INVESTIGATING THE POTENTIAL RISK OF UNTARGETED DAILY
ORAL IRON SUPPLEMENTATION IN CAMBODIAN WOMEN OF
REPRODUCTIVE AGE

by
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Investigating the Potential Risk of Untargeted Daily Oral Iron Supplementation in Cambodian Women of Reproductive Age

submitted by Shannon Steele in partial fulfillment of the requirements for
the degree of Master of Science in Human Nutrition

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Abstract

Background: There is limited evidence regarding the potential risk of untargeted daily oral iron supplementation in women of reproductive age, especially in countries where genetic hemoglobinopathies are common and iron deficiency is not the major cause of anemia. Excess iron exposure can cause increased production of reactive oxygen species (ROS), which can lead to cellular (e.g. lipid, DNA) damage.

Objective: The aim of this research was to retrospectively assess the effect of daily oral iron supplementation with 60 mg of elemental iron for 12 weeks, compared to placebo, on relative leukocyte telomere length (LTL) and mitochondrial DNA (mtDNA) content.

Methods: In a double-blind randomized controlled trial, non-pregnant Cambodian women aged 18–45 years received 60 mg of elemental iron as ferrous sulphate (n = 201) or a placebo (n = 200) for 12 weeks. Relative LTL and mtDNA content were quantified in buffy coat collected at baseline and endline by monochrome multiplex quantitative polymerase chain reaction (MMqPCR) and the change in relative LTL and mtDNA content was determined.

Results: Iron supplementation was not associated with an adjusted absolute or percent change in relative LTL after 12 weeks, compared to placebo (β-coefficient: −0.04 [95% CI: −0.16, 0.08]; P = 0.50 and β-coefficient: −0.96 [95% CI: −2.69, 0.77]; P = 0.28, respectively). However, iron supplementation was associated with a significantly smaller adjusted absolute and percent
increase in mtDNA content after 12 weeks, compared to placebo (β-coefficient: −11 [95% CI: −20, −2]; \( P = 0.02 \) and β-coefficient: −11 [95% CI: −20, −1]; \( P = 0.02 \), respectively).

Conclusions: Our findings suggest that daily oral iron supplementation with 60 mg of elemental iron for 12 weeks, as per the World Health Organization (WHO) global policy, may be associated with altered mitochondrial homeostasis. This is concerning and more research is needed to ascertain if there is potential risk associated with untargeted daily oral iron supplementation, to ultimately inform the safety of the WHO policy.
Lay Summary

There is limited research on the potential risk of daily oral iron supplementation when provided in untargeted (blanket) programs. Global guidelines recommend daily oral iron supplementation for all non-pregnant women aged 15–49 years in countries where more than 40% of women have anemia. This guideline may be a waste of resources in countries where the major cause of anemia is not iron deficiency, such as in Cambodia. Worse, the supplements could expose women to too much iron, which may be harmful. This research looked at the effect of 12 weeks of daily oral iron supplementation, compared to no treatment, in non-pregnant Cambodian women aged 18–45 years. Measures of risk were measured before and after women received the iron or no treatment. The findings from this study will help inform safe iron supplementation practices for non-pregnant women worldwide.
Preface

This thesis work is a secondary analysis of specimens previously collected in a randomized controlled trial carried out by my supervisor, Dr. Crystal Karakochuk. It contains only my original and unpublished work, which will be submitted to partially fulfill the requirement for a Master of Science in Human Nutrition at the University of British Columbia.

The design of this study was a joint effort between Dr. Crystal Karakochuk and myself. My committee members, Drs. Hélène Côté and Angela Devlin, also provided substantial input regarding study design and methodology. Laboratory measurement of the biomarkers of cellular damage in this study (relative leukocyte telomere length and mitochondrial DNA content) were carried out by Izabelle Gadawski, from the Côté Lab, and myself. The plan for statistical analyses was a joint effort between Drs. Crystal Karakochuk, Hélène Côté, and Angela Devlin, and myself. I carried out all statistical analyses and led the interpretation of the findings.

Ethics approval was obtained from The University of British Columbia Research Ethics Board in Canada (H17-02650) and the National Ethics Committee for Health Research in Cambodia.
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<tbody>
<tr>
<td>8-Oxo-dG</td>
<td>8-Oxo-2’-deoxyguanosine</td>
</tr>
<tr>
<td>8-OxoG</td>
<td>8-Oxoguanine</td>
</tr>
<tr>
<td>AGP</td>
<td>α-1-Acid Glycoprotein</td>
</tr>
<tr>
<td>aTL</td>
<td>Absolute Telomere Length</td>
</tr>
<tr>
<td>BMI</td>
<td>Body Mass Index</td>
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<tr>
<td>CDHS</td>
<td>Cambodia Demographic Health Survey</td>
</tr>
<tr>
<td>CRP</td>
<td>C-Reactive Protein</td>
</tr>
<tr>
<td>CV</td>
<td>Coefficient of Variation</td>
</tr>
<tr>
<td>Ct</td>
<td>Cycle Threshold</td>
</tr>
<tr>
<td>DMT1</td>
<td>Divalent Metal Transporter 1</td>
</tr>
<tr>
<td>dNTP</td>
<td>Deoxynucleotide Triphosphate</td>
</tr>
<tr>
<td>EGRac</td>
<td>Erythrocyte Glutathione Reductase Activity Coefficient</td>
</tr>
<tr>
<td>FAC</td>
<td>Ferric Ammonium Citrate</td>
</tr>
<tr>
<td>Fe2+</td>
<td>Ferrous Iron</td>
</tr>
<tr>
<td>Fe3+</td>
<td>Ferric Iron</td>
</tr>
<tr>
<td>FFQ</td>
<td>Food Frequency Questionnaire</td>
</tr>
<tr>
<td>Flow-FISH</td>
<td>Flow Fluorescent In-Situ Hybridization</td>
</tr>
<tr>
<td>GDP</td>
<td>Gross Domestic Product</td>
</tr>
<tr>
<td>GHI</td>
<td>Global Hunger Index</td>
</tr>
<tr>
<td>GNI</td>
<td>Gross National Income</td>
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</tbody>
</table>
HbE: Hemoglobin E
HDI: Human Development Index
HEIRS: Hemochromatosis and Iron Overload Screening
NHANES: National Health and Nutrition Examination Survey
HO-1: Heme-Oxygenase 1
HPLC: High Performance Liquid Chromatography
IC: Internal Control
IDA: Iron Deficiency Anemia
IFA: Iron and Folic Acid
IFNγ: Interferon-γ
IL-1: Interleukin-1
IL-6: Interleukin-6
IL-10: Interleukin-10
IPM: Iron Polymaltose
LTL: Leukocyte Telomere Length
MCH: Mean Corpuscular Hemoglobin
MCV: Mean Corpuscular Volume
MgCl₂: Magnesium Chloride
MMN: Multiple Micronutrients
MMqPCR: Monochrome Multiplex Quantitative Polymerase Chain
mtDNA: Mitochondrial DNA
NaFeEDTA: Sodium Iron EDTA
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>nDNA</td>
<td>Nuclear DNA</td>
</tr>
<tr>
<td>PM2.5</td>
<td>Fine Particulate Matter</td>
</tr>
<tr>
<td>Q-FISH</td>
<td>Quantitative Fluorescent In-Situ Hybridization</td>
</tr>
<tr>
<td>QC</td>
<td>Quality Control</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative Polymerase Chain Reaction</td>
</tr>
<tr>
<td>RBP</td>
<td>Retinol Binding Protein</td>
</tr>
<tr>
<td>RDW</td>
<td>Red Cell Distribution Width</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>S</td>
<td>Single-Copy Nuclear Gene Signal</td>
</tr>
<tr>
<td>STELA</td>
<td>Single Telomere Length Analysis</td>
</tr>
<tr>
<td>sTfR</td>
<td>Soluble Transferrin Receptor</td>
</tr>
<tr>
<td>T</td>
<td>Telomere Signal</td>
</tr>
<tr>
<td>TBARS</td>
<td>Thiobarbituric Acid Reactive Substances</td>
</tr>
<tr>
<td>TIBC</td>
<td>Total Iron Binding Capacity</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor Necrosis Factor α</td>
</tr>
<tr>
<td>TRF</td>
<td>Telomere Restriction Fragment</td>
</tr>
<tr>
<td>TSAT</td>
<td>Transferrin Saturation</td>
</tr>
<tr>
<td>UNDP</td>
<td>United Nations Development Program</td>
</tr>
<tr>
<td>WBC</td>
<td>White Blood Cell</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
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Acknowledgements

I would like to express my sincere gratitude to my supervisor, Dr. Crystal Karakochuk. Crystal, you have been an incredible mentor to me and have truly enhanced my research knowledge and skills. I thank you for your unconditional support, brilliant insight and endless patience. It has truly been a pleasure to be your graduate student.

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On that note, I would like to extend my appreciation to Izabelle Gadawski, who performed the MMqPCR assay for the measurement of relative LTL and trained me in the MMqPCR assay for the measurement of mtDNA content. I would also like to thank the Côté Lab members for their help and encouragement with my research.

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and Manjula Desai Prize in Human Nutrition, the Graduate Student Travel Award and the Ursula Knight Abbott Travel Scholarship in Agricultural Sciences.

Last, but not least, I would like to thank my family and friends. To my parents, words cannot express how grateful I am for your unconditional support and encouragement. You have challenged me to strive for excellence in everything that I do. To Charles, I am also forever grateful for your unwavering support and encouragement. You never failed to boost my morale.
To My Parents.
Chapter 1: Introduction

1.1 Introduction

Anemia, defined as hemoglobin < 120 g/L, affects approximately 500 million non-pregnant women of reproductive age (15–49 years) globally.\(^1\) It is a serious public health problem, with consequences that include poor maternal health outcomes,\(^2\) poor birth outcomes,\(^2\) and reduced productivity.\(^3\) In 2012, the World Health Organization (WHO) set a global nutrition target to achieve a 50% reduction in anemia cases among women of reproductive age.\(^4\) As iron deficiency is reported to be the predominant cause of anemia globally,\(^1,5\) the WHO recommends untargeted daily oral iron supplementation with 60 mg of elemental iron for three consecutive months per year, for all non-pregnant women of reproductive age in regions where anemia prevalence is ≥ 40\%.\(^6\)

One such region is Cambodia, where the estimated prevalence of anemia is 43% among non-pregnant women of reproductive age.\(^1\) In contrast, recent reports have suggested that the prevalence of iron deficiency, based on ferritin concentrations ≤ 15 µg/L, is ≤ 10% among non-pregnant Cambodian women of reproductive age.\(^7-10\) There are other possible causes of anemia, including other micronutrient deficiencies,\(^11\) inflammation,\(^12,13\) excessive blood loss,\(^14\) and genetic hemoglobin disorders.\(^15\) In Cambodian women of reproductive age, the prevalence of genetic hemoglobin disorders has been reported to be > 50\%.\(^7,9,16\)
As iron deficiency does not seem to be a predominant cause of anemia among non-pregnant Cambodian women, untargeted daily oral iron supplementation may be ineffective and a waste of resources in the effort to reduce anemia prevalence. While there is strong evidence for the efficacy of iron supplementation in treating women who have completely depleted iron stores (i.e. ferritin < 15 µg/L), providing iron supplementation to iron-replete women may increase the adverse effects of excess iron. Women with certain genetic hemoglobin disorders, such as those with hemoglobin E (HbE) homozygosity or HbE/β-thalassemia heterozygosity, may be at even greater risk for excess iron accumulation, because of altered iron metabolism. Excess iron has the potential to be harmful, as free-iron may catalyze the formation of reactive oxygen species (ROS), via the Fenton reaction. Increased production of ROS can cause oxidative stress, which can cause cellular damage, such as damage to membrane lipids and DNA.

To date, there is minimal data on the potential risk of untargeted daily oral iron (60 mg/d elemental iron) supplementation. A double-blind randomized controlled trial was conducted to assess the effect of 12 weeks of untargeted daily oral iron supplementation, with or without multiple micronutrients (MMN), compared to placebo, on hemoglobin concentrations in n = 809 non-pregnant Cambodian women aged 18–45 years. For this research, secondary analysis of specimens from women who completed the trial in the iron (n = 191) and placebo (n = 186) treatment groups were conducted. Two biomarkers of cellular damage, relative leukocyte telomere length (LTL) and mitochondrial DNA (mtDNA) content, were quantified inuffy coats using monochrome multiplex quantitative polymerase chain reaction (MMqPCR). These biomarkers of cellular damage were selected because this assay can be conducted on stored
specimens. Further, oxidative stress has been shown to be associated with decreased LTL and altered mtDNA content.\textsuperscript{25,26}

1.2 Cambodia

Cambodia is a country located in Southeast Asia, bordered by Vietnam, Laos, and Thailand, as well as the Gulf of Thailand (\textbf{Figure 1-1}).\textsuperscript{16} It has a population of approximately 16.2 million people, of which 80.5\% reside in rural areas.\textsuperscript{16} The remaining population live in urban areas, including the capital city of Phnom Penh.\textsuperscript{16} The work outlined in this thesis was conducted using specimens collected during a 2015 randomized controlled trial in Kampong Chhnang province in Cambodia.\textsuperscript{24}

\textbf{Figure 1-1.} Map of Cambodia.

(Source: Cambodia Demographic and Health Survey (CDHS) 2014. Phnom Penh, Cambodia and Rockville, Maryland, USA: National Institute of Statistics, Directorate General for Health, and ICF International; 2015.)
Cambodia gained independence in 1953, after many years as a protectorate of France. Following a period of civil war from 1970–1975, the Khmer Rouge regime took rule of Cambodia and carried out the horrific genocide of over 2 million people. In 1979, the Socialist Republic of Vietnam gained control over the Khmer Rouge regime. Finally, in 1991, the Paris Peace Accords ended the Cambodian-Vietnamese war. Since the signing of the agreement, Cambodia has made important advancements and boasts one of the fastest growing economies in Asia. Despite this, Cambodia remains one of Asia’s poorest and underdeveloped countries. The World Bank classifies Cambodia as a lower middle income country, with an estimated gross national income (GNI) per capita of only $1,230 USD in 2017. The United Nations Development Program (UNDP) reported that Cambodia had a Human Development Index (HDI) of 0.543 (HDI = 1 is the highest score) for 2012. Additionally, the 2017 Global Hunger Index (GHI) results report that Cambodia has a “serious” state of hunger with a GHI of 22.2/100.

