	Drive back to PP
Green Car	Drives to SR from PP	Drives samples from SR to PP	Drives back to SR from PP	Drives blood to PP	Drives back to SR	Drives blood to PP	Drives back to SR	Drives blood to PP

Appendix E: Blood sampling procedure at baseline and 12 months



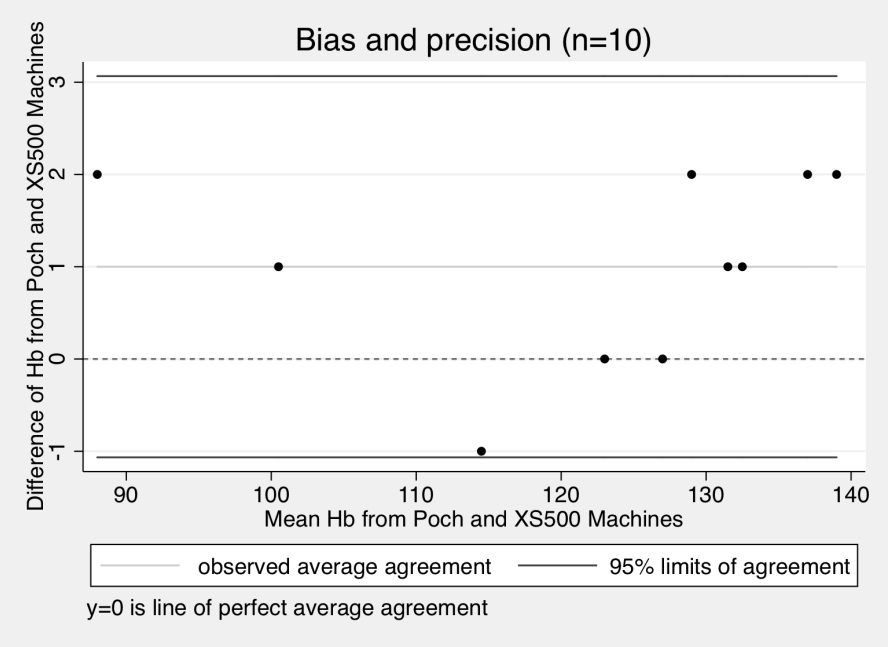
Appendix F: Blood sampling procedure at 6 months



Appendix G: Comparing two Sysmex machines at 6 months

Even though only 10 samples were run on both machines, in order to account for differences between the 2 Sysmex machines agreement and precision was determined. Agreement was calculated using Bland and Altman's bias and the precision was measured using limits of agreement (95% CI of the bias), and is reported as ± 1.96 SD. Bias is reported using the difference in means for Hemoglobin concentration with the pocH-100i compared to the Sysmex XS-500i. Methods are interpreted to be equivalent when bias is small, and the precision is narrow (111,112). At 6 months, 10 random samples on both machines to determine if the two machines were comparable. I used Bland and Altman's limits of agreement to determine the agreement and precision of the two hematology analyzers Figure 4. The two machines are considered to be equivalent when bias is small and precision is narrow. Bias was low at 1.0 g/L and the 95% CI are narrow as well.

Figure 4: Bias and precision plot for hemoglobin concentration on 2 sysmex machines



Appendix H: Questionnaires utilized in data collection

BASELINE QUESTIONNAIRE

A randomized control trial of the Lucky Iron Fish™ to improve hemoglobin concentration in women in Preah Vihear, Cambodia

CONFIDENTIAL
All information collected in this survey is strictly confidential and will be used for statistical purposes only.

IDENTIFICATION INFORMATION	
Geographic Identification	**Interviewer Record**
Province: _____Preah Vihear_____ District: _____Roveing_____ Commune: _____ ☐☐ Village: _____ ☐☐ Subject ID: __ __ __	Interviewer Name: _____ Signature: _____ Remarks:
Date of Interview: (DD/MM/YYYY) ___ ___ / ___ ___ / ___ ___ ___ ___	

INFORMED CONSENT	
This participant, Subject ID __ __ __ __ __, has read/been read and understands the consent form, and has given voluntary, informed verbal consent to participate in this study.	1. Yes 2. No → Do **not** proceed ☐

MODULE 1: PARTICIPANT INFORMATION	
1. How old are you?	_____ years ☐☐
2. How many people currently live in your household (defined as eating from the same pot each day)?	_____ people ☐☐
3. How many children have you given birth to?	_____ children, ☐☐
4. If you have children, how long has it been since you gave birth? (Not pregnancy – actual birth)	_____ Month ☐☐ _____ Year ☐☐
5. Have you completed any schooling?	1. Yes 2. **No → skip to Q7** ☐
6. What is the highest level of school you attended?	1. Primary school 2. Lower Secondary school 3. Upper Secondary school

	4. Higher education 5. Non formal Literacy Course
7. Did your husband/partner complete any schooling?	1. Yes 2. **No → skip to Q9** **8.** N/A **→ skip to Q9**
8. What is the highest level of schooling your husband/partner attended?	1. Primary school 2. Lower Secondary school 3. Upper Secondary school 4. Higher education 5. Non formal Literacy Course
9. What was the income for your household last **month**?	US$_____
10. Is this income your typical monthly income?	1. Yes 2. No
11. What was the income for your household in the past **12 months**? If the woman only knows her income ask her to estimate the total income – if she cant estimate ask her to tell you her income or how much she knows exactly and then make a note that it is only her income and include who else earns money in the family but that she doesn't know how much they earn	US$_____
12. How much time did you spend sleeping yesterday?	Hours _____ Minutes _____
13. How much time did you spend working yesterday? *Note: work includes any paid or unpaid duties, and can include tasks such as childcare and household management.*	Hours _____ Minutes _____
14. Is this pattern a typical routine?	1. Yes → skip to Q16 2. No
15. If this pattern is atypical, how many hours do you usually spend sleeping and working per day?	**Sleeping:** Hours _____ Minutes _____ **Working:** Hours _____ Minutes _____

MODULE 2: FOOD INTAKE

16. I would like to ask you about the foods and drinks that you may have consumed **yesterday** during the day or night. I want to know how often you ate the food, approximately how much of the food you ate in a typical serving, and how it was consumed.

Food Type	Consumed yesterday	If yes, how often?	Amount per serving (g or mL)	Consumed alone, or with other foods?

A. Bread, rice, noodles, porridge, or other foods made from grains?	1. Yes ☐ 2. No → code, then proceed to next row	1. Once 2. Twice 3. Three times 4. >3 times _____ ☐		☐ 1. Consumed alone 2. Consumed with other foods; describe:	
B. Dark leafy green vegetables (eg. kang kong)	1. Yes ☐ 2. No → code, then proceed to next row	1. Once 2. Twice 3. Three times 4. >3 times _____ ☐		☐ 1. Consumed alone 2. Consumed with other foods; describe:	
C. Ripe mangoes, papayas or any other yellow or orange fruits?	1. Yes ☐ 2. No → code, then proceed to next row	1. Once 2. Twice 3. Three times 4. >3 times _____ ☐		☐ 1. Consumed alone 2. Consumed with other foods; describe:	
D. Other fruits or vegetables?	1. Yes ☐ 2. No → code, then proceed to next row	1. Once 2. Twice 3. Three times 4. >3 times _____ ☐		☐ 1. Consumed alone 2. Consumed with other foods; describe:	
E. Liver, kidney, heart, or other organ meats?	1. Yes ☐ 2. No → code, then proceed to next row	1. Once 2. Twice 3. Three times 4. >3 times _____ ☐		☐ 1. Consumed alone 2. Consumed with other foods; describe:	
F. Meat, such as beef, pork, lamb, goat, chicken, or duck?	1. Yes ☐ 2. No → code, then proceed to next row	1. Once 2. Twice 3. Three times 4. >3 times _____ ☐		☐ 1. Consumed alone 2. Consumed with other foods; describe:	
G. Eggs?	1. Yes ☐ 2. No → code, then proceed to next row	1. Once 2. Twice 3. Three times 4. >3 times ☐		☐ 1. Consumed alone 2. Consumed with other foods; describe:	
H. Fresh or dried fish and shellfish (including clams, snails, crab, squid)?	1. Yes ☐ 2. No → code, then proceed to next row	1. Once 2. Twice 3. Three times 4. >3 times _____ ☐		☐ 1. Consumed alone 2. Consumed with other foods; describe:	
I. Foods made from beans, peas,	1. Yes ☐	1. Once 2. Twice 3. Three ☐		☐ 1. Consumed alone 2. Consumed with other foods; describe:	

lentils, or nuts?	2. No → code, then proceed to next row	times 4. >3 times _____		
J. Foods made with oil, fat, or butter?	1. Yes ☐ 2. No → code, then proceed to next row	1. Once 2. Twice 3. Three times 4. >3 times ☐ _____		☐ 1. Consumed alone 2. Consumed with other foods; describe:
K. Tea or Coffee	1. Yes ☐ 2. No → code, then proceed to next row	1. Once 2. Twice 3. Three times 4. >3 times ☐ _____		☐ 1. Consumed alone 2. Consumed with other foods; describe:
L. Iron fortified Fish Sauce	1. Yes ☐ 2. No → code, then proceed to next row	1. Once 2. Twice 3. Three times 4. >3 times ☐		☐ 1. Consumed alone 2. Consumed with other foods; describe:
M. Iron fortified Soy Sauce	1. Yes ☐ 2. No → code, then proceed to next row	1. Once 2. Twice 3. Three times 4. >3 times ☐		☐ 1. Consumed alone 2. Consumed with other foods; describe:

17. Did you use an iron pot for cooking yesterday? If yes, how many times and for how long did you cook with the pot?	1. Yes ☐ 2. No *If yes:* Number of uses: _____ **Total** cooking time: Minutes _____
18. How many meals and snacks did you consume yesterday?	_____ meals, **and** _____ snacks
19. Was yesterday's food intake typical/usual for you?	**1. Yes→ skip to Q21** ☐ 2. No
20. If no, why was it not typical/usual? CHECK ALL THAT APPLY.	1. I was ill 2. I was not hungry ☐ 3. There was not enough food 4. It was a celebration (ate more or differently) 5. Other – specify: _____
21. Who is primarily responsible for food preparation in your household?	1. Self (woman) 2. Husband/partner ☐ 3. Grandparent