Agriculture is the primary economic livelihood in Cambodia, of which rice production accounts for the majority of agricultural efforts. The 2014 Cambodia Demographic and Health Survey (CDHS), a nationally-representative sample of Cambodian women and men aged 15–49 years, indicated that 44% of working women and 51% of working men hold jobs in the agricultural sector. Those who live in rural areas are more likely to take part in agricultural work and small-scale subsistence farming, compared to those living in urban areas. Garment factories and tourism services are also important economic activities, particularly in urban settings. As an important product of agriculture, rice makes up the majority of a Cambodian diet, along with
limited fruit, vegetable and animal product intake.\textsuperscript{32} The diet is typically low in energy, fat and important micronutrients, such as iron and vitamin A.\textsuperscript{32}

1.3 Anemia

Anemia is a condition characterized by a low hemoglobin concentration or a reduction in erythrocytes (i.e. red blood cells), that causes a reduction in the oxygen-carrying capacity in the body.\textsuperscript{33} For non-pregnant women of reproductive age (15–45 years), such as our study population in Cambodia, anemia is defined as hemoglobin < 120 g/L.\textsuperscript{33} In this population, mild, moderate and severe anemia is defined as hemoglobin concentration of 110–119 g/L, 80–109 g/L, and <80 g/L, respectively.\textsuperscript{33} Hemoglobin concentrations are affected by age and sex, but also altitude, cigarette smoking, race, and stages of pregnancy.\textsuperscript{33} These factors must be considered when selecting a hemoglobin cut-off to define anemia status. Residing at an altitude > 1,000 m above sea level and cigarette smoking are both associated with elevated hemoglobin concentrations, while pregnancy or African descent are associated with a lower hemoglobin concentrations.\textsuperscript{11}

1.3.1 Prevalence of Anemia

In 2011, it was estimated that approximately 500 million non-pregnant women had anemia globally.\textsuperscript{1} This translates to a global anemia prevalence of 29\% (95\% CI: 24–35).\textsuperscript{34} The 2014 CDHS reports an even greater anemia prevalence of 45\% for a nationally-representative sample of \(~12,000\) Cambodian women aged 15–49 years.\textsuperscript{16} Anemia was defined as a hemoglobin
concentration < 120 g/L (or < 110 g/L for pregnant women). Women residing in Kampong Chhnang had one of the highest prevalence rates of anemia at 53%, compared to other provinces. Based on the WHO anemia classification system, Cambodia is a region with “severe” anemia prevalence (≥ 40%). Since the 2005 CDHS, there has been little-to-no reduction in overall anemia prevalence.

1.3.2 Consequences of Anemia

Anemia is a significant public health problem, particularly for women and children. In particular, maternal anemia is associated with poor maternal outcomes and poor birth outcomes, including increased risk of perinatal mortality, low birth weight and preterm birth. Further, maternal postpartum anemia is associated with tiredness, breathlessness, heart palpitations, and increased infections. Additionally, postpartum anemia contributes to an increased risk of postpartum depression, reduced cognitive performance, and increased risk of maternal death. These adverse health outcomes are a serious concern for women of reproductive age who may become pregnant.

Anemia can also lead to a reduction in aerobic capacity, and therefore endurance capacity and energetic efficiency. This is likely due to the decrease in oxygen transport. Anemia is also associated with reduced work productivity. Given that a large percentage of women work in the agricultural sector in Cambodia, which requires manual labor, anemia may have negative consequences on Cambodia’s gross domestic product (GDP). The impact of reduced physical
productivity on GDP was demonstrated in 2003, when physical productivity losses in South Asia (e.g. India, Bangladesh, Pakistan) accounted for an estimated annual loss of $4.2 billion.  

1.3.3 Etiology of Anemia

There are many causes of anemia, including iron deficiency and other micronutrient deficiencies (e.g. vitamin A, vitamin B-12, folate),  

11 infection and disease (e.g. malaria),  

12,13 excessive blood loss,  

14 and genetic hemoglobin disorders.  

1.3.3.1 Iron

Iron is a dietary essential mineral required for many biologic functions, including erythropoiesis (i.e. red blood cell production), oxygen transport, DNA synthesis, respiration, and electron transport.  

37 Food sources contain two major forms of iron.  

Non-heme iron exists in plant (e.g. legumes and leafy greens), as well as in animal food sources (e.g. meat and poultry).  

37 Heme iron exists only in animal food sources.  

1.3.3.1.1 Iron Metabolism

After consumption of non-heme iron, insoluble ferric iron (Fe³⁺) is reduced to ferrous iron (Fe²⁺) when it reaches the proximal duodenum because of the gastric acid that lowers the pH in this environment.  

38 Divalent metal transporter 1 (DMT1) then transports Fe²⁺ into intestinal epithelial cells (enterocytes) of the duodenum and upper jejunum.  

38 The bioavailability of non-heme iron
varies from <1% to >90%, as its uptake into the enterocyte is influenced by the presence of dietary components that can enhance or inhibit absorption.\textsuperscript{37} Vitamin C is an enhancer of iron absorption, whereas phytic acid, polyphenols and calcium are inhibitors of iron absorption.\textsuperscript{37} Unlike non-heme iron, heme iron remains protected in the heme complex after consumption, preventing the oxidation of Fe\textsuperscript{2+} into insoluble Fe\textsuperscript{3+}, until it is transferred from the intestinal lumen into the enterocytes by heme oxygenase 1 (HO-1), at which point Fe\textsuperscript{2+} is liberated from the heme molecule.\textsuperscript{38} The bioavailability of heme iron is less affected by other dietary components, as the heme complex structure prevents Fe\textsuperscript{2+} from being chelated by other food constituents.\textsuperscript{37} The absorption of both non-heme and heme iron is also influenced by the an individual’s iron status.\textsuperscript{20} Once within the enterocyte, Fe\textsuperscript{2+} is bound by ferritin or exists as free-iron.\textsuperscript{20}

Ferroportin, an iron exporter, allows Fe\textsuperscript{2+} stored within the enterocyte to enter the bloodstream.\textsuperscript{38} Upon entering the bloodstream, Fe\textsuperscript{2+} is re-oxidized to Fe\textsuperscript{3+} and rapidly bound by transferrin, which is a glycoprotein responsible for the transport of iron in the bloodstream to various tissues.\textsuperscript{38} Depending on the saturation of transferrin, Fe\textsuperscript{2+} entering the bloodstream may also exist as non-transferrin bound iron (NTBI).\textsuperscript{21}

The majority of iron is used to produce erythrocytes in bone marrow, which is a process known as erythropoiesis.\textsuperscript{38} Iron that is not used for erythropoiesis is bound to the iron-storage protein, ferritin, and stored primarily in the liver, spleen and bone marrow.\textsuperscript{38} Iron may also be exist as free-iron in the intracellular space.\textsuperscript{20}
Once erythrocytes die, they are recycled by macrophages.\textsuperscript{37} In this process, the macrophage will liberate the iron contained in the erythrocyte.\textsuperscript{37} Depending on iron requirements, the liberated iron will either be stored as ferritin or transported through ferroportin channels on the surface of the macrophage back into circulation.\textsuperscript{37}

Iron losses are estimated to be very minimal and occur via the loss of iron-containing cells in the epithelial surfaces of the skin, genitourinary and gastrointestinal tracts.\textsuperscript{38} Iron losses are typically only major when there is substantial blood loss caused by excessive menstruation, or other bleeding.\textsuperscript{38} As there is no physiological mechanism for iron excretion, systemic iron homeostasis is predominantly regulated at the stage of absorption by hepcidin, a peptide hormone secreted by the liver.\textsuperscript{37} Hepcidin maintains iron homeostasis by binding and degrading ferroportin, the transport protein located on the surface of intestinal enterocytes and macrophages; thus, preventing the absorption of iron into the bloodstream from the enterocyte or from macrophages.\textsuperscript{37} In an individual with adequate or high iron stores, or in instances of an inflammatory response (due to stress, inflammation, or acute injury), hepcidin expression is increased in order to prevent further absorption of iron in the gut or release of iron from the macrophage into the bloodstream.\textsuperscript{37} Therefore, hepcidin is considered a major regulator of iron absorption. In an individual with low iron stores, hepcidin expression is decreased to allow for the absorption and release of iron into the bloodstream.\textsuperscript{37}

While there is no physiological mechanism for iron excretion, it is important to note that iron that is not absorbed by intestinal enterocytes of the duodenum and upper jejunum can pass into
the lumen of the colon, where it will eventually be excreted in feces.\textsuperscript{39} This iron may exist as free-iron, unless it is bound by other dietary components.

### 1.3.3.1.2 Iron Deficiency

Ferritin concentration, a biomarker of iron stores, is the most commonly used indicator of iron deficiency. For adults, a normal ferritin concentration is 15–300 µg/L, with a concentration < 15 µg/L indicating completely depleted iron stores (iron deficiency).\textsuperscript{37} Soluble transferrin receptor (sTfR) concentration is a biomarker of tissue iron stores. Conversely to ferritin, values of sTfR increase in the state of iron deficiency. A sTfR concentration of > 8.3 mg/L indicates depleted tissue iron stores (iron deficiency).\textsuperscript{40} These biomarkers may be measured in serum or plasma. Iron deficiency can be categorized by stages, according to progression (Table 1-1).\textsuperscript{37}

<table>
<thead>
<tr>
<th>Storage iron, ferritin</th>
<th>Stage I: iron depletion</th>
<th>Stage II: iron depletion with impaired erythropoiesis</th>
<th>Stage III: iron deficiency anemia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tissue iron, sTfR</td>
<td>low</td>
<td>low</td>
<td>low</td>
</tr>
<tr>
<td>TSAT</td>
<td>normal</td>
<td>elevated</td>
<td>elevated</td>
</tr>
<tr>
<td>Hemoglobin</td>
<td>Normal</td>
<td>low</td>
<td>low</td>
</tr>
</tbody>
</table>

sTfR, soluble transferrin receptor; TSAT, transferrin saturation.

Inadequate dietary iron intake, impaired dietary iron absorption, increased iron requirements (e.g. pregnancy, growth periods), and excessive iron losses (e.g. blood loss caused by excessive menstruation or hookworm infection) may all lead to iron deficiency by creating a negative iron balance.\textsuperscript{17} In Cambodia, it is often assumed that anemia is caused by inadequate dietary iron
intake. These findings are consistent with the fact that traditional Cambodian diets predominantly consist of rice and lack iron-rich animal-source foods. However, the 2014 CDHS showed that plasma ferritin concentration was < 15 µg/L in only 2.6% of women included in the survey.

1.3.3.2 Other Micronutrient Deficiencies

Deficiencies of vitamin A, vitamin B-12, folate, and riboflavin may also contribute to anemia. There are several ways in which vitamin A deficiency may lead to anemia. Retinoids (i.e. metabolites of vitamin A) play a role in the differentiation and proliferation of erythroid progenitor cells during erythropoiesis, and vitamin A deficiency could lead to ineffective erythropoiesis. Rat models of vitamin A deficiency have demonstrated decreases in erythropoietin (a hormone that stimulates erythropoiesis) expression, which may also lead to ineffective erythropoiesis. Further, a double-blind randomized controlled trial of Moroccan school children (n = 81) with poor vitamin A and iron status found that vitamin A supplementation was associated with a significant increase in erythropoietin. Vitamin A is also involved in immune function, thus deficiency may lead to reduced immunity. Reduced immunity may lead to an increased risk of infection and the development of anemia of inflammation. Additionally, vitamin A deficiency has been associated with increased iron stores in the liver and spleen, as well as poor mobilization of iron stores, which shows that it has the capacity to disturb iron metabolism. Due to these disruptions in iron metabolism, anemia caused by vitamin A deficiency is characterized by increased ferritin concentrations. This is unlike iron deficiency anemia, which is characterized by decreased ferritin concentrations. Other markers of vitamin
A deficiency anemia include reduced serum iron, low total iron binding capacity (TIBC) and low TSAT.\textsuperscript{44} However, vitamin A deficiency is an unlikely cause of anemia in this Cambodian population, as the 2014 CDHS found that < 3.2\% of Cambodian women included in the survey had vitamin A deficiency, based on a plasma retinol binding protein (RBP) concentration of < 1.05 µmol/L.\textsuperscript{16} This finding has been confirmed by other surveys that report a low prevalence of vitamin A deficiency among Cambodian women.\textsuperscript{7,8}

Vitamin B-12 and folate are required for DNA synthesis, which is important for the maturation of erythroblasts (the precursor to an erythrocyte) during erythropoiesis.\textsuperscript{45} Folate or vitamin-B12 deficiency can result in the production of abnormally large erythroblasts, known as megaloblasts, impairing erythropoiesis and leading to megaloblastic macrocytic anemia.\textsuperscript{46} Pernicious anemia, which is a type of megaloblastic macrocytic anemia, occurs because of defective absorption of vitamin B-12.\textsuperscript{46} Increased folate status, due to supplementation or food fortification, may mask pernicious anemia.\textsuperscript{47} This is an issue as vitamin B-12 deficiency may go undetected and result in cognitive impairment.\textsuperscript{47} It is also suspected that riboflavin deficiency may contribute to the development of anemia by altering iron metabolism.\textsuperscript{48} Riboflavin deficiency has been shown to impair the mobilization of iron from tissue stores, impair iron absorption and increase the rate of iron loss from the gastrointestinal tract.\textsuperscript{48} A double-blind randomized controlled trial of Chinese pregnant women \((n = 366)\) with anemia found that riboflavin supplementation combined with iron and folic acid supplementation resulted in a significantly larger increase in hemoglobin concentration, compared to iron and folic acid supplementation alone.\textsuperscript{49} The 2014 CDHS and other surveys have reported a low prevalence of B-12 deficiency (plasma vitamin B-12 concentration < 150 pmol/L) in the Cambodian population.\textsuperscript{7,16} However, the prevalence of folate
deficiency (plasma folate concentration < 10 nmol/L) was reported to be ~19.2% in Cambodian women.\textsuperscript{16} Most concerning is the high prevalence of riboflavin deficiency, defined as an erythrocyte glutathione reductase activity coefficient (EGRac) of ≥1.3, estimated at 89% and 92% in a sample of Cambodian women from Phnom Penh (n = 146) and Prey Veng (n = 156), respectively.\textsuperscript{50}

1.3.3.3 Inflammation (Infection & Disease)

Anemia of inflammation, also referred to as anemia of chronic disease, is considered the second most common cause of anemia worldwide.\textsuperscript{13} There are many underlying causes of anemia of inflammation, including infection (e.g. viral, bacterial, parasitic, fungal), cancer, and autoimmune disease (e.g. rheumatoid arthritis).\textsuperscript{13} In Cambodia, intestinal parasitic hookworm infection is relatively common; the 2014 CDHS reported that an estimated 15% of women aged 15–49 years were infected.\textsuperscript{16} Hookworms (i.e. Necator americanus and Ancylostoma duodenale) latch onto villi in the small intestine in order to feed on host blood.\textsuperscript{51} Moderate and heavy hookworm infection can lead to blood loss and contribute to anemia.\textsuperscript{51}

Aside from blood loss, infection and disease can also lead to inflammatory responses that initiate the release of cytokines, such as interferon-\(\gamma\) (IFN\(\gamma\)), tumor necrosis factor \(\alpha\) (TNF-\(\alpha\)), interleukin-1 (IL-1), interleukin-6 (IL-6) and interleukin-10 (IL-10).\textsuperscript{13} Interleukin-6 stimulates the liver to increase hepcidin release, which prevents the absorption of iron from the duodenum and iron export from macrophages.\textsuperscript{12,13} Interferon-\(\gamma\) also inhibits iron export from macrophages by decreasing the expression of the iron transporter, ferroportin-1.\textsuperscript{13} Expression of macrophage
DMT1 is upregulated by IFNγ, which stimulates Fe2+ uptake. Ferritin expression is stimulated by TNF-α, IL-1, IL-6 and IL-10, which enables the macrophage to store and retain the incoming iron. Tumor necrosis factor α also damages erythrocytes, which signals macrophages to phagocytose and degrade the erythrocytes in order to recycle iron. Further, IL-10 increases transferrin receptor mediated uptake of transferrin bound iron into monocytes by increasing the expression of transferrin receptor. In the kidneys, TNF-α and IFNγ decrease erythropoietin production. These cytokines, along with IL-1, also act in the bone marrow to prevent the differentiation and proliferation of erythroid progenitor cells. All of these events lead to decreased iron concentration in the blood (i.e. hypoferremia), increased ferritin concentrations, and decreased erythropoietin, which significantly decreases the amount of available iron for erythropoiesis. Ultimately, this decrease in erythrocyte production, and therefore decrease in hemoglobin concentration, can result in anemia.