	4. Male children 5. Female children 6. Other – specify: _____

MODULE 3: PHYSICAL ACTIVITY STATUS	
22. Does your work involve vigorous activity, which increases your heart rate and causes heavy breathing? (For example, carrying heavy loads, or digging)	1. Yes **2. No → Skip to Q24** ☐
23. How many days in a week do you engage in work that is vigorous?	_____ Days ☐
24. How much time do you spend walking or riding a bicycle during the day?	Hours _____ ☐☐ Minutes _____ ☐☐

MODULE 4: WATER	
25. Do you treat your water in any way to make it safer to drink?	1. Yes 2. **No → Skip to Q28** ☐
26. What is the main way you make water safer to drink? ***If 4 selected, proceed to Q27*** ***If other, proceed to Q28***	1. Boil 2. Add bleach/chlorine 3. Strain it 4. Use a water filter (sand, ceramic, composite, etc.) 5. Other, specify _____ 6. Don't know ☐
27. Is all the water utilized in your household treated?	1. Yes 2. **No → Skip to Q28** ☐

MODULE 5: KNOWLEDGE OF IRON and IRON DEFICIENCY ANEAMIA	
28. Have you ever heard of iron?	1. Yes 2. No ☐
29. Have you ever heard of iron deficiency, specifically iron deficiency aneamia?	1. Yes 2. **No → code, and proceed to Q33** _____ ☐
30. Do you know any symptoms of iron deficiency aneamia?	1. Yes 2. **No → code, and proceed to Q33**
31. What are the symptoms of iron deficiency aneamia?	☐
32. How do you treat iron deficiency aneamia?	
33. Do you know what foods are good sources of iron?	1. Yes **2. No → code, and proceed to Q35**
34. What foods are good dietary sources of iron?	

MODULE 6: KNOWLEDGE OF MALARIA	
35. Have you heard of Malaria?	1. Yes 2. No → proceed to Q44
36. Do you know the cause of Malaria?	1. Yes 2. No → proceed to Q38
37. What is the cause of malaria?	_____
38. Do you know the symptoms for malaria?	1. Yes 2. No → proceed to Q40
39. What are the symptoms of malaria?	_____
40. Do you know how to treat malaria if symptoms persist?	1. Yes 2. No → proceed to Q42
41. What is the treatment for malaria?	_____
42. Do you know how to prevent malaria?	1. Yes 2. No → proceed to Q44
43. How do you prevent malaria?	_____
44. Do you sleep under treated bed nets?	1. Yes 2. No → Proceed to 46
45. How many nights a week	1. Once a week 2. Twice a week 3. Five days a week 4. Everyday 5. Other

MODULE 7: PRECEPTIONS OF SUPPLEMENT USE	
46. Have you ever taken medicine, or vitamins or minerals in the form of pills/tablets?	1. Yes 2. **No → Skip to Q50**
47. If yes, where did you retrieve these pills/tablets?	1. Health Centre 2. Hospital 3. Village Health Volunteer 4. Vendor/Store 5. Other: _____
48. Did you take the pills/tablets according to instructions?	1. Yes 2. **No → Skip to Q50**
49. If no, why?	1. Did not remember to take pills/tablets 2. Disliked swallowing pills/tablets 3. Side effects of pills/tablets

	4. Stigma of taking pills/tablets 5. No change in health, so discontinued use 6. Other: _____

MODULE 8: ANTHROPOMETRIC MEASUREMENTS	
Anthropometrics of Participant	
50. Height of **Participant**	1) __ __ __ . __ cm 2) __ __ __ . __ cm 3) __ __ __ . __ cm
51. Weight of **Participant**	1) __ __ . __ __ kg 2) __ __ . __ __ kg 3) __ __ . __ __ kg
52. Hemocue, Hemoglobin Level	_____ g/L

Thank you so much for your time!

MIDLINE QUESTIONNAIRE

CONFIDENTIAL
All information collected in this survey is strictly confidential and will be used for statistical purposes only.

IDENTIFICATION INFORMATION	
Geographic Identification	**Interviewer Record**
Province: _____PREAH VIHEAR_____ District: _____Rovieng_____ Commune: _____ Village: _____ Subject ID: __ __ __ __ __	Interviewer Name: _____ Signature: _____ Remarks:
Date of Interview: (DD/MM/YYYY) ___ ___ / ___ ___ / ___ ___ ___ ___	

Are you willing to provide a fingerpick blood sample today?	1. Yes 2. No → Do **not** proceed ☐

MODULE 1: PARTICIPANT INFORMATION	
1. How many people currently live in your household (defined as eating from the same pot each day)?	_____ people ☐☐
2. What was the income for your household last **month**?	US$_____ ☐☐☐☐
3. How much time did you spend sleeping yesterday?	Hours _____ ☐☐ Minutes _____ ☐☐
4. How much time did you spend working yesterday? *Note: work includes any paid or unpaid duties, and can include tasks such as childcare and household management.*	Hours _____ ☐☐ Minutes _____ ☐☐
5. Is this pattern a typical routine?	1. Yes → skip to Q16 2. No ☐
6. If this pattern is atypical, how many hours do you usually spend sleeping and working per day?	**Sleeping:** Hours _____ ☐☐

	Minutes ____ ☐☐ **Working:** Hours _____ ☐☐ Minutes _____ ☐☐

MODULE 2: FOOD INTAKE

7. I would like to ask you about the foods and drinks that you may have consumed **yesterday** during the day or night. I want to know how often you ate the food, approximately how much of the food you ate in a typical serving, and how it was consumed.

Food Type	Consumed yesterday	If yes, how often?	Amount per serving (g or mL)	Consumed alone, or with other foods?
A. Bread, rice, noodles, porridge, or other foods made from grains?	1. Yes ☐ 2. No → code, then proceed to next row	1. Once 2. Twice 3. Three times 4. >3 times _____ ☐		☐ 1. Consumed alone 2. Consumed with other foods; describe:
B. Dark leafy green vegetables (eg. kang kong)	1. Yes ☐ 2. No → code, then proceed to next row	1. Once 2. Twice 3. Three times 4. >3 times _____ ☐		☐ 1. Consumed alone 2. Consumed with other foods; describe:
C. Ripe mangoes, papayas or any other yellow or orange fruits?	1. Yes ☐ 2. No → code, then proceed to next row	1. Once 2. Twice 3. Three times 4. >3 times _____ ☐		☐ 1. Consumed alone 2. Consumed with other foods; describe:
D. Other fruits or vegetables?	1. Yes ☐ 2. No → code, then proceed to next row	1. Once 2. Twice 3. Three times 4. >3 times _____ ☐		☐ 1. Consumed alone 2. Consumed with other foods; describe:
E. Liver, kidney, heart, or other organ meats?	1. Yes ☐ 2. No → code, then proceed to next row	1. Once 2. Twice 3. Three times 4. >3 times _____ ☐		☐ 1. Consumed alone 2. Consumed with other foods; describe:
F. Meat, such as beef, pork, lamb, goat, chicken, or duck?	1. Yes ☐ 2. No → code, then proceed to next row	1. Once 2. Twice 3. Three times 4. >3 times _____ ☐		☐ 1. Consumed alone 2. Consumed with other foods; describe:

G. Eggs?	1. Yes ☐ 2. No → code, then proceed to next row	1. Once 2. Twice 3. Three times 4. >3 times ☐		☐ 1. Consumed alone 2. Consumed with other foods; describe:
H. Fresh or dried fish and shellfish (including clams, snails, crab, squid)?	1. Yes ☐ 2. No → code, then proceed to next row	1. Once 2. Twice 3. Three times 4. >3 times _____ ☐		☐ 1. Consumed alone 2. Consumed with other foods; describe:
I. Foods made from beans, peas, lentils, or nuts?	1. Yes ☐ 2. No → code, then proceed to next row	1. Once 2. Twice 3. Three times 4. >3 times _____ ☐		☐ 1. Consumed alone 2. Consumed with other foods; describe:
J. Foods made with oil, fat, or butter?	1. Yes ☐ 2. No → code, then proceed to next row	1. Once 2. Twice 3. Three times 4. >3 times _____ ☐		☐ 1. Consumed alone 2. Consumed with other foods; describe:
K. Snake, frog, rats, or insects?	1. Yes ☐ 2. No → code, then proceed to next row	1. Once 2. Twice 3. Three times 4. >3 times ☐		☐ 1. Consumed alone 2. Consumed with other foods; describe:
L. Tea or Coffee	1. Yes ☐ 2. No → code, then proceed to next row	1. Once 2. Twice 3. Three times 4. >3 times _____ ☐		☐ 1. Consumed alone 2. Consumed with other foods; describe:
M. Iron fortified Fish Sauce	1. Yes ☐ 2. No → code, then proceed to next row	1. Once 2. Twice 3. Three times 4. >3 times ☐		☐ 1. Consumed alone 2. Consumed with other foods; describe:
N. Iron fortified Soy Sauce	1. Yes ☐ 2. No → code, then proceed to next row	1. Once 2. Twice 3. Three times 4. >3 times ☐		☐ 1. Consumed alone 2. Consumed with other foods; describe:

8. Did you use an iron pot for cooking yesterday? If yes, how many times and for how long did you cook with the pot?	1. Yes ☐ 2. No *If yes:*

	Number of uses: _____ **Total** cooking time: Minutes _____
9. How many meals and snacks did you consume yesterday?	_____ meals, **and** _____ snacks
10. Was yesterday's food intake typical/usual for you?	**1. Yes→ skip to Q21** 2. No
11. If no, why was it not typical/usual? CHECK ALL THAT APPLY.	1. I was ill 2. I was not hungry 3. There was not enough food 4. It was a celebration (ate more or differently) 5. Other – specify: _____
12. Who is primarily responsible for food preparation in your household?	1. Self (woman) 2. Husband/partner 3. Grandparent 4. Male children 5. Female children 6. Other – specify: _____

MODULE 3: KNOWLEDGE OF IRON and IRON DEFICIENCY ANEAMIA	
13. Have you ever heard of iron?	1. Yes 2. No
14. Have you ever heard of iron deficiency, specifically iron deficiency aneamia?	1. Yes 2. **No → code, and proceed to Q19**
15. Do you know any symptoms of iron deficiency aneamia?	1. Yes 2. **No → code, and proceed to Q17**
16. What are the symptoms of Iron deficiency aneamia?	
17. Do you know how to treat iron deficiency anemia?	
18. How do you treat iron deficiency aneamia?	
19. Do you know what foods are good sources of iron?	3. Yes **4. No → code, and proceed to Q21**
20. What foods are good dietary sources of iron?	