The increase in ferritin concentration as a result of anemia of inflammation potentially results in the underestimation of iron deficiency, as low ferritin concentration is a common marker of iron deficiency. To deal with this problem, correction factors have been proposed to adjust ferritin concentrations with the use of two acute phase proteins that become elevated during inflammation: C-reactive protein (CRP) and α-1-acid glycoprotein (AGP). Application of these correction factors can help to increase the accuracy of ferritin concentrations to diagnose iron deficiency in the presence of inflammation.
1.3.3.4 Genetic Hemoglobin Disorders

Hemoglobin is an oxygen-binding protein that is produced by erythrocytes in bone marrow. Adult hemoglobin is comprised of two α-globin chains and two β-globin chains. Bound to each of these chains is a heme molecule, each of which contains a ferrous iron (Fe2+) molecule that can bind an oxygen molecule. Each hemoglobin molecule can bind a total of four oxygen molecules. Genetic variations in the globin chains that affect the structure, function and/or production of hemoglobin result in genetic hemoglobin disorders (i.e. hemoglobinopathies), which may result in anemia or other serious health conditions. There are two main types of hemoglobinopathies: thalassemia syndromes and/or hemoglobin variants. Thalassemias results from a defect in the production of one of the globin chains causing disordered hemoglobin production. In α-thalassemia, a defect in the synthesis of the α-globin chain typically occurs because of a partial or total deletion of the α-globin genes and less frequently because of a mutation in one or more of the four α-globin genes. In β-thalassemia, a defect in the synthesis of the β-globin chain occurs because of a variant in one or both β-globin genes. On the other hand, hemoglobin variants (e.g. hemoglobin E or sickle cell) occur due to a structural defect in one of the globin chains, typically due to a single nucleotide polymorphism. Individuals may carry one or more forms of a hemoglobinopathy. Additionally, individuals may be heterozygous or homozygous for a given disorder, the latter usually characterized by more severe clinical symptoms (e.g. more severe anemia). Hemoglobinopathies can be detected by protein-based analytic methods (e.g. hemoglobin electrophoresis, isoelectric focusing), high performance liquid chromatography (HPLC), or DNA sequencing.
In regions like Southeast Asia, hemoglobinopathies are present in up to 60% of the population. Most individuals are carriers of HbE. In fact, the CDHS 2014 reported that 28% of women (aged 15–49 years) were heterozygotes for HbE, 6% were homozygotes for HbE, and 23% were affected by other forms of hemoglobin disorders. Hemoglobin E heterozygosity (i.e. HbE trait) is associated with mild, microcytic hypochromic anemia. Hemoglobin E homozygosity (i.e. HbE disease) is associated with moderate, microcytic hypochromic anemia. A cross-sectional study of $n=450$ Cambodian women aged 18–45 years from Prey Veng province, reported that women who were homozygous for HbE had significantly lower hemoglobin concentrations (mean ± SD: 109 ± 7.3 g/L), compared to women without a hemoglobin disorder (130 ± 8.9 g/L). Additionally, the authors found that serum ferritin concentrations were significantly higher among women who were homozygous for HbE (mean ± SD: 129 ± 90 µg/L), as compared to women without a hemoglobin disorder (95.8 ± 56.2 µg/L). Serum sTfR was also found to be elevated among women who were homozygous for HbE, compared to women without a hemoglobin disorder. Thus, a hemoglobin disorder can result in anemia and altered concentrations of ferritin and sTfR, regardless of iron status.

Along with HbE variants, $\alpha$-thalassemias are also common in Cambodian women. Another study by Zimmermann et al. reported that Thai women that were heterozygous for $\alpha$-thalassemia had significantly higher median [IQR] serum ferritin concentrations (28 [1, 142] µg/L), compared to those without a hemoglobin disorder (15 [1, 148] µg/L). Women that were heterozygous for $\alpha$-thalassemia also had significantly higher median [IQR] body iron stores (5.3 [−9.8, 9.6] mg elemental iron/kg body wt), compared to those without a hemoglobin disorder (2.1 [−11.4, 10.5] mg elemental iron/kg body wt).
Some hemoglobinopathies, including compound HbE/β-thalassemia heterozygosity, can cause ineffective erythropoiesis and decreased hepcidin expression, which can result in increased iron absorption.\textsuperscript{18} This occurs regardless of iron status, leaving individuals with hemoglobinopathies at higher risk of accumulating excess iron within the body. However, a survey of \( n = 441 \) Cambodian women of reproductive age found no women with HbE E/Beta-thalassemia,\textsuperscript{7} potentially because it is a very severe form of the disease and many carriers do not survive to adulthood without treatment.

### 1.3.3.5 Blood Loss

For women of reproductive age, heavy menstrual bleeding (i.e. menorrhagia) is a common cause of iron deficiency anemia.\textsuperscript{14} Menorrhagia is characterized by blood loss of \( \geq 80 \text{ mL/d} \) for \( \geq 7 \) consecutive days.\textsuperscript{14} Heavy menstrual bleeding can cause iron deficiency anemia when dietary iron intakes are not high enough to replete iron lost through menstruation.\textsuperscript{14} A study of \( n = 105 \) women aged 20–45 years reported a strong correlation between menstrual loss (measured by a standardized pads’ weight method) and menstrual iron loss (measured by the alkaline haematin method) \((P < 0.001)\).\textsuperscript{56} Further, the authors found a significant inverse association between menstrual loss and serum ferritin concentrations in the \( n = 20 \) women with menorrhagia \((P = 0.005)\).\textsuperscript{56} Similarly, Harvey et al. investigated the association between menstrual iron losses (measured by alkaline haematin method) and iron deficiency in \( n = 90 \) healthy women aged 18–45 years.\textsuperscript{57} Once again, the authors also found that menstrual iron loss was significantly inversely
correlated with serum ferritin concentrations ($P < 0.001$), suggesting that heavy menstrual bleeding was associated with low iron stores.$^{57}$

### 1.3.4 Iron Supplementation Policies, Interventions and Exposures

Iron deficiency anemia accounts for the majority of anemia cases worldwide.$^{5,6,58}$ In 2011, the WHO predicted (through statistical modeling) that iron supplementation should result in an 8.6 g/L (95% CI: 3.9–13.4) increase in hemoglobin concentration among non-pregnant women (15–49 years) and that approximately 49% (95% CI: 43–53) of anemia cases in non-pregnant women (15–49 years) would be amenable to iron supplementation.$^1$ This prediction was based on a meta-analysis of 257 surveys conducted between 1990 and 2012, most of which were nationally representative.$^1$ As such, the WHO developed iron supplementation guidelines in order to meet their global nutrition target, which is to achieve a 50% reduction in anemia prevalence among women of reproductive age by 2025.$^1$ In regions where the prevalence of anemia is ≥40% (e.g. Cambodia), it is recommended that women of reproductive age receive daily oral iron supplementation with 60 mg of elemental iron for three consecutive months of the year.$^1$

In 2007, before the development of the 2016 WHO guideline, the Cambodia Ministry of Health set national guidelines for the use of iron and folic acid supplementation to prevent anemia in non-pregnant women of reproductive age (15–49 years)$^{32}$ The guideline recommends a weekly dose of 60 mg of elemental iron and 2.8 mg of folic acid.$^{32}$ Further, women in Cambodia are also exposed to dietary iron through iron-fortified fish and soy sauce (a nationally mandated fortification program).$^{59}$ Although it is not a policy or intervention, groundwater may also be
another potential source of dietary iron. In a survey of Cambodian households located in Prey Veng province, elevated iron levels (iron concentration > 300 µg/L) were reported in 72.7% \( (n = 16/22) \) of the household groundwater samples, according to the 2011 WHO water quality guidelines.\(^\text{60}\) It is therefore possible that naturally existing iron in groundwater may contribute to dietary iron intakes among Cambodian women, as has been reported in other regions in Asia.\(^\text{61}\) Due to recent reports of a low prevalence of iron deficiency among women in Cambodia,\(^\text{7–10,16}\) the Cambodia Ministry of Health has put the iron and folic acid supplementation guideline on hold until further evaluation can be conducted.

1.3.4.1 Efficacy of Iron Supplementation in the Treatment of Anemia

While there is strong evidence for the efficacy and rationale for iron supplementation in iron-deplete women,\(^\text{17}\) recent data suggests that a large majority of anemia cases in Cambodia may not be caused by iron deficiency.\(^\text{7–10,16}\) In fact, multiple studies report that the prevalence of iron deficiency (based on ferritin concentration \( \leq 15 \) µg/L) is \( \leq 10\% \) among Cambodian women.\(^\text{7–10}\) In the randomized controlled trial in Cambodia, only \( \sim 24\% \) of women in the iron supplementation group responded with an increase in hemoglobin > 10 g/L after 12 weeks.\(^\text{24}\)

1.4 Iron and Oxidative Stress

While iron is essential for many physiological functions, excess iron has the potential to be harmful.\(^\text{20}\) Consumption of excess iron can lead to an increase in free-iron, which is iron that is not bound to ferritin or transferrin (i.e. NTBI).\(^\text{20}\) Free-iron can accumulate within the intestinal
lumen, enterocytes, within cells of organs (i.e. liver, spleen and bone marrow) where iron is typically stored and as NTBI in the bloodstream.\textsuperscript{37}

Several studies have demonstrated a positive association between oral iron supplementation and NTBI concentration in humans. Brittenham et al. carried out a randomized controlled crossover trial in order to determine the effects of oral iron supplementation on the production of NTBI.\textsuperscript{62} The trial included $n = 32$ women aged 18–39 without anemia who were students or staff at the University of Zürich in Switzerland, of whom $n = 16$ women were iron-replete (serum ferritin concentration > 25 μg/L) and $n = 16$ women were iron-deplete (serum ferritin concentration ≤ 25 μg/L). The following three interventions were provided to each woman in a random sequence on separate days: 60 mg of elemental iron as ferrous sulphate with water, 60 mg of elemental iron as ferrous sulphate with a standard test meal (i.e. white rice and vegetable sauce) and 6 mg of elemental iron as ferrous sulphate with the same standard test meal. Blood specimens were collected before the administration of each intervention, as well as at 1, 2, 4, 6 and 8 hours after administration. A final blood specimen was collected at 14 days after the last intervention was administered. Following the 60 mg dose of elemental iron as ferrous sulphate, an increase in serum NTBI concentration above lower limits of detection (i.e. 0.1 μmol/L) was observed among both iron-replete and iron-deplete women.\textsuperscript{62}

In another randomized controlled crossover trial by Schümann et al., the effects of four different interventions on NTBI production were investigated among $n = 10$ iron-deplete (serum ferritin concentration < 30 μg/L), Guatemalan women aged 22–49 years without anemia.\textsuperscript{63} The following four treatments were provided to each woman in a random sequence on separate days:
plain water (placebo), 100 mg of elemental iron as ferrous sulphate with water, 100 mg of elemental iron as sodium iron EDTA (NaFeEDTA) with water, and 100 mg of elemental iron as iron polymaltose (IPM). Blood specimens were collected before the intervention administration, and at 90, 180 and 270 minutes after the intervention was administered. A 100 mg dose of elemental iron as ferrous sulphate resulted in an increase in serum NTBI concentration that was significantly greater in comparison to the water, NaFeEDTA and IPM interventions ($P < 0.001$).

Hutchinson et al. also investigated the effect of 65 mg of elemental iron as ferrous sulphate with food (i.e. bread, margarine and honey) and without food on the production of NTBI among $n = 7$ iron-deplete (mean $\pm$ SD serum ferritin concentration = $11.4 \pm 2 \mu g/L$) women with anemia, who were recruited from King’s College London in the United Kingdom. Blood specimens were taken before the administration of the intervention and at 4 hours after administration. There was a mean $\pm$ SE increase in serum NTBI concentration of $4.6 \pm 0.5 \mu m/L$ (without food) and $3.9 \pm 1.1 \mu m/L$ (with food) 4 hours after administration of 65 mg of elemental iron as ferrous sulphate, compared to serum NTBI values before administration ($P < 0.001$).

The accumulation of free-iron and NTBI is potentially harmful, as free-iron and NTBI can participate in the Fenton reaction and produce ROS. Under normal physiological conditions, ROS are produced in small concentrations. When free-iron or NTBI concentrations increase, ROS production also increases, which can disrupt the physiological balance of oxidants and antioxidants, leading to a condition known as oxidative stress. Oxidative stress can cause cellular damage, such as lipid peroxidation and DNA damage. Further, oxidative stress has
been implicated in a number of diseases (e.g. cancer, type 2 diabetes, neurodegenerative disorders).\textsuperscript{65–68}

In addition to studies that demonstrate a positive association between iron supplementation and NTBI concentration, many studies have investigated the association between iron supplementation and biomarkers of oxidative stress in humans. A study by Orozco et al., assessed the effects of 100 mg of elemental iron as ferrous sulphate, 100 mg of elemental iron as NaFeEDTA, and 100 mg of elemental iron as IPM on the generation of fecal ROS.\textsuperscript{69} The study included \( n = 10 \) iron-replete (serum ferritin concentration > 40 μg/L) men aged 18–56 years without anemia, who were recruited from the Universidad del Valle in Guatemala. Each intervention was administered for 6 days, with a 10-day washout period in between each intervention. Fecal iron content and ROS responses were measured during the last 3 days of each intervention and during the washout periods. Fecal ROS were significantly greater during periods of iron supplementation with ferrous sulphate and IPM, compared to periods before iron supplementation \((P<0.01)\).\textsuperscript{69} Thus, 100 mg elemental iron as these forms for 6 days was sufficient to cause an oxidative stress response in these adult men.