MODULE 4: WATER	
21. Do you treat your water in any way to make it safer to drink?	1. Yes 2. **No → Skip to Q24**

22. What is the main way you make water safer to drink? ***If 4 selected, proceed to Q23*** ***If other, proceed to Q24***	1. Boil 2. Add bleach/chlorine 3. Strain it 4. Use a water filter (sand, ceramic, composite, etc.) 5. Other, specify _____ 6. Don't know ☐
23. How many times in the last week have you used your water filtration system?	Times ☐

MODULE 5: PHYSICAL ACTIVITY STATUS	
24. In the last week has your work involved vigorous activity, which increases your heart rate and causes heavy breathing? (For example, carrying heavy loads, or digging)	1. Yes 2. No → Proceed to Q26 ☐
25. How many times last week did you engage in work that is vigorous?	☐☐ Days
26. How much time do you spend walking or riding a bicycle during the day on average last week?	☐☐ Hours ☐☐ Minutes

MODULE 6: PRECEPTIONS OF SUPPLEMENT USE	
27. Have you ever taken, or vitamins or minerals in the form of pills/tablets?	1. Yes 2. **No → Skip to 31** ☐
28. If yes, where did you retrieve these pills/tablets?	1. Health Centre ☐ 2. Hospital ☐ 3. Village Health Volunteer ☐ 4. Vendor/Store ☐ 5. Pharmacy ☐ 6. Other: _____ ☐
29. Did you take the pills/tablets according to instructions?	1. Yes→ **Skip to 31** 2. **No**
30. If no, why?	1. Did not remember to take pills/tablets ☐ 2. Disliked swallowing pills/tablets 3. Side effects of pills/tablets 4. Stigma of taking pills/tablets 5. No change in health, so discontinued use 6. Other: _____ ☐

MODULE 7: SMOKING	
31. Do you smoke?	1. Yes 2. **No → skip to 35** ☐
32. How long have you been smoking for?	1. 0 – 6 months 2. 7 – 12 months 3. 13 – 24 months 4. 25 – 48 months 5. 48 months + ☐
33. In the last 24 hours how many cigarettes did you smoke?	1. 0 → skip to 35 2. 1 - 2 3. 3 - 5 4. 6 – 9 5. 10+ ☐
34. When you smoke is it tobacco?	1. Yes 2. No 3. Other: _____ ☐
35. Do you currently use any (other) type of tobacco aside from smoking?	1. None → skip to 37 2. Pipe 3 Chewing Tobacco 4. Snuff 5. Other: _____ ☐
36. In the last 24 hours how many times did you use tobacco other than smoking?	1. 0 2. 1 - 2 3. 3 - 5 4. 6 – 9 5. 10+ ☐
37. Is this tobacco routine typical for you smoking and other tobacco types?	1. Yes → skip to 38 2. No 8. N/A ☐ Please specify why: a. I was ill b. I did not have any c. It was a celebration (routine was different) d. Other – specify: _____ ☐

MODULE 8: ANTHROPOMETRIC MEASUREMENTS	
Anthropometrics of Participant	
38. Height of **Participant**	4) __ __ __ . __ cm 5) __ __ __ . __ cm 6) __ __ __ . __ cm
39. Weight of **Participant**	4) __ __ . __ __ kg 5) __ __ . __ __ kg 6) __ __ . __ __ kg
40. Hemocue, Hemoglobin Level	301: _____ g/L

Thank you so much for your time!

ENDLINE QUESTIONNAIRE

A randomized control trial of the Lucky Iron Fish™ to improve hemoglobin concentration in women in Preah Vihear, Cambodia

CONFIDENTIAL
All information collected in this survey is strictly confidential and will be used for statistical purposes only.

IDENTIFICATION INFORMATION	
Geographic Identification	**Interviewer Record**
Province: _____PREAH VIHEAR_____ District: _____ROVIENG_____ Commune: _____ Village: _____ Subject ID: __ __ __ __ __	Interviewer Name: _____ Signature: _____ Remarks:
Date of Interview: (DD/MM/YYYY) ___ ___ / ___ ___ / ___ ___ ___ ___	

MODULE 1: PARTICIPANT INFORMATION	
1. How many people currently live in your household (defined as eating from the same pot each day)?	_____ people
2. What was the income for your household last **month**?	US$_____
3. What was the income for your household in the past **12 months**?	US$_____
4. How much time did you spend sleeping yesterday?	Hours _____ Minutes _____
5. How much time did you spend working yesterday? *Note: work includes any paid or unpaid duties, and can include tasks such as childcare and household management.*	Hours _____ Minutes _____
6. Is this pattern a typical routine?	1. Yes → skip to Q8 2. No
7. If this pattern is atypical, how many hours do you usually spend sleeping and working per day?	**Sleeping:** Hours _____ Minutes ____

	Working: Hours _____ ☐☐ Minutes ____ ☐☐ _____

MODULE 2: FOOD INTAKE

8. I would like to ask you about the foods and drinks that you may have consumed **yesterday** during the day or night. I want to know how often you ate the food, approximately how much of the food you ate in a typical serving, and how it was consumed.

Food Type	**Consumed yesterday**	**If yes, how often?**	**Amount per serving (g or mL)**	**Consumed alone, or with other foods?**
A. Bread, rice, noodles, porridge, or other foods made from grains?	1. Yes ☐ 2. No → code, then proceed to next row	1. Once 2. Twice 3. Three times 4. >3 times _____ ☐		☐ 1. Consumed alone 2. Consumed with other foods; describe:
B. Dark leafy green vegetables (eg. kang kong)	1. Yes ☐ 2. No → code, then proceed to next row	1. Once 2. Twice 3. Three times 4. >3 times _____ ☐		☐ 1. Consumed alone 2. Consumed with other foods; describe:
C. Ripe mangoes, papayas or any other yellow or orange fruits?	1. Yes ☐ 2. No → code, then proceed to next row	1. Once 2. Twice 3. Three times 4. >3 times _____ ☐		☐ 1. Consumed alone 2. Consumed with other foods; describe:
D. Other fruits or vegetables?	1. Yes ☐ 2. No → code, then proceed to next row	1. Once 2. Twice 3. Three times 4. >3 times ☐ _____		☐ 1. Consumed alone 2. Consumed with other foods; describe:
E. Liver, kidney, heart, or other organ meats?	1. Yes ☐ 2. No → code, then proceed to next row	1. Once 2. Twice 3. Three times 4. >3 times _____ ☐		☐ 1. Consumed alone 2. Consumed with other foods; describe:

F. Meat, such as beef, pork, lamb, goat, chicken, or duck?	1. Yes ☐ 2. No → code, then proceed to next row	1. Once 2. Twice 3. Three times 4. >3 times _____ ☐		☐ 1. Consumed alone 2. Consumed with other foods; describe:
G. Eggs?	1. Yes ☐ 2. No → code, then proceed to next row	1. Once 2. Twice 3. Three times 4. >3 times ☐		☐ 1. Consumed alone 2. Consumed with other foods; describe:
H. Fresh or dried fish and shellfish (including clams, snails, crab, squid)?	1. Yes ☐ 2. No → code, then proceed to next row	1. Once 2. Twice 3. Three times 4. >3 times _____ ☐		☐ 1. Consumed alone 2. Consumed with other foods; describe:
I. Foods made from beans, peas, lentils, or nuts?	1. Yes ☐ 2. No → code, then proceed to next row	1. Once 2. Twice 3. Three times 4. >3 times _____ ☐		☐ 1. Consumed alone 2. Consumed with other foods; describe:
J. Foods made with oil, fat, or butter?	1. Yes ☐ 2. No → code, then proceed to next row	1. Once 2. Twice 3. Three times 4. >3 times _____ ☐		☐ 1. Consumed alone 2. Consumed with other foods; describe:
K. Tea or Coffee	1. Yes ☐ 2. No → code, then proceed to next row	1. Once 2. Twice 3. Three times 4. >3 times _____ ☐		☐ 1. Consumed alone 2. Consumed with other foods; describe:
L. Iron fortified Fish Sauce	1. Yes ☐ 2. No → code, then proceed to next row	1. Once 2. Twice 3. Three times 4. >3 times ☐		☐ 1. Consumed alone 2. Consumed with other foods; describe:
M. Iron fortified Soy Sauce	1. Yes ☐ 2. No → code, then proceed to next row	1. Once 2. Twice 3. Three times 4. >3 times ☐		☐ 1. Consumed alone 2. Consumed with other foods; describe:

9. Did you use an iron pot for cooking yesterday? If yes, how many times and for how long did you cook with the pot?	1. Yes ☐ 2. No

	If yes: Number of uses: _____ **Total** cooking time: Minutes _____
10. How many meals and snacks did you consume yesterday?	_____ meals, **and** _____ snacks
11. Was yesterday's food intake typical/usual for you?	**1. Yes→ skip to Q21** 2. No
12. If no, why was it not typical/usual? CHECK ALL THAT APPLY.	1. I was ill 2. I was not hungry 3. There was not enough food 4. It was a celebration (ate more or differently) 5. Other – specify: _____
13. Who is primarily responsible for food preparation in your household?	1. Self (woman) 2. Husband/partner 3. Grandparent 4. Male children 5. Female children 6. Other – specify: _____

MODULE 3: KNOWLEDGE OF IRON and IRON DEFICIENCY ANEAMIA	
14. Have you ever heard of iron?	1. Yes 2. No
15. Have you ever heard of iron deficiency, specifically iron deficiency aneamia?	1. Yes 2. **No → code, and proceed to Q19**
16. Do you know any symptoms of iron deficiency aneamia?	1. Yes 2. **No → code, and proceed to Q18**
17. What are the symptoms of Iron deficiency aneamia?	
18. How do you treat iron deficiency aneamia?	
19. Do you know what foods are good sources of iron?	5. Yes **6. No → code, and proceed to Q21**
20. What foods are good dietary sources of iron?	

MODULE 4: WATER	
21. Do you treat your water in any way to make it safer to drink?	1. Yes 2. **No → Skip to Q24**
22. What is the main way you make water safer to drink? ***If 4 selected, proceed to Q23*** ***If other, proceed to Q24***	1. Boil 2. Add bleach/chlorine 3. Strain it 4. Use a water filter (sand, ceramic, composite, etc.)