Similarly, Lund et al. measured total, free and weakly chelated fecal iron, as well as free radical production (e.g. ROS) in fecal specimens, after 2 weeks of supplementation with 19 mg/d of elemental iron as ferrous sulphate.\textsuperscript{70} This study included \( n = 18 \) adults aged 21–54 years without anemia, who were recruited from Norwich Research Park in the United Kingdom. Unfortunately, no information about participant’s baseline iron store status was provided, which is a limitation as a person’s iron store status plays a role in determining how much iron is absorbed after
consumption. The authors reported a significant increase in total iron concentration in feces after 2 weeks of supplementation ($P < 0.05$), compared to before supplementation. Further, there was a significant increase in the free radical generating capacity of the fecal specimens after 2 weeks of supplementation ($P < 0.001$), compared to before supplementation. The authors concluded that iron that is not absorbed into enterocytes has the potential to cause damage to mucosal cells or increase production of carcinogens in the colon, due to the increase in free radical production.

In addition to studies conducted with iron-replete populations without anemia, findings in populations that have iron deficiency anemia also support the association between oral iron supplementation and increased biomarkers of oxidative stress. King et al. investigated the effect of 98 mg/d of elemental iron as ferrous sulphate for 8 weeks on biomarkers of lipid peroxidation. Plasma malondialdehyde (MDA), measured in fasting venous blood specimens, and breath ethane exhalation rates (BEER) were assessed among $n = 12$ iron-deplete (plasma ferritin concentration $\leq 20 \mu g/L$) women aged 18–30 years with anemia, who were recruited in America. Both MDA and BEER had increased by $>40\%$ after 6 weeks of iron supplementation, compared to the women’s MDA and BEER measurements pre-supplementation ($P < 0.05$). These findings suggest that daily oral iron supplementation led to an increase in lipid peroxidation in iron-deplete women.

Further, in a randomized controlled trial by Aksu et al., leukocyte DNA damage after supplementation with an oral ferric preparation of 5–6 mg/kg/d for 12 weeks was measured. The comet assay was used to assess biomarkers of leukocyte DNA damage (strand breaks and
Fgp-sensitive sites) in $n = 27$ children with iron deficiency anemia and $n = 20$ age-matched healthy controls. There was a significant increase in both DNA strand breaks and Fgp-sensitive sites in children with iron deficiency anemia after supplementation, compared to baseline ($P < 0.01$). Children with iron deficiency anemia had a significantly higher mean ± SD number of Fgp-sensitive sites after supplementation (36±7 arbitrary units) compared to the control group (32±3 arbitrary units), but there was no significant difference in the number of DNA strand breaks. The authors concluded that these increases were likely a result of increased oxidative stress related to the iron supplementation.

Clearly, the published literature provides evidence of a positive association between iron supplementation and biomarkers associated with oxidative stress in iron-replete populations without anemia and in populations with iron deficiency anemia. Given that ROS have the propensity to damage DNA, the change in relative leukocyte telomere length (LTL) and the change in mitochondrial DNA (mtDNA) content after 12 weeks of daily oral iron supplementation with 60 mg elemental iron as ferrous sulphate represent potential biomarkers of iron-induced cellular damage. These biomarkers will be discussed in Sections 1.4.1 and 1.4.2, respectively.

### 1.4.1 Iron and Relative Leukocyte Telomere Length

The majority of the human genome is encoded by nuclear DNA (nDNA), which is organized into thread-like structures, known as chromosomes, within the nucleus of all eukaryotic cells. At each
end of a chromosome exists a repetitive sequence of DNA (TTAGGG), known as a telomere. Telomeres are non-coding regions that serve to protect DNA located within coding regions of chromosomes from damage during the replication process of cell division. Due to the nature of DNA replication, telomeric DNA is lost during each round of cell division. This phenomenon is known as telomere attrition (i.e. telomere shortening) and occurs with each cell division until the cell reaches the Hayflick limit and the cell becomes senescent. Thus, telomere length is often used as a biomarker of biological age, as telomere attrition occurs with age. Further, telomere length has now emerged as a potential biomarker for several age-related diseases, such as cardiovascular disease. As ROS have the potential to damage telomeric DNA, ROS may also cause accelerated telomere attrition. Experimental and correlational studies have shown that oxidative stress is associated with decreased LTL.

Numerous methods can be used to measure telomere length. Telomere restriction fragment (TRF) analysis is the ‘gold standard’, as it was the first method developed to measure telomere length. This method provides a base-pair length estimate for the telomere length in a specimen. A limitation of this method that it is low throughput and it requires greater quantities of DNA to evaluate telomere length. Also, the measure is an average of telomere length, as opposed to measures for individual telomeres of cells, in a specimen. This is a limitation as it does not allow for the assessment of telomere length heterogeneity between individual cells. Other commonly used methods include conventional monoplex quantitative polymerase chain reaction (qPCR), monochrome multiplex qPCR (MMqPCR) and absolute telomere length (aTL) quantification. Both qPCR and MMqPCR provide a measure for the relative telomere length in a specimen, which is the ratio of telomere fluorescent signals (T) normalized to that of a
single-copy nuclear gene (S), otherwise known as the T/S ratio. This measure is denoted as relative because it is proportional to absolute telomere length. For monoplex qPCR, the two genes are measured in separate wells, whereas MMqPCR allows for the measurement of the two genes within the same well. The aTL method provides a base-pair length estimate for the telomere length in a specimen. Similar to TRF analysis, PCR methods are limited in that they only provide a measure of the average telomere length in a specimen, as opposed to measures for individual telomeres of cells in a specimen. Further, the PCR methods only provide a measure of relative telomere length, as opposed to absolute telomere length. However, MMqPCR is relatively cost-effective and suitable for high-throughput testing. One method that measures individual telomeres is single telomere length analysis (STELA), however, STELA is labour intensive and technically challenging. Other methods used to measure telomere length are quantitative fluorescence in situ hybridization (Q-FISH) and flow-FISH. It is important to note that these methods measure telomere length in live cell specimens, not DNA specimens, and therefore cannot be done on archive specimens. The PCR methods are the most commonly used methods in studies to investigate the association between iron status or iron intake and relative telomere length.

A number of cross-sectional studies have investigated the association between biomarkers of iron status and relative LTL. A cross-sectional study of \( n = 1174 \) Korean adults aged 40–69 years from the Korean Genome Epidemiology Study reported that relative LTL, measured by monoplex qPCR, was inversely associated with TSAT (a percent indicator of the amount of serum iron bound to transferrin). Participants with high-normal concentrations (30–45%) and abnormally high concentrations (> 45%) of TSAT had significantly shorter relative LTL
compared to participants with low-normal TSAT concentrations (<30%) \( (P < 0.05) \). While genetics play a role in determining an individual’s TSAT, the authors concluded that it is also important to pay more attention to excessive iron consumption (i.e. using iron supplements) in order to keep TSAT within the low-normal concentration range, as higher concentrations may lead to biological aging, such as telomere attrition, and age-related diseases.  

Another cross-sectional study by Mainous et al. included \( n = 1009 \) multiracial-ethnic adults aged ≥25 years from the United States and Canada who were participants in the Hemochromatosis and Iron Overload Screening (HEIRS) study. In this study, individuals were considered to have an elevated iron phenotype if their TSAT was ≥45–50% and if their serum ferritin concentration was ≥200–300 \( \mu \)g/L. Bivariate analyses revealed that the elevated iron phenotype was associated with shorter relative LTL \( (P < 0.001) \). The odds of an individual with an elevated iron phenotype having shorter relative LTL were 2.71 (95% CI: 1.39–3.39) times the odds of an individual without an elevated iron phenotype in a logistic regression model adjusted for age, sex, and race-ethnicity. 

In a separate analyses of \( n = 669 \) participants from the HEIRS cohort, individuals with elevated TSAT (≥60%) had significantly shorter relative LTL in an unadjusted model, compared to individuals with non-elevated TSAT (<50% for males; <45% for females) \( (P < 0.01) \). These differences remained significant in a model adjusted for demographic factors (age, sex, race-ethnicity, usual source of medical care, education and health insurance), but not in a model adjusted for demographic factors and diseases (liver disease, diabetes, heart disease or failure, arthritis, impotence/fertility problems, and subject-reported iron overload). It may be that these
diseases, as opposed to elevated iron status, are associated with shorter relative LTL. Of note, both studies by Mainous et al. measured relative LTL using a monoplex qPCR.\textsuperscript{82,83}

Recently, Liu et al. assessed the association between serum ferritin concentration and relative LTL, assessed by monoplex qPCR, using data from \( n = 7336 \) adults aged \( \geq 20 \) years living in the United States that were a part of the National Health and Nutrition Examination Survey (NHANES) 1999-2002.\textsuperscript{84} Adults with high serum ferritin concentrations (> 200 μg/L) had significantly shorter relative LTL compared to adults with normal serum ferritin concentrations (30–200 μg/L) \( (P<0.05) \), in all three linear regression models adjusted for age, sex, race/ethnicity, and other factors.

Other studies have investigated the association between estimated supplementary or dietary or iron intake and relative LTL. A cross-sectional study by Xu et al. of \( n = 586 \) women aged 35–74 years from the Sister Study examined the association between multivitamin use and relative LTL, measured by monoplex qPCR.\textsuperscript{85} The authors found that iron supplement users \( (n = 41) \) had significantly shorter relative LTL compared to non-users \( (n = 527) \) \( (P=0.007) \).\textsuperscript{85} A limitation of this study is that no information was made available for the type or dose of iron supplements used, which limits the ability to compare the findings to other studies. Further, no information was made available for the duration of iron supplement usage, as participants were simply classified as ‘users’ or ‘non-users’.

Lee et al. investigated \( n = 1958 \) Korean men and women aged 40–69 years from the Korean Genome Epidemiology Study, in order to assess the associations between dietary micronutrient
intakes, measured by a validated food frequency questionnaire (FFQ), and relative LTL, measured by monoplex qPCR.\textsuperscript{86} No significant differences in iron intake across LTL tertiles and no linear association between iron intake and relative LTL were found in any of the adjusted models.\textsuperscript{86} This inconsistent finding may be due to a number of factors. First, a major limitation of the study was that estimated dietary intakes of iron among participants was assessed 10 years prior to the measurement of relative LTL. Therefore, the dietary intake of iron used in the study may not reflect the intake of iron at the time of study, unless the participants’ diet remained consistent over 10 years. Further, the estimated mean daily intakes of dietary iron reported to be consumed were between only 10.6–10.8 mg. This low intake of iron would not be expected to induce oxidative stress in a healthy individual, as it falls below the recommended nutrient intakes suggested by the WHO and the Food and Agricultural Organization (FAO) of the United Nations.\textsuperscript{87} Thus, it is not surprising that iron intake would not be associated with relative LTL in this population.

Similarly, Mazidi et al. evaluated $n=10568$ American adults aged $\geq18$ years from the NHANES, in order to assess the association between dietary components and relative LTL measured by monoplex qPCR.\textsuperscript{88} Unexpectedly, the mean ± SD daily iron intake was highest (15.45 ± 0.34 mg) among participants in the fourth quartile (i.e. participants with the longest relative LTL).\textsuperscript{88} Further, there was a significant positive linear association between iron intake and relative LTL ($P=0.014$).\textsuperscript{88} However, higher vitamin C intake was also significantly associated with longer relative LTL ($P=0.032$).\textsuperscript{88} Vitamin C is a powerful antioxidant, which has the potential to mitigate oxidative stress that could be caused by excess iron intakes.\textsuperscript{89,90} Similar to the study by Lee et al.\textsuperscript{86}, mean daily iron intake in Mazidi et al.\textsuperscript{88} population was relatively low (14.32–15.45
mg) and would not be expected to be associated with shorter relative LTL. Despite this, the conflicting results for iron and vitamin C intakes and their association with relative LTL in this population make it difficult to ascertain if either nutrient is independently associated with relative LTL.

No experimental studies have explored the direct effects of iron exposure on LTL. However, Brown et al. examined the effects of iron overload on telomere length and telomerase (an enzyme that can add TTAGGG sequences to the ends of chromosomes) activity in rat hepatocytes (i.e. liver cells). Rats \((n = 5)\) were injected with iron dextran every 2 weeks. The dose of the first four injections was 50 mg iron dextran, followed by one dose of 100 mg. Control rats \((n = 5)\) were injected with an equivalent dose of dextran (without iron) according to the same schedule. The authors determined liver telomere length using TRF. Telomerase activity was also measured. After 6 months of treatment, no difference in mean telomere length was found between the iron-loaded livers and control livers. However, telomerase activity was found to be increased threefold in the iron-loaded livers, compared to the control livers \((P < 0.05)\). The authors hypothesized that during instances of iron overload in the liver, telomerase activity is increased in effort to protect the chromosome from oxidative stress. This phenomenon would explain why no difference was seen in telomere length between the iron-loaded and control livers. Although, the findings from the aforementioned observational studies suggest that telomerase activity is not increased in leukocytes in response to iron loading, as increased iron status or intake has been associated with shortened relative LTL in humans. Further, telomerase is not typically highly active in leukocytes. Therefore, it is difficult to make comparisons between these rodent studies using liver cells and human studies using leukocytes.
In summary, it appears that the overall evidence mainly supports an inverse association between iron status or iron intake and relative LTL.

1.4.2 Iron and Mitochondrial DNA Content

Mitochondria are organelles that are essential for cell viability. Mitochondria contain their own DNA (i.e. mtDNA), which is double-stranded, circular and 16.6 kilobases in length. Mitochondria are the main source of intracellular ROS, due to their role in energy metabolism (e.g. oxidative phosphorylation, ATP synthesis). Mitochondria also play a role in cell proliferation and differentiation, synthesis of compounds (e.g. steroids, heme, iron-sulfur clusters), and regulation of apoptosis. Mitochondria are also a major site of iron metabolism, as they help transform iron into its bioactive form via heme and iron-sulfur cluster synthesis pathways. Iron levels within the mitochondria are tightly regulated, as free-iron has the potential to react with the abundant oxygen present in the mitochondria and lead to overproduction of ROS. Thus, free-iron is bound to mitochondrial ferritin and frataxin. However, excess iron consumption may result in the saturation of these storage proteins, leading to the presence of free-iron in the mitochondrial matrix. The presence of free-iron can lead to the overproduction of ROS in the mitochondria, which can induce damage to mtDNA. Since mtDNA is located near the electron transport chain, and does not have a robust repair mechanism, it is especially susceptible to damage by ROS. This damage can result in mtDNA deletion or mtDNA mutations, both of which can cause mitochondrial dysfunction.
Mitochondrial dysfunction is an emerging biomarker of age-related diseases (i.e. neurodegenerative diseases). Damage to mtDNA can lead to increased mitophagy (i.e. mitochondrial autophagy) in order to eliminate dysfunctional mitochondria, resulting in a decrease in mtDNA content. However, mtDNA damage can also lead to increased mitochondrial biogenesis, in order to compensate for dysfunctional mitochondria, resulting in an increase in mtDNA content. Experimental studies have shown that oxidative stress can indeed alter mtDNA content. Thus, altered mtDNA content can serve as a biomarker of mtDNA damage and mitochondrial dysfunction.