	5. Other, specify _____ 6. Don't know
23. How many times in the last week have you used your water filtration system?	_____ Times ☐☐

MODULE 5: PHYSICAL ACTIVITY STATUS	
24. In the last week has your work involved vigorous activity, which increases your heart rate and causes heavy breathing? (For example, carrying heavy loads, or digging)	1. Yes 2. No → Proceed to Q26 ☐
25. How many times last week did you engage in work that is vigorous?	_____ Days ☐
26. How much time do you spend walking or riding a bicycle during the day on average last week?	Hours _____ ☐☐ Minutes _____ ☐☐

MODULE 6: ANTHROPOMETRIC MEASUREMENTS	
Anthropometrics of Participant	
27. Height of **Participant**	7) __ __ __ . __ cm 8) __ __ __ . __ cm 9) __ __ __ . __ cm
28. Weight of **Participant**	29. __ __ . __ __ kg 30. __ __ . __ __ kg 31. __ __ . __ __ kg
32. Hemocue, Hemoglobin Level	301: _____ g/L

Thank you so much for your time!

Appendix I: National Nutrition Program education

Training topics included:

- An introduction to the trial, and an overview of the study
- An introduction on iron including its basic physiology
- Discussion surrounding the importance of iron and a balanced diet
- An explanation on the differences between iron deficiency, IDA, and anemia
- What anemia is
- The impact of anemia and why it is an important topic for women of reproductive age
- We discussed the causes of IDA and iron deficiency
- We taught VHSG to recognize symptoms of anemia
- The 3 food groups, iron food sources, and iron deficiency prevention

A sample of resources utilized can be found on the following two pages:

ជាតិដែក

និយមន័យនៃជាតិដែក

គឺជាវ៉ែនធីជមួយប្រភេទ ដែលរាងកាយមនុស្សយើងត្រូវការជាចាំបាច់ សំរាប់បង្កើតគ្រាប់ឈាមក្រហម។

រូបវិទ្យានៃជាតិដែក

- ជាតិដែកបានមកពីចំណីអាហារត្រូវបាន បឺតស្រូបតាមពោះវៀនតូច
- ជាតិដែកចំនួន ៦០-៧០% បានតោងជាប់នឹងគ្រាប់ឈាមក្រហម
- ជាតិដែកចំនួន ៣០-៣៥% ត្រូវស្តុកទុកនៅក្នុងថ្លើម
- ជាតិដែកបញ្ចេញចោលតាម: ទឹកនោម លាមក ស្បែក

សារៈសំខាន់នៃជាតិដែក

- ជាតិដែកមានតួនាទីបង្កើតអេម៉ូក្លូប៊ីន
- ជួយជំរុញក្នុងការបង្កើតអង្គបដិបក្ខប្រាណ
- ជួយបំលែងការ៉ូទីនឱ្យក្លាយទៅជាជីវជាតិអា
- ស្ត្រីមានផ្ទៃពោះត្រូវការជាតិដែកច្រើនជាងគេសំរាប់រក្សាគភ៌
- ជួយការពារ/លប់បំបាត់ភាពស្លេកស្លាំងដោយសារកង្វះជាតិដែក
- ការកើនឡើងនូវចំនួនអេម៉ូក្លូប៊ីន ១ក្រាម/ដេស៊ីលីត្រ អាចកាត់បន្ថយអត្រាស្លាប់របស់ម្តាយបាន ២០% និងកុមារបាន ១៦%

អាហារសំបូរជីវជាតិសេ: ដើម្បីសំរួលដល់ការបិតស្រូបយកជាតិដែកមានដូចជា:

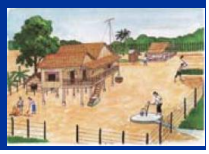
- ក្រូចពោធិសាត់,ក្រូចឆ្មារ
- ត្រប៉ែក,ទៀប**ចាកំង**,ស្វាយ,ប៉េងចាំ:... ។ ល។

អាហារដែលរារាំងការបិតស្រូបជាតិដែក:

- តែ , កាហ្វេ ។ល។

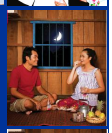
វិធីការពារភាពស្លេកស្លាំងដោយសារបញ្ហាកង្វះជាតិដែក

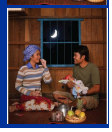
- *បំបៅកូនដោយទឹកដោះម្តាយតែមួយមុខគត់ពីកើតដល់អាយុ ៦ខែ*
- *ត្រូវផ្តល់អាហារបន្ថែមសមស្របដល់កុមារចាប់ពីអាយុ៦ខែឡើងទៅ រួមជាមួយការបំបៅដោះកូនរហូតដល់អាយុ២ឆ្នាំ ឬលើស*
- *បរិភោគអាហារដែលសំបូរជាតិដែក និង អាហារដែលសំបូរជីវជាតិសេ*
- *អនុវត្តន៍វិធានមើយស្អាតបីប្រការ*
- *ការពារជំងឺដែលបង្កដោយជំងឺដង្កូវក្រួន និង ការបង្កភោគផ្សេងៗទៀត*



វិធីការពារភាពស្លេកស្លាំងដោយសារបញ្ហាកង្វះជាតិដែក

- *ត្រូវផ្តល់ថ្នាំទម្លាក់ព្រូន ១ ដូស នៅរៀងរាល់៦ខែម្តង ដល់កុមារដែលមានអាយុចាប់ពី១២ខែឡើង*
- *ស្ត្រីក្នុងវ័យបន្តពូជត្រូវលេបគ្រាប់ថ្នាំជាតិដែក ប្រចាំសប្តាហ៍ ១គ្រាប់រៀងរាល់សប្តាហ៍*
- *ស្ត្រីមានផ្ទៃពោះ ត្រូវលេបថ្នាំជាតិដែក/អាស៊ីដហ្វូលីកឱ្យបាន ៩០គ្រាប់ និងថ្នាំទំលាក់ព្រូន១គ្រាប់នៅត្រីមាសទី២*
- *ស្ត្រីក្រោយសម្រាល ត្រូវលេបថ្នាំជាតិដែក/អាស៊ីដហ្វូលីក ឱ្យបាន ៤២គ្រាប់ និងថ្នាំទំលាក់ព្រូន១គ្រាប់។*
- *ត្រូវបញ្ចូលជាតិដែកទៅក្នុងអាហារ ហើយបរិភោគជាប្រចាំ*
- *ត្រូវមានសូនដំណាំគ្រួសារ និងការចិញ្ចឹមសត្វ*





Appendix J: Monitoring and evaluation forms

Commune:	Interviewers name:	Subject ID: ☐☐☐
Village:	Interviewers signature:	In the last month have you become pregnant? 1. Yes → do not proceed 2. No

Visit	1. In the last month did you spend time away from your home?	2. Yesterday did you use the LIF? If yes, how much?	3. Most of the time, how do you use the fish?	4. Was this typical for the last month?	5. Did you share or sell the LIF?	6. Were there positive health outcomes?	7. Were these positive outcomes from the LIF?	8. Were there negative health outcomes?	9. Other Remarks
	1. Yes 2. No ☐ How many days? ☐☐ Did you bring the LIF with you? 1. Yes 2. No ☐ 8. N/A	1. Yes 2. No - next Q ☐ How many times? ☐ 1. Once 2. Twice 3. Three 4. More than 3 times	1. Cooking 2. Boiling Water 3. Both cooking and drinking water ☐	1. Yes ☐ 2. No Why:	1. Yes 2. No → Q7 ☐ Explain:	1. Yes 2. No → next Q ☐ What?	1. Yes ☐ 2. No Explain?	1. Yes ☐ 2. No → next Q What?	

Visit	10. Does the LIF change the colour of your food?	11. Did the LIF change the taste of your food?	12. If the LIF changes the colour or taste of the food, does this change the amount you use it?	13. How long do you usually cook the LIF for?	14. Yesterday did you add lime when if you used the LIF?	15. After you use the LIF how do you feel?	16. Does the LIF change your Bowel Movements?
	1. Yes → describe then Q11 2. No → Q11 ☐ Please explain:	1. Yes → describe then Q12 2. No → Q13 Please explain: ☐	1. Yes → describe then Q13 2. No → Q13 Please explain: ☐	1. Less than 10 minutes 2. 10 minutes 3. 20 minutes 4. 30 minutes 5. 40 minutes 6. 50 minutes 7. 60 minutes 8. More than 1 hour ☐	1. Yes 2. No 3. NA ☐		1. Yes 2. No ☐ Explain:

(Questions 10 – 16 are only asked at 3 months, 6 months, and 12 months)

Commune:	Interviewers name:	Subject ID: ☐☐☐
Village:	Interviewers signature:	In the last month have you become pregnant? 1. Yes → do not proceed 2. No

Number of pills left in old bottle	**Date (when given new bottle)**

Visit	**1. In the last month did you spend time away from your home?**	**2. Did you take the pills everyday?**	**3. Did you have a problem opening the pill bottle?**	**4. Did you share or sell the pills? If yes with whom?**	**5. Were there positive health outcomes?**	**6. Were these positive outcomes from the IS?**	**7. Were there negative health outcomes?**	**8. Other Remarks**
	1. Yes 2. No ☐ How many days? ☐☐ Did you bring the IS with you? 1. Yes 2. No 8. N/A ☐	1. Yes 2. No ☐ Why:	1. Yes ☐ 2. No Explain:	1. Yes 2. No ☐ - Q Specify & Why:	1. Yes 2. No → Q7 ☐ What?	1. Yes 2. No ☐ Explain?	1. Yes 2. No ☐ → next Q What?	

Visit	9. Do the IS change your Bowel Movements?	10. After you use the IS how do you feel?	11. Yesterday, if you took the IS what did you take it with?
	1. Yes 2. No ☐ Explain:		1. Water 2. Food 3. Both food and water ☐ Please explain:

(Questions 9 – 11 were only asked at 3 months, 6 months, and 12 months)

Commune:	Interviewers name:	Subject ID: ☐☐☐
Village:	Interviewers signature:	In the last month have you become pregnant? 1. Yes → do not proceed 2. No

Visit	**1. In the last month did you spend time away from your home?**	**2. Yesterday did you consume any multivitamins or iron supplements?**	**3. Yesterday did you consume any foods that are high in iron**	**4.Yesterday did you consume iron fortified fish or soy sauce?**	**5. Was this intake typical for you over the last month?**	**6. Were there positive health outcomes?**	**7. Were these positive outcomes from the change in diet?**	**8. Were there negative health outcomes?**	**9. Other Remarks**
	1. Yes 2. No ☐ How many days?	1. Yes ☐ 2. No	1. Yes ☐ 2. No → Q4 What:	1. Yes ☐ 2. No → Q5 Quantity:	1. Yes ☐ 2. No Explain:	1. ☐ Yes 2. No – Q8 What?	1. Yes ☐ 2. No Explain?	1. Yes ☐ 2. No –Q9 What?	