PCR methods are considered the ‘gold standard’ used to measure mtDNA content. These methods include conventional monoplex qPCR and MMqPCR, both of which provide a measure for the absolute mtDNA content in a specimen, which is the ratio of the copy number of mtDNA normalized to the copy number of a single-copy nuclear gene (nDNA), otherwise known as the mtDNA/nDNA ratio. The PCR methods are relatively low-cost and are suitable for high-throughput testing.

There are no currently published randomized controlled trials investigating the effects of iron supplementation on mtDNA content in humans. However, cross-sectional studies that investigated the association between iron status and mtDNA content have been published in healthy populations. Liu et al. investigated redox factors in plasma that may contribute to alterations in mtDNA content in human leukocytes. Healthy adults (n = 156) aged 25–80 years were recruited in Taiwan. Leukocyte DNA was isolated from buffy coat and monoplex qPCR was used to determine the mtDNA content. A multiple linear regression model showed that
plasma ferritin levels were positively associated with leukocyte mtDNA content (β-coefficient: 0.063, *P* = 0.049). Unfortunately, a 95% CI was not provided for this β-coefficient. Plasma ferritin was also significantly positively associated with plasma thiobarbituric acid reactive substances (TBARS, an indicator of lipid peroxidation) (*P* = 0.002). Both models were appropriately adjusted for factors such as age, sex, BMI and cigarette smoking. However, one limitation is that plasma ferritin concentrations in this studied population were not adjusted for levels of inflammation. This could have resulted in plasma ferritin concentrations being falsely elevated, which could have introduced bias into the interpretation of the results.

Notwithstanding this, the studied population was thought to be only inclusive of ‘healthy’ adults, so the levels of inflammation in this population are likely relatively low. Therefore, the authors concluded that oxidative stress-mediated leukocyte mtDNA content increase may have resulted from elevated level of free-iron in plasma.

Transfusion-dependent subjects with thalassemia have also been included in several cross-sectional studies, as blood transfusions can lead to the accumulation of excess iron within the body. Lal et al. compared the mtDNA content in *n* = 38 transfusion-dependent subjects with thalassemia aged 4–53 years, recruited from an American thalassemia clinic, and *n* = 24 healthy controls aged 19–46 years. The mtDNA content was measured in DNA extracted from peripheral blood mononuclear cells (PBMCs) using MMqPCR and a two-tailed student’s *t* test was used to compare the mtDNA content between the subjects with thalassemia and healthy controls. The authors found that the mean mtDNA content in the subjects with thalassemia was 41% greater than in the healthy control group (*P* = 0.026). This suggests that excess iron accumulation within the body may contribute to an increase in mtDNA content, as also
suggested by Liu et al.\textsuperscript{103} No associations between the mean mtDNA content and serum ferritin concentrations were found.\textsuperscript{104} A major limitation of this study is that the healthy control group was not age- or sex-matched to the subjects with thalassemia. Further, no adjustments were made for age in the comparison of mean mtDNA content between the subjects with thalassemia and the healthy controls. The mean mtDNA content could have been higher in the subjects with thalassemia, compared to the healthy controls, as the subjects with thalassemia contained younger individuals and mtDNA content is reported to be inversely associated with age.\textsuperscript{105}

Kim et al. performed a similar cross-sectional study including \(n = 129\) healthy Korean women aged > 60 years.\textsuperscript{106} The aim of the study was to investigate the association between mtDNA content and relative LTL, however the association between serum ferritin concentration and mtDNA content was also assessed. Both mtDNA content and relative LTL were measured by monoplex qPCR and a multivariable regression model was used to assess the association. The model was adjusted for multiple factors, including age, body mass index (BMI), cholesterol levels, alcohol consumption, current smoking, regular exercise and various diseases. They found no significant association between serum ferritin concentration and mtDNA content (\(\beta\)-coefficient: \(-0.056, P = 0.06\)).\textsuperscript{106}

In addition to cross-sectional human studies, a number of animal models have examined the association between iron exposure and mtDNA damage. In an experimental animal model, Gao et al. exposed H9c2 rat cardiac myocytes (i.e. heart muscle cells) to 300 \(\mu\)M ferric ammonium citrate (FAC) for 7 and 14 days.\textsuperscript{107} Long-range PCR was used to measure the mtDNA content in the rat cardiac myocytes. Exposure to 300 \(\mu\)M FAC for 7 and 14 days resulted in a significant
reduction in the amount of near full-length (~16 kilobases) mtDNA content, while the content of similar-length nDNA was unchanged. Thus, the authors concluded that long-term iron-mediated cell damage is associated with the progressive damage of mtDNA in rats.

While animal models have been helpful in determining mechanisms that account for the role of iron in mtDNA damage, it is difficult to extrapolate these findings to human subjects for a variety of reasons. Iron exposure in cultured cells is likely much greater than the exposure experienced by humans via iron supplementation or due to a genetic predisposition for a state of excess iron accumulation within the body (e.g. due to a genetic hemoglobin disorder). This may be why iron-mediated damage in cell cultures has rapid onset, while damage in humans who have accumulated excess iron takes much longer. Oxygen concentration in the cell culture may also not reflect physiological oxygen concentration, as higher oxygen concentrations are often used to support the survival of the culture cells. Increased oxygen concentration promotes ROS formation, which could lead to a magnification of the pro-oxidant effects of iron exposure that would typically occur at a physiological oxygen concentration. Nevertheless, the aforementioned study depicts a strong association between iron exposure and mtDNA damage in an animal model.

In another study, Gao et al. created a murine model of iron overload cardiomyopathy in which they randomized male B6D2F1 mice to either receive iron dextran injections of 10 mg/d elemental iron as ferrous sulphate or saline injections. Injections were administered 5 d/wk for 4 weeks. Similar to their previous study, long-range PCR was used to measure mtDNA content in extracted DNA from the cardiac myocytes of mice. Among the mice that received iron
supplementation, they observed a significant loss of full-length (~16 kilobases) mtDNA content, compared to mice that received saline.\textsuperscript{109} No change was observed in the content of nDNA.\textsuperscript{109} Again, this finding suggests iron-mediated damage specific to mtDNA in mice.

In summary, it appears that the evidence from cross-sectional human studies mainly supports a positive association between iron status or iron exposure and mtDNA content. In contrast, a decrease in mtDNA content is typically observed in response to iron exposure in experimental studies of animal models. More research is needed in humans to understand if these biological effects of iron exposure are replicable beyond these animal models. The association between iron supplementation and mtDNA content in humans has emerged as an important topic of research in order to determine the potential risk of iron supplementation.

1.5 Research Objectives and Hypotheses

The primary aim of this research was to retrospectively assess the effect of daily oral iron supplementation with 60 mg of elemental iron for 12 weeks, compared to placebo, in a population of non-pregnant Cambodian women of reproductive age on two selected cellular biomarkers.

Objective 1: To assess relative LTL change in women who received 60 mg of elemental iron as ferrous sulphate ($n = 191$) or a placebo ($n = 186$) for 12 weeks.

- Hypothesis: Women in the iron supplementation group will have a greater decrease in relative LTL after 12 weeks, compared to women in the placebo group.
Objective 2: To assess mtDNA content change in women who received 60 mg of elemental iron as ferrous sulphate ($n = 191$) or a placebo ($n = 186$) for 12 weeks.

- Hypothesis: Women in the iron supplementation group will have a greater change (decrease or increase) in mtDNA content after 12 weeks, compared to women in the placebo group.

Objective 3: To assess the combined effect of baseline iron status (i.e. baseline serum ferritin concentration, μg/L) and iron supplementation on the change in relative LTL and mtDNA content.

- Hypothesis: Among women in the iron supplementation group, those with higher baseline iron status (i.e. higher baseline serum ferritin concentration, μg/L) will have a greater decrease in relative LTL or a greater change (decrease or increase) in mtDNA content after 12 weeks.

Objective 4: To assess the combined effect of the presence of a genetic hemoglobin disorder and iron supplementation on the change in relative LTL and mtDNA content.

- Hypothesis: Among women in the iron supplementation group, those with any variant of a genetic hemoglobin disorder will have a greater decrease in relative LTL or a greater change (decrease or increase) in mtDNA content after 12 weeks.
Chapter 2: Research Design & Methods

In this chapter, I provide a brief summary of the randomized controlled trial from which the specimens for this study were sourced, as well as detailed description of the research design and methods. Thereafter, I provide a brief summary and a detailed description of the research design and methods for the secondary analysis study that encompasses my thesis work.

2.1 The Previous Trial

2.1.1 Summary and Rationale

The specimens used in this thesis research were collected in a $2 \times 2$ factorial double-blind randomized controlled trial conducted in rural Cambodia by my supervisor, Dr. Crystal Karakochuk. The aim of the first trial was to assess the effect of 12 weeks of untargeted daily oral iron supplementation with or without MMN, compared to placebo, on hemoglobin concentration and hemoglobin response in $n = 809$ non-pregnant Cambodian women aged 18–45 years. Ethics approval was obtained from the University of British Columbia Clinical Research Ethics Board in Canada (H15-00933) and the National Ethics Committee for Health Research in Cambodia (110-NECHR). The randomized controlled trial was registered at clinicaltrials.gov (NCT-02481375).
2.1.2 Methods

2.1.2.1 Participants and Recruitment

In order to determine the efficacy of the micronutrient interventions at increasing hemoglobin concentration and hemoglobin response (i.e. whether or not an increase of hemoglobin concentration of $\geq 10$ g/L was observed after 12 weeks), the planned study population was anemic non-pregnant women of reproductive age from Kampong Chhnang province, Cambodia. The rural Kampong Chhnang province was selected for this trial as the prevalence of anemia is one of the highest in Cambodia, at 53%.$^{16}$

Women were eligible for the study if they met the inclusion criteria and were able to provide written informed consent prior to enrolment. Inclusion criteria included: healthy, non-pregnant, aged 18–45 years, and hemoglobin concentration $\leq 117$ g/L determined by a finger prick capillary blood specimen using a HemoCue® Hb 301 (HemoCue AB, Sweden). A lower hemoglobin cut-off was selected, instead of the typical $< 120$ g/L cut-off recommended by the WHO,$^{110}$ in order to recruit more women capable of a hemoglobin response $\geq 10$ g/L. Women were excluded if they were taking medications or food supplements in the three months prior recruitment.

The G*Power Statistical Program v.3.1.9.2 for Mac (Heinrich Heine University of Düsseldorf) was used to estimate the sample size, assuming 90% power to detect a mean difference in hemoglobin (the primary outcome) of 3 g/L, considering an SD for hemoglobin of 10 g/L and a
2-tailed significance level of 0.05. The amount of 3 g/L has previously been reported as a clinically important difference in hemoglobin. A total sample size of $n = 628$ women ($n = 157$/group) was calculated. In order to account for possible participant dropout or loss to follow-up, a total of $n = 800$ ($n = 200$/group) women were required for the trial.

2.1.2.2 Procedures

The trial started with screening and recruitment on July 5, 2015. Enrolment then continued on a weekly-rolling basis for five weeks, until completion of the trial on November 5, 2015. Study implementation, data collection, monitoring and counseling were supervised by one study coordinator, two managers and four research officers.

A list of all villages in Kampong Tralack and Sameakki Mean Chey districts was provided by the provincial health authority in Cambodia. From the list, a computer-generated list of random numbers was used to select 26 villages. Community sensitization was provided in these villages through consultations with health center staff, and then village chiefs and village health volunteers. The women in these villages were then recruited via a convenience sample method. In order to determine eligibility for the study, recruited healthy, non-pregnant women aged 18–45 years were invited for hemoglobin screening. Eligible women were enrolled and stratified by anemia severity (mild [hemoglobin 110–117 g/L], moderate [hemoglobin 80–110 g/L], and severe [hemoglobin <80 g/L]) and by village. Each stratum was then separately randomized, using a computer-generated randomization list prepared by the study coordinator, to one of four
treatment groups in equal allocation on a weekly-rolling basis. The list was concealed until the
time of randomization.

The four treatment groups included 12 weeks of daily oral iron (60 mg elemental iron as ferrous
sulphate; iron group), 14 other micronutrients not including iron (MMN group), iron and 14
other micronutrients (iron+MMN group), and a placebo group that received maltodextrin
capsules. All capsules were manufactured by DSM Nutritional Products Ltd. (Isando, South
Africa) in January 2015. The gel capsules were identical in size and colour, only differentiated
by a code on the capsule package. The DSM Nutritional Products Ltd. project coordinator carried
out the blinding and did not share the code until statistical analysis of the primary outcome (i.e.
mean hemoglobin concentration at 12 weeks) was complete. Investigators, research staff, and
participants were all blinded to group assignment. The national policy in Cambodia, according to
the Ministry of Health, is to treat anemia (hemoglobin concentration <120 g/L) with 60 mg
elemental iron twice daily.32 A single 60 mg daily oral dose of elemental iron was chosen for the
formulation in this trial, as a higher quantity may have caused unnecessary side effects and a
lower dose may not have elicited a hemoglobin response.

Eligible women who were enrolled attended a total of six study visits: at screening, baseline, 1,
4, 8, and 12 weeks. Village chiefs and village health volunteers helped mobilize women during
screening and study visits. Regular monitoring was conducted by research staff and village
health volunteers throughout the study. At screening, one deworming tablet (500 mg
Mebendazole) was provided to women for the preventive treatment of helminth infection. At
baseline, socio-demographic and health data were collected at each woman’s household by a
research staff administered questionnaire. Phlebotomists collected a morning fasting venous blood specimen at baseline, as well as at 1 and 12 weeks, in the village at a central location. Blood was collected in a 6 mL trace element-free tube, a 6 mL evacuated tube containing EDTA, and a 2 mL tube containing EDTA (Becton Dickinson, Franklin Lakes, NJ, USA). Racks in a covered icebox were used to transport the blood to the National Institute of Public Health Laboratory for processing within 2–4 hours of collection. After baseline blood collection, and at 4 and 8 weeks, research officers provided the women with one bottle of 30 treatment capsules. Capsule counts were conducted at 4, 8, and 12 weeks to monitor adherence, which was based on the average of the three capsule counts. Women were considered adherent if they consumed ≥ 80% of the capsules.

Plasma, serum and buffy coat specimens were stored at –80°C in 2 mL vials prior to shipment on dry ice to Canada or other laboratories for analysis. An automated hematology analyzer (Sysmex XN-1000, Sysmex Corp, Kobe, Japan) was used to perform a complete blood count, in order to measure hemoglobin (g/L), mean corpuscular volume (MCV; fL), mean corpuscular hemoglobin (MCH; pg), and red cell distribution width (RDW; % of RBC). A sandwich-ELISA was used to assess serum for serum ferritin (μg/L), sTfR (mg/L), AGP (g/L), and CRP (mg/L). Serum ferritin concentrations were adjusted for inflammation using the Thurnham et al. correction factors. Serum hepcidin (nmol/L) was measured with a Hepcidin-25 Bioactive immunoassay kit (DRG International Inc., Springfield Township, NJ, USA). DNA was extracted from buffy coat using a QiaAmp Blood DNA kit (Qiagen Ltd., Hilden, Germany) in the Devlin Lab at BC Children’s Hospital Research Institute and then frozen at -80°C. The DNA extractions for baseline specimens were performed in December 2015 by one technician, while DNA
extractions for endline specimens were performed in March 2017 by a different technician. The same DNA extraction protocol was used by both technicians. This extracted DNA was assessed for 21 α-globin gene deletions and point mutations using the α-globin StripAssay® kit (ViennaLab Diagnostics, Vienna, Austria). Detection of hemoglobin variants (E, CS, H, Bart, or F) in the extracted DNA was also conducted using hemoglobin electrophoresis.

2.2 Secondary Analyses

2.2.1 Summary and Rationale

The research reported in this thesis was a secondary analysis of specimens collected in the $2 \times 2$ factorial double-blind randomized controlled trial$^{24}$ described in Section 2.1. Given that there is limited evidence regarding the potential risk of 12 weeks untargeted daily oral iron supplementation with 60 mg of elemental iron (as per the 2016 WHO policy), the primary aim of this research was to retrospectively assess the effect of this iron supplementation guideline in non-pregnant Cambodian women aged 18–45 years. Two biomarkers of cellular damage, relative LTL and mtDNA content were measured using MMqPCR in buffy coats from women in the iron ($n=191$) and placebo ($n=186$) treatment groups of the trial. Women who received MMN with or without iron during the trial were not included in the current study, as the aim was to assess the potential risk of iron alone. Ethics approval for this study was obtained from The University of British Columbia Research Ethics Board in Canada (H17-02650) and the National Ethics Committee for Health Research in Cambodia. The measurement of relative LTL and mtDNA
content were conducted using validated methods in the Côté Lab in the Department of Pathology and Laboratory Medicine at the University of British Columbia.

2.2.2 Methods

2.2.2.1 Monochrome Multiplex Quantitative PCR Assay

An MMqPCR assay, adapted from that developed by Cawthon et al., was used to measure both relative LTL and mtDNA content in buffy coat. This assay was optimized in the Côté Lab by Hsieh et al. for the measurement of both relative LTL and mtDNA content. Relative LTL and mtDNA content were measured in the baseline (n = 400) and endline (n = 376) DNA specimens that were extracted from buffy coat, as described in Section 2.1.2.2. The relative LTL and mtDNA content were not measured in n = 1 baseline specimen that was missing. The relative LTL and mtDNA content were only measured in specimens from women who received 60 mg elemental iron as ferrous sulphate or a placebo for 12 weeks in the trial.

2.2.2.1 Specimen Preparation

Extracted DNA specimens were thawed, vortexed using a Vortex-Genie 2 (Scientific Industries, Bohemia, NY), and spun on a Galaxy Mini Centrifuge (VWR International, Radnor, PA). The specimens were then diluted (1:10) by combining the extracted DNA with Buffer AE (Qiagen, Hilden, Germany) in 1.7 mL ultra-clear microtubes. The diluted specimens were again vortexed and spun. Extracted DNA specimens were only thawed, diluted and prepared immediately before
they were analyzed for relative LTL or mtDNA content. However, the specimens underwent multiple freeze-thaw cycles, including for the DNA extraction, for the relative LTL assay and for the mtDNA content assay.

2.2.2.1.2 Procedures

To prepare the assay mix, one tube of LightCycler® 480 SYBR Green I Master (product number 04887352001, F. Hoffmann-La Roche AG, Basel, Switzerland) was removed from the −20°C freezer and thawed at room temperature for 10 minutes. The LightCycler® 480 SYBR Green I Master contains FastStart Taq DNA Polymerase, SYBR Green I (a double-strand-specific DNA dye), reaction buffer, deoxyribonucleotide triphosphate (dNTP) mix, and magnesium chloride (MgCl₂). For the relative LTL assay, the following reagents were then added to a labeled 1.7 ml snap-cap RNAse-, DNAs-e-free microtube to create the assay mix: 1X LightCycler® 480 SYBR Green I Master, 1.2 mM EDTA (product number E7889, Sigma-Aldrich Canada Co., Oakville, Canada), 0.9 μM albumin primers (AlbuF, AlbdR) and 0.9 μM telomere primers (TelgF, TelcR). The same reagents were used for the mtDNA content assay, except 0.9 μM D-loop primers (D-loop_MPLX_F, D-loop_MPLX_R) were used instead of the telomere primers. The primer sequences are presented in Table 2-1. The mix contents were then gently mixed by pipetting the contents up and down several times with a clean no-DNA P200 pipette and then quickly spun down. The assay mix was only prepared immediately before use.
Table 2-1. Primer sequences.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Nucleotide Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>AlbuF</td>
<td>5’-CGGC CGCGCGCGCGCGCGCGCGCTGGGCGGAAATGCTGCA CAGAATCCTTG-3’</td>
</tr>
<tr>
<td>AlbdR</td>
<td>5’-GCCCGCGCGCGCGCGCGCGCTGGGCGGAAAGCATGGGTCGCCTGTT-3’</td>
</tr>
<tr>
<td>TelgF</td>
<td>5’-ACA CTAAG GGT TTGGGTTGGTTGGTTGGGTTGGTGTAGTGT-3’</td>
</tr>
<tr>
<td>TelcR</td>
<td>5’-TGT TAGG TAT CTC TAT CTC TAT CTC TAT CTC TAT CTC TAT CTC TCCTAACA-3’</td>
</tr>
<tr>
<td>D-loop_MPLX_F</td>
<td>5’-ACGCTCGACACACAGCACTTAAACACATCTCTGTGTCCT-3’</td>
</tr>
<tr>
<td>D-loop_MPLX_R</td>
<td>5’-GC TCGAGTTCACAGTATGGGAGTGRGAGGGRAAAA-3’</td>
</tr>
</tbody>
</table>

Bold nucleotides represent non-complementary GC clamps. Underlined nucleotides represent non-complementary bases added to increase amplicon melting temperature. Degenerate bases (50% A and 50% G) are represented by R.

To prepare the LightCycler® 480 96-well plate (product number 0472969200, F. Hoffmann-La Roche AG, Basel, Switzerland), 8 μL of assay mix and 2 μL of diluted DNA specimen were pipetted into each well. A total of 40 DNA specimens were included in duplicate on each plate. Baseline and endline specimens were pipetted into adjacent wells on the same plate. A standard curve, which was previously prepared in the Côté Lab, was included in the center of the plate.

For the relative LTL assay, the standard curve consisted of a serial dilution (1:2) of whole blood pooled from 24 healthy individuals. It ranged from 0.16 to 21 ng/μL of DNA across 8 standards, with a 128-fold linear range (r > 0.99). For the mtDNA content assay, the standard curve consisted of a serial dilution (1:5) of a mixture of two pCR® 2.1-Topo® plasmids containing the two amplicons amplified in the assay, that were mixed at a 1:50 (Alb:D-loop) ratio. It ranged from 3250 to 1.27×10⁹ copies of D-loop and 65 to 2.5×10⁷ copies of Alb across 8 standards, with
a 3125-fold linear range \((r > 0.99)\). The plate also contained two positive internal controls (ICs) and a negative control, included in duplicate on each plate. For the relative LTL assay, the positive ICs consisted of a long telomere IC (DNA extracted from pooled whole blood) and a short telomere IC (DNA extracted from K562 bone marrow lymphoblast cell cultures). For the mtDNA content assay, the positive ICs consisted of a high mtDNA IC (DNA extracted from SKBR3 mammary gland epithelial cell cultures) and a low mtDNA IC (DNA extracted from pooled whole blood). The negative control consisted of Buffer AE (Qiagen, Hilden, Germany). A depiction of the plate template is represented in Figure 2-1.

**Figure 2-1.** Plate template for the MMqPCR assay.

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
</tr>
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<tbody>
<tr>
<td>A</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>7</td>
<td>8</td>
<td>8</td>
<td>Std3</td>
<td>14</td>
<td>14</td>
<td>22</td>
</tr>
<tr>
<td>B</td>
<td>1</td>
<td>1</td>
<td>9</td>
<td>9</td>
<td>Std4</td>
<td>15</td>
<td>15</td>
<td>23</td>
<td>23</td>
<td>31</td>
<td>31</td>
<td>38</td>
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<tr>
<td>C</td>
<td>2</td>
<td>2</td>
<td>10</td>
<td>10</td>
<td>Std5</td>
<td>16</td>
<td>16</td>
<td>24</td>
<td>24</td>
<td>32</td>
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<tr>
<td>D</td>
<td>3</td>
<td>3</td>
<td>Positive IC (long/high)</td>
<td>Positive IC (long/high)</td>
<td>Std6</td>
<td>17</td>
<td>17</td>
<td>25</td>
<td>25</td>
<td>33</td>
<td>33</td>
<td>39</td>
</tr>
<tr>
<td>E</td>
<td>4</td>
<td>4</td>
<td>Positive IC (short/low)</td>
<td>Positive IC (short/low)</td>
<td>Std7</td>
<td>18</td>
<td>18</td>
<td>26</td>
<td>26</td>
<td>34</td>
<td>34</td>
<td>39</td>
</tr>
<tr>
<td>F</td>
<td>5</td>
<td>5</td>
<td>11</td>
<td>11</td>
<td>Std8</td>
<td>19</td>
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<td>27</td>
<td>27</td>
<td>35</td>
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<tr>
<td>G</td>
<td>6</td>
<td>6</td>
<td>12</td>
<td>12</td>
<td>Std9</td>
<td>20</td>
<td>20</td>
<td>28</td>
<td>28</td>
<td>36</td>
<td>36</td>
<td>40</td>
</tr>
<tr>
<td>H</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>7</td>
<td>13</td>
<td>13</td>
<td>Std10</td>
<td>21</td>
<td>21</td>
<td>29</td>
</tr>
</tbody>
</table>

A total of \(n = 40\) DNA specimens could be included on each plate in duplicate. IC, internal control; Std, standard. This figure was adapted from a figure created by the Côté Lab in the Department of Pathology and Laboratory Medicine at the University of British Columbia.
After completing the delivery of DNA, standards, and controls to the 96-well plate, it was sealed using LightCycler® 480 Sealing Foil (product number 04729757001, F. Hoffmann-La Roche AG, Basel, Switzerland). The plate was then centrifuged for 2 minutes at 1500 g in a centrifuge 5810R (Eppendorf Canada, Mississauga, ON) until no bubbles were present in the wells. After centrifugation, the plate was placed in a LightCycler480® Instrument II (product number 05015278001, F. Hoffmann-La Roche AG, Basel, Switzerland), which carries out a thermal cycling program. The thermal cycling programs for the relative LTL and mtDNA content assays are presented in Table 2-2 and Table 2-3, respectively.

Table 2-2. LightCycler® 480 thermal cycling program for the relative LTL assay.

<table>
<thead>
<tr>
<th>Program</th>
<th>Cycles</th>
<th>Program</th>
<th>Temp. (°C)</th>
<th>Signal Acquisition Mode</th>
<th>Hold Time (mm:ss)</th>
<th>Temp. Ramp Rate (°C/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-Incubation</td>
<td>1</td>
<td>1</td>
<td>95</td>
<td>None</td>
<td>15:00</td>
<td>4.4</td>
</tr>
<tr>
<td>Amplification</td>
<td>2</td>
<td>2</td>
<td>94</td>
<td>None</td>
<td>00:15</td>
<td>2.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>49</td>
<td>None</td>
<td>00:15</td>
<td>2.2</td>
</tr>
<tr>
<td>Amplification</td>
<td>40</td>
<td>3</td>
<td>94</td>
<td>None</td>
<td>00:15</td>
<td>4.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>62</td>
<td>None</td>
<td>00:10</td>
<td>2.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>74</td>
<td>Single</td>
<td>00:15</td>
<td>4.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>84</td>
<td>None</td>
<td>00:10</td>
<td>4.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>88</td>
<td>Single</td>
<td>00:15</td>
<td>4.4</td>
</tr>
<tr>
<td>Melting Curve</td>
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<td>4</td>
<td>95</td>
<td>None</td>
<td>01:00</td>
<td>4.4</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td>45</td>
<td>None</td>
<td>00:01</td>
<td>2.0</td>
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<td></td>
<td></td>
<td></td>
<td>95</td>
<td>Continuous</td>
<td>--</td>
<td>0.11</td>
</tr>
<tr>
<td>Cooling</td>
<td>1</td>
<td>5</td>
<td>40</td>
<td>None</td>
<td>00:00:01</td>
<td>1.5</td>
</tr>
</tbody>
</table>
The thermal cycling program for the relative LTL assay is as follows: during the pre-incubation period (program 1), there is complete activation of the FastStart Taq DNA Polymerase, which is the enzyme contained in the LightCycler® 480 SYBR Green I Master. The first amplification period (program 2) is comprised of two segments. Segment 1 (94°C, 15 seconds) allows for template DNA dissociation and segment 2 (49°C, 15 seconds) allows for only the TelgF primer to bind to and amplify the template DNA, creating a TelgF primer extension product. The AlbuF and AlbdR primers also bind and amplify the template DNA during segment 2. The second amplification period (program 3) is comprised of five segments. Segment 1 (94°C, 15 seconds) allows for DNA dissociation. In segment 2 (62°C, 10 seconds), both the TelgF and TelcR primers bind to the dissociated TelgF primer extension product that was created in program 2. The AlbuF and AlbdR primers bind to the dissociated template DNA. During segment 3 (74°C, 15 seconds), all primers amplify and a fluorescent signal for both the telomere and albumin amplicons (i.e. amplification products) is acquired. A cycle threshold (Ct) value, which is the cycle number (1–40) when the fluorescence of an amplicon can be detected above the
background fluorescent signal, can be obtained during this segment. Note that both the telomere and albumin primers are amplified, therefore the fluorescent signal is a sum of telomere and albumin amplicons. However, since the quantity of albumin at this segment’s C<sub>t</sub> is negligible, the C<sub>t</sub> value from this signal can be used to calculate the telomere copy number only. In segment 4, the telomere amplicons dissociate. Then in segment 5, a fluorescent signal for only the albumin amplicons is acquired. The C<sub>t</sub> at segment 5 can be used to calculate the albumin copy number.

Throughout the cycling process, the temperature ramping rates are 4.4°C/s, with the exception of program 2 and segment 2 (62°C, 10 seconds) of program 3, which have temperature ramping rates of 2.2°C/s.

The thermal cycling program for the mtDNA content assay is as follows: during the pre-incubation period (program 1), there is complete activation of the FastStart Taq DNA Polymerase, which is the enzyme contained in the LightCycler® 480 SYBR Green I Master. There is only one amplification period (program 3), which is comprised of five segments. Segment 1 (94°C, 15 seconds) allows for template DNA dissociation. In segment 2 (62°C, 10 seconds), both the D-loop_MPLX_F and D-loop_MPLX_R primers bind to the dissociated template DNA. The AlbuF and AlbdR primers also bind to the dissociated template DNA. During segment 3 (74°C, 15 seconds), all primers amplify and a fluorescent signal for both the D-loop and albumin amplicons (i.e. amplification products) is acquired. A C<sub>t</sub> value can be obtained during this segment. Note that both the D-loop and albumin primers are amplified, therefore the fluorescent signal is a sum of D-loop and albumin amplicons. However, since the quantity of albumin at this segment’s C<sub>t</sub> is negligible, the C<sub>t</sub> value from this signal can be used to calculate the D-loop copy number only. In segment 4, the D-loop amplicons dissociate. Then in
segment 5, a fluorescent signal for only the albumin amplicons is acquired. The C_t at segment 5 can be used to calculate the albumin copy number. Throughout the cycling process, the temperature ramping rates are 4.4°C/s, with the exception of segment 2 (62°C, 10 seconds) of program 3, which has a temperature ramping rate of 2.2°C/s.

For both the relative LTL and mtDNA content assay, a melting curve analysis (program 4) occurs after the amplification period. The melting curve analysis serves to ensure the purity and specificity of the amplicons. Then, the plate is briefly cooled down in program 5.

Once a run was complete, the LightCycler® 480 software version 1.5.1.62 SP2 (F. Hoffmann-La Roche AG, Basel, Switzerland) was used to acquire data from the run. The data was exported as a text file and then imported into Microsoft Excel (Microsoft Corp., Redmond, WA). Within Excel, data for the signal acquisition at 74°C (program 2, segment 3) and 88°C (program 2, segment 5) were then sorted according to these program and segment markers. This sorted data was then exported from excel in two separate text files, one for the signal acquisition at 74°C and one for the signal acquisition at 88°C. The LC480 Conversion software 2.0. (Heart Failure Research Centre, Amsterdam, Netherlands) was used to convert the text files into a grid format, which were then saved back into the excel document. The grid format data was then used in LinRegPCR version 2012.1 (Heart Failure Research Centre, Amsterdam, Netherlands) to obtain baseline fluorescence corrections and C_T calculations. In order to calculate the C_T for each specimen, the software aligns the exponential phase of the fluorescence curve for each individual well on the plate, which establishes a common window of linearity. One cycle below the upper bound of the window of linearity is then determined to be the C_T. The software determines the
PCR efficiency (i.e. the increase in amplicon per cycle) for each well on the plate, however, these values were not used here. Instead, the PCR efficiency for each acquisition was calculated from the standard curve on the plate. This data is imported into excel and generates values for the relative LTL or mtDNA content in each well of the plate. The relative LTL was represented by the ratio of the telomere fluorescent signals normalized to the fluorescent signals of a single-copy nuclear gene (T/S ratio). The mtDNA content is defined as the copy number of mtDNA normalized to the copy number of a single-copy nuclear gene (mtDNA/nDNA ratio).

2.2.2.1.3 Quality Control

Quality control (QC) practices for these assays have been established by the Côté Lab. No more than one of the following run-specific QC criteria can be violated in order for a run to pass QC:

1. All collected data must lie within the bounds of the standard curve.
2. The signal from the negative control must be absent or extremely low (> 3 Ct below the last standard).
3. The IC measurements must be within 2 SDs of IC measurements from previous runs performed under the same conditions.
4. The PCR efficiencies of each gene must be between 90–100% (between 1.80- and 2.00-fold amplification per cycle)
5. The differences between the PCR efficiencies between the two genes must be < 2.5% (< 0.05-fold amplification per cycle)
6. The mean of the absolute difference in the ratios of duplicate measurements for all specimens in a run must be < 10%.
If the run fails more than one of these QC criteria, it is rejected and all specimens must be run again. If the run is accepted, there are also specimen-specific QC practices (variation between specimen duplicates), which are as follows:

1. The data for a specimen is accepted if the ratios of the duplicate measurement vary by < 15%.

If any duplicates fail this QC criteria, the duplicates must be run again. If the duplicates fail QC criteria after being repeated, the mean and standard deviation of all four ratios is taken. If the standard deviation between ratios is < 10%, then the mean of the ratios of the four replicates is accepted as the ratio for the specimen.

2.2.3 Statistical Analyses

In this exploratory study, relative LTL and mtDNA content were measured in all available specimens from women who received iron or a placebo in the randomized controlled trial. A total of \( n=376 \) women were included in the relative LTL analyses and a total of \( n=370 \) women were included in the mtDNA content analyses. Power calculations for the trial are summarized in Section 2.1.2.1. The change in relative LTL (i.e. the change in the T/S ratio) and the change in mtDNA content (i.e. the change in the mtDNA/nDNA ratio) after 12 weeks were calculated using the equations outlined in Table 2-4.
Table 2-4. Primary outcome equations.

<table>
<thead>
<tr>
<th>Primary Outcome</th>
<th>Equation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absolute relative LTL change</td>
<td>endline relative LTL − baseline relative LTL</td>
</tr>
<tr>
<td>Percent relative LTL change</td>
<td>( \frac{\text{endline relative LTL} - \text{baseline relative LTL}}{\text{baseline relative LTL}} \times 100 )</td>
</tr>
<tr>
<td>Absolute mtDNA content change</td>
<td>endline mtDNA content − baseline mtDNA content</td>
</tr>
<tr>
<td>Percent mtDNA content change</td>
<td>( \frac{\text{endline mtDNA content} - \text{baseline mtDNA content}}{\text{baseline mtDNA content}} \times 100 )</td>
</tr>
</tbody>
</table>

LTL, leukocyte telomere length; mtDNA, mitochondrial DNA.

Intention-to-treat analysis was conducted using generalized linear mixed-effects models to predict the primary outcomes: the mean change in relative LTL and mtDNA content across iron and placebo treatment groups after 12 weeks. The same models were used to conduct a per-protocol analysis that only included adherent women (those who consumed ≥ 80% of the capsules). The relative LTL model was adjusted for age and baseline relative LTL (fixed-effects) and village clusters (random-effects). Age was adjusted for as it is widely reported to be inversely associated with LTL.113 Similarly, baseline relative LTL was adjusted for as telomere length is reported to be positively associated with the rate of telomere attrition.113 The mtDNA content model was adjusted for age (fixed-effect) and village clusters (random-effects). Age was adjusted for as it is reported to be inversely associated with mtDNA content.105 The following variables were also tested in the mtDNA content model, however, they were not included in the final model: baseline mtDNA content, baseline platelet count and endline platelet count. Baseline mtDNA content was tested in the model to assess if it was associated with the rate of change in mtDNA content. Further, baseline and endline platelet counts were tested in the model as platelets contain small amounts of mtDNA, but not nDNA. This could skew the mtDNA content
(i.e. the mtDNA/nDNA ratio) if the specimens contain platelets (i.e. buffy coat).¹¹⁴ These variables were not included in the final model as their inclusion did not significantly change the coefficients as observed in the unadjusted model. Further, the platelet counts were not significantly and independently associated with the outcome variable (i.e. absolute or percent change in mtDNA content).

Generalized linear-mixed effects models were also used to assess the secondary outcomes, as follows:

- The interaction effect of baseline iron status (baseline inflammation-adjusted serum ferritin concentration, μg/L) and iron supplementation on both relative LTL and mtDNA content.
- The interaction effect of the presence of a genetic hemoglobin disorder (any variant) and iron supplementation on both relative LTL and mtDNA content.

A $P$-value of $\alpha < 0.05$ was considered statistically significant. All statistical analyses were conducted using Stata IC v.15.1 (Stata Corp., Texas, USA).
Chapter 3: Results

In this chapter, I present the flow chart of enrolment, adherence rates to the supplement protocol, reported side effects, baseline characteristics, and the key findings for my primary and secondary objectives.

3.1 Flow Chart of Enrolment

Figure 3-1 depicts the flow chart of trial enrolment from the trial, for specimens included in the current study. In the trial, a total of \( n = 2846 \) women were screened among the 26 villages. Of these women, \( n = 2037 \) were excluded. A total of \( n = 809 \) women were randomized to one of the four treatment groups: \( n = 201 \) allocated to the iron supplementation group and \( n = 200 \) allocated to the placebo group. The trial had a retention rate of 94%; \( n = 760/809 \) randomized women completed the 12-week trial. Among the women in the iron and placebo treatment groups, \( n = 191 \) women and \( n = 186 \) women completed the 12-week trial, respectively.
Figure 3-1. Flow chart of trial enrolment from the trial, for specimens included in the current study.

- Excluded (n=2,037)
  - Did not meet eligibility criteria: 1,889
  - Declined to participate: 52
  - Loss to follow-up after screening: 91
  - Pregnancy after screening: 4
  - Illness after screening: 1

- enrolled & randomized (n=809)
  - allocated to men (n=203)
    - Lost to follow-up (n=2)
    - Migrated: 2
    - Discontinued intervention (n=8)
    - Pregnancy: 3
    - Severe illness: 1
    - Withdrew: 1
    - Unable to collect blood: 3
    - women who completed the trial (n=191)
      - Failed QC criteria for mtDNA assay (n=4)
      - Missing baseline sample (n=1)
      - LTL analyzed (n=190)
      - mtDNA analyzed (n=186)

- allocated to MMN (n=202)
  - Lost to follow-up (n=4)
    - Migrated: 4
    - Discontinued intervention (n=10)
    - Pregnancy: 5
    - Adverse side effects: 1
    - Withdrew: 1
    - Unable to collect blood: 3
    - women who completed the trial (n=186)
      - Failed QC criteria for mtDNA assay (n=2)
      - LTL analyzed (n=186)
      - mtDNA analyzed (n=184)

MMN, multiple micronutrients; QC, quality control; LTL, leukocyte telomere length; mtDNA, mitochondrial DNA.

3.2 Adherence and Adverse Side Effects

The adherence rates and reported side effects for the women included in the relative LTL and mtDNA content analyses are presented in Table 3-1 and Table 3-2.
Table 3-1. Adherence rates and reported side effects for women included in the relative LTL analyses.

<table>
<thead>
<tr>
<th></th>
<th>Iron</th>
<th>Placebo</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total enrolled, n (%)</td>
<td>190 (51)</td>
<td>186 (49)</td>
</tr>
<tr>
<td>Adherence rate (≥80% capsules consumed), n (%)</td>
<td>162 (85)</td>
<td>163 (88)</td>
</tr>
<tr>
<td>Reported side effects, n (%)</td>
<td>92 (48)</td>
<td>82 (44)</td>
</tr>
<tr>
<td>Nausea, n (%)</td>
<td>30 (33)</td>
<td>23 (28)</td>
</tr>
<tr>
<td>Stomach cramps, n (%)</td>
<td>25 (27)</td>
<td>18 (22)</td>
</tr>
<tr>
<td>Headache, % n (%)</td>
<td>20 (22)</td>
<td>19 (23)</td>
</tr>
</tbody>
</table>

Total n = 376. Values are n (%).

Table 3-2. Adherence rates and reported side effects for women included in the mtDNA content analyses.

<table>
<thead>
<tr>
<th></th>
<th>Iron</th>
<th>Placebo</th>
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</thead>
<tbody>
<tr>
<td>Total enrolled, n (%)</td>
<td>186 (50)</td>
<td>184 (50)</td>
</tr>
<tr>
<td>Adherence rate (≥80% capsules consumed), n (%)</td>
<td>159 (85)</td>
<td>162 (88)</td>
</tr>
<tr>
<td>Reported side effects, n (%)</td>
<td>90 (48)</td>
<td>80 (43)</td>
</tr>
<tr>
<td>Nausea, n (%)</td>
<td>30 (33)</td>
<td>22 (28)</td>
</tr>
<tr>
<td>Stomach cramps, n (%)</td>
<td>24 (27)</td>
<td>18 (23)</td>
</tr>
<tr>
<td>Headache, % n (%)</td>
<td>19 (21)</td>
<td>19 (24)</td>
</tr>
</tbody>
</table>

Total n = 376. Values are n (%).

The remainder of the results chapter is divided into two sections, outlining the baseline characteristics and key findings with respect to the two major outcomes of the study: buffy coat relative LTL and mtDNA content.

3.3 Buffy Coat Relative LTL Analyses

3.3.1 Baseline Characteristics of Women Included in the Relative LTL Analyses

Baseline characteristics are presented in Table 3-3. A total of n = 376 specimens were included in the relative LTL analyses, as n = 1 baseline specimens was missing. The mean ± SD age at baseline was 31 ± 8 years and the mean ± SD household size was 4.7 ± 1.7 people. A total of 65% of women were married, 53% of women had completed primary school, and 37% of women had
1 or 2 children. Among women who had ≥1 child (72%), 77% of women reported previously taking any quantity and/or duration of iron folic acid supplements during their last pregnancy. At baseline, 17% and 31% of women were lactating and taking birth control, respectively.

Table 3-3. Baseline characteristics of women included in the relative LTL analyses by supplement group.

<table>
<thead>
<tr>
<th></th>
<th>Iron</th>
<th>Placebo</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total enrolled, n (%)</td>
<td>190 (51%)</td>
<td>186 (49%)</td>
</tr>
<tr>
<td>Age, y (mean ± SD)</td>
<td>31 ± 8</td>
<td>30 ± 8</td>
</tr>
<tr>
<td>Household size (mean ± SD)</td>
<td>4.8 ± 1.7</td>
<td>4.7 ± 1.6</td>
</tr>
<tr>
<td>Marital status, n (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Single</td>
<td>47 (25)</td>
<td>48 (26)</td>
</tr>
<tr>
<td>Married</td>
<td>126 (66)</td>
<td>120 (65)</td>
</tr>
<tr>
<td>Widow</td>
<td>10 (5)</td>
<td>6 (3)</td>
</tr>
<tr>
<td>Separated/divorced</td>
<td>7 (4)</td>
<td>12 (6)</td>
</tr>
<tr>
<td>Completed education, n (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>25 (13)</td>
<td>28 (15)</td>
</tr>
<tr>
<td>Primary (grades 1–5)</td>
<td>96 (51)</td>
<td>104 (56)</td>
</tr>
<tr>
<td>Lower secondary (grades 6–9)</td>
<td>64 (34)</td>
<td>42 (23)</td>
</tr>
<tr>
<td>Upper secondary (grades 10–12)</td>
<td>5 (3)</td>
<td>11 (6)</td>
</tr>
<tr>
<td>Higher education/university</td>
<td>0 (0)</td>
<td>1 (&lt;1)</td>
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<tr>
<td>Parity, n (%)</td>
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<td></td>
</tr>
<tr>
<td>0</td>
<td>52 (27)</td>
<td>55 (30)</td>
</tr>
<tr>
<td>1–2</td>
<td>69 (36)</td>
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</tr>
<tr>
<td>3–4</td>
<td>52 (27)</td>
<td>42 (23)</td>
</tr>
<tr>
<td>≥5</td>
<td>17 (9)</td>
<td>18 (10)</td>
</tr>
<tr>
<td>Women with parity ≥1 who reported to receive, n (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFA during last pregnancy</td>
<td>106/138 (77)</td>
<td>101/131 (77)</td>
</tr>
<tr>
<td>Deworming during last pregnancy</td>
<td>74/138 (54)</td>
<td>73/131 (56)</td>
</tr>
<tr>
<td>Lactating, n (%)</td>
<td>28 (15)</td>
<td>36 (19)</td>
</tr>
<tr>
<td>Taking birth control, n (%)</td>
<td>52 (27)</td>
<td>63 (34)</td>
</tr>
</tbody>
</table>

Table 3-4 presents baseline hematological, nutrition and inflammation indicators. Not presented in the table are the median (IQR) endline values for serum ferritin concentration and TSAT. At endline, after 12 weeks of the intervention, the median (IQR) TSAT was 27.93% (20.56, 36.81) in the iron supplementation group and 19.79% (12.74, 28.91) in the placebo group. Further, the
median (IQR) endline inflammation-adjusted serum ferritin concentration was 89.59 μg/L (50.86, 123.05) in the iron supplementation group and 34.08 μg/L (14.58, 64.50) in the placebo group.

Table 3-4. Baseline hematological, nutrition and inflammation indicators of women included in the relative LTL analyses by supplement group.

<table>
<thead>
<tr>
<th></th>
<th>Iron</th>
<th>Placebo</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total enrolled, n (%)</td>
<td>190 (51)</td>
<td>186 (49)</td>
</tr>
<tr>
<td>Hematological indicators</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hb, g/L</td>
<td>116 ± 14</td>
<td>117 ± 13</td>
</tr>
<tr>
<td>Hepcidin, nmol/L</td>
<td>5.6 (1.9, 11.2)</td>
<td>5.5 (1.6, 10.8)</td>
</tr>
<tr>
<td>Hematocrit, %</td>
<td>36.0 ± 3.6</td>
<td>36.3 ± 3.2</td>
</tr>
<tr>
<td>MCV, fL</td>
<td>76.0 ± 10.4</td>
<td>77.5 ± 9.4</td>
</tr>
<tr>
<td>MCH, pg</td>
<td>24.4 ± 3.9</td>
<td>24.9 ± 3.5</td>
</tr>
<tr>
<td>RDW, %</td>
<td>14.2 (13.2, 15.9)</td>
<td>13.9 (13.0, 15.6)</td>
</tr>
<tr>
<td>Nutrition indicators</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Storage iron, ferritin(^a), μg/L</td>
<td>39.1 (18.0, 81.4)</td>
<td>37.1 (15.1, 61.0)</td>
</tr>
<tr>
<td>Tissue iron, sTfR, mg/L</td>
<td>6.0 (4.8, 8.3)</td>
<td>5.9 (4.7, 7.7)</td>
</tr>
<tr>
<td>TSAT, %</td>
<td>22.5 (14.0, 29.3)</td>
<td>21.3 (12.3, 28.9)</td>
</tr>
<tr>
<td>Inflammation markers</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CRP, mg/L</td>
<td>0.37 (0.18, 0.87)</td>
<td>0.43 (0.23, 1.03)</td>
</tr>
<tr>
<td>AGP, mg/L</td>
<td>0.55 (0.45, 0.72)</td>
<td>0.56 (0.46, 0.70)</td>
</tr>
</tbody>
</table>

Total \(n=376\). Values are mean ± SD, median (IQR), or \(n\) (%). Hb, hemoglobin; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; RDW, red cell distribution width; sTfR, soluble transferrin receptor; TSAT, transferrin saturation; CRP, C-reactive protein; AGP, \(\alpha\)-1-acid glycoprotein.

\(^a\)Thurnham et al.\(^{12}\) correction factors were used to correct values for inflammation.

The baseline prevalence of anemia, iron deficiency, genetic hemoglobin disorders and inflammation are outlined in **Table 3-5**. Baseline anemia prevalence was 56% \((n=210/376)\).

This was despite enrolling only women who were screened as having a hemoglobin concentration of ≤117 g/L, based on a capillary finger prick blood specimen measured by the HemoCue®. Baseline prevalence of iron deficiency was 22% \((n=84/375)\) based on a serum ferritin concentration of <15 μg/L and 24% \((n=90/375)\) based on a sTfR concentration of >8.3 mg/L.
A total of 74% \((n = 280/376)\) of women had some form of genetic hemoglobin disorder (either a hemoglobin variant and/or \(\alpha\)-thalassemia). The prevalence of an abnormal hemoglobin variant and \(\alpha\)-thalassemia was 57% \((n = 213/376)\) and 43% \((n = 159/374)\), respectively. The most common hemoglobin variant was hemoglobin E, with a prevalence of 52% \((n = 196/376)\). Of the women with a hemoglobin E variant, 80% \((n = 156/196)\) were heterozygous (hemoglobin E trait) and 20% \((n = 40/196)\) were homozygous (hemoglobin E disease). Among the women with \(\alpha\)-thalassemia, the most common trait was \(\alpha^{3.7}\)-thalassemia, which accounted for 57% \((n = 90/159)\) of \(\alpha\)-thalassemia cases.

**Table 3-5.** Prevalence rates of anemia, iron deficiency, genetic hemoglobin disorders and inflammation among women included in the relative LTL analyses by supplement group.

<table>
<thead>
<tr>
<th></th>
<th>Iron</th>
<th>Placebo</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total enrolled, (n) (%)</strong></td>
<td>190 (51)</td>
<td>186 (49)</td>
</tr>
<tr>
<td><strong>Anemia prevalence</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anemia, Hb &lt; 120 g/L</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IDA, ferritin&lt;sub&gt;a&lt;/sub&gt; &lt; 15 µg/L and Hb &lt; 120 g/L</td>
<td>114 (60)</td>
<td>96 (52)</td>
</tr>
<tr>
<td>IDA, sTfR &gt; 8.3 mg/L and Hb &lt; 120 g/L</td>
<td>33/189 (17)</td>
<td>38 (20)</td>
</tr>
<tr>
<td><strong>Iron deficiency prevalence</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ferritin&lt;sub&gt;a&lt;/sub&gt; &lt; 15 µg/L</td>
<td></td>
<td></td>
</tr>
<tr>
<td>sTfR &gt; 8.3 mg/L</td>
<td>38/189 (20)</td>
<td>46 (25)</td>
</tr>
<tr>
<td><strong>Genetic hemoglobin disorders</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Any (Hb variant or (\alpha)-thalassemia)</td>
<td>150 (79)</td>
<td>130 (70)</td>
</tr>
<tr>
<td>Hb variant (E, CS, H, Bart, or F)</td>
<td>115 (61)</td>
<td>98 (53)</td>
</tr>
<tr>
<td>(\alpha)-thalassemia mutation</td>
<td>83/189 (44)</td>
<td>76/185 (41)</td>
</tr>
<tr>
<td><strong>Inflammation</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acute inflammation, CRP &gt; 5 mg/L</td>
<td>9/189 (5)</td>
<td>4 (2)</td>
</tr>
<tr>
<td>Chronic inflammation, AGP &gt; 1 g/L</td>
<td>15/189 (8)</td>
<td>14 (8)</td>
</tr>
</tbody>
</table>

<sup>Total \(n = 376\). Values are \(n\) (%). Hb, hemoglobin; IDA, iron deficiency anemia; sTfR, soluble transferrin receptor; CRP, C-reactive protein; AGP, \(\alpha\)-1-acid glycoprotein.</sup>

<sup>aThurnham et al.\(^{32}\) correction factors were used to correct values for inflammation.</sup>
3.3.2 Variability in the Relative LTL Assay

The assay coefficient of variation (CV) was calculated as the $\frac{\text{SD} \times 100}{\text{mean}}$ of the T/S ratios. The CV for the average value of duplicate measurements of a positive IC across multiple runs is considered the inter-assay variability. Among all accepted runs included in this secondary analysis ($n = 20$), the inter-assay variability was 0.2% for both the long and short telomere ICs. The intra-assay CV, which is the CV for the average value of duplicate measurements of a positive IC across the same run, has been reported as 4.2–6.2% for the long telomere IC.\textsuperscript{112}

3.3.3 Unadjusted Absolute and Percent Change in Relative LTL After 12 Weeks

At baseline, the median (IQR) unadjusted relative LTL was 7.1 (6.5, 7.7) in the iron supplementation group and 7.1 (6.5, 7.8) in the placebo group. The median (IQR) unadjusted relative LTL at endline was 7.0 (6.4, 7.7) in the iron supplementation group and 7.1 (6.1, 7.8) in the placebo group. \textbf{Figure 3-2} and \textbf{Figure 3-3} show the unadjusted absolute and percent change in relative LTL after 12 weeks. The median (IQR) unadjusted absolute change in relative LTL after 12 weeks was $-0.2 (-0.4, 0.2)$ for women in the iron supplementation group and $-0.1 (-0.5, 0.3)$ for women in the placebo group. This translates to a median (IQR) unadjusted percent change of $-2.1% (-6.1, 3.3)$ in the iron supplementation group and $-1.4% (-5.9, 4.8)$ in the placebo group, signifying an overall decrease in relative LTL for both treatment groups.
Figure 3-2. The median (IQR) unadjusted absolute change in relative LTL.

LTL, leukocyte telomere length.
**Figure 3-3.** The median (IQR) unadjusted percent change in relative LTL.

LTL, leukocyte telomere length.
3.3.4 Adjusted Absolute and Percent Change in Relative LTL After 12 Weeks

For all women included in the relative LTL analyses ($n = 376$), iron supplementation was not significantly associated with an adjusted absolute or percent change in relative LTL after 12 weeks, compared to placebo ($\beta$-coefficient: $-0.04$ [95% CI: $-0.16, 0.08$]; $P = 0.50$ and $\beta$-coefficient: $-0.96$ [95% CI: $-2.69, 0.77$]; $P = 0.28$, respectively). Further, after restricting analyses to only adherent women ($n = 325$), there remained no significant association with iron supplementation with respect to both adjusted absolute ($\beta$-coefficient: $-0.01$ [95% CI: $-0.14, 0.12$]; $P = 0.86$) and percent change ($\beta$-coefficient: $-0.53$ [95% CI: $-2.41, 1.34$]; $P = 0.58$) in relative LTL after 12 weeks. Age was significantly associated with adjusted absolute and percent change in relative LTL for the models that included all ($P = 0.01$ and $P = 0.02$) and adherent women ($P = 0.01$ and $P = 0.01$). Similarly, baseline relative LTL was significantly associated with adjusted absolute and percent change in relative LTL for the models that included all ($P < 0.001$) and adherent women ($P < 0.001$). The coefficients and 95% CIs obtained from the models of adjusted absolute and percent change in relative LTL after 12 weeks are presented in Figure 3-4 and Figure 3-5, respectively.
**Figure 3-4.** The adjusted effect of iron or placebo on absolute change in relative LTL among women.

![Graph showing the adjusted effect of iron or placebo on absolute change in relative LTL among women.](image)

LTL, leukocyte telomere length.

**Figure 3-5.** The adjusted effect of iron or placebo on percent change in relative LTL among women.

![Graph showing the adjusted effect of iron or placebo on percent change in relative LTL among women.](image)

LTL, leukocyte telomere length.
3.3.5 The Potential Interaction Effect of Baseline Iron Status on Absolute and Percent Change in Relative LTL After 12 Weeks

For women included in the relative LTL analyses ($n=375$), baseline inflammation-adjusted serum ferritin concentration did not significantly modify the effect of iron supplementation on the adjusted absolute or percent change in relative LTL after 12 weeks ($\beta$-coefficient: $-0.0000003$ [95% CI: $-0.003, 0.003$]; $P=1.00$ and $\beta$-coefficient: $-0.0002$ [95% CI: $-0.04, 0.04$]; $P=0.99$, respectively). Further, after restricting analyses to only adherent women included in the relative LTL analyses ($n=324$), baseline inflammation-adjusted serum ferritin concentration did not significantly modify the effect of iron supplementation on the adjusted absolute or percent change in relative LTL after 12 weeks ($\beta$-coefficient: $0.0004$ [95% CI: $-0.003, 0.003$]; $P=0.82$ and $\beta$-coefficient: $-0.006$ [95% CI: $-0.04, 0.05$]; $P=0.80$, respectively). Due to a missing value for baseline serum ferritin concentration, $n=1$ specimen was excluded from the above interaction models. Age and baseline relative LTL were both significantly associated with adjusted absolute and percent change in relative LTL after 12 weeks in the above interaction models ($P<0.05$).

3.3.6 The Potential Interaction Effect of Having a Genetic Hemoglobin Disorder on Absolute and Percent Change in Relative LTL After 12 Weeks

For all women included in the relative LTL analyses ($n=376$), the presence of any genetic hemoglobin disorder variant did not significantly modify the effect of iron supplementation on the adjusted absolute or percent change in relative LTL after 12 weeks ($\beta$-coefficient: $-0.006$ [95% CI: $-0.04, 0.05$]; $P=0.80$, respectively).
[95% CI: −0.29, 0.28]; \( P = 0.97 \) and \( \beta \)-coefficient: −0.14 [95% CI: −4.24, 3.97]; \( P = 0.95 \), respectively). Further, after restricting analyses to only adherent women included in the relative LTL analyses \( (n = 325) \), the presence of any genetic hemoglobin disorder variant did not significantly modify the effect of iron supplementation on the adjusted absolute or percent change in relative LTL after 12 weeks (\( \beta \)-coefficient: −0.04 [95% CI: −0.35, 0.27]; \( P = 0.80 \) and \( \beta \)-coefficient: −0.54 [95% CI: −4.98, 3.90]; \( P = 0.81 \), respectively). Age and baseline relative LTL were both significantly associated with adjusted absolute and percent change in relative LTL after 12 weeks in the above interaction models \( (P < 0.05) \).

3.4 Buffy Coat mtDNA Content Analyses

3.4.1 Baseline Characteristics of Women Included in the Relative mtDNA Content Analyses

Baseline characteristics are presented in Table 3-6. A total of \( n = 370 \) specimens were included in the mtDNA content analyses; \( n = 1 \) baseline specimen was missing and \( n = 6 \) specimens failed to meet the QC criteria outlined in Section 2.2.2.1.3. The mean ± SD age at baseline was 31 ± 8 years and the mean ± SD household size was 4.7 ± 1.7 people. The majority of women were married (65%), 54% had completed primary school, and 37% had 1 or 2 children. Among women who had ≥ 1 child (72%), 77% of women reported previously taking any quantity and/or duration of iron folic acid supplements during their last pregnancy. At baseline, 17% and 31% of women were lactating and taking birth control, respectively.
Table 3-6. Baseline characteristics of women included in the mtDNA content analyses by supplement group.

<table>
<thead>
<tr>
<th></th>
<th>Iron</th>
<th>Placebo</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total enrolled, n (%)</td>
<td>186 (50)</td>
<td>184 (50)</td>
</tr>
<tr>
<td>Age, y (mean ± SD)</td>
<td>31 ± 8</td>
<td>30 ± 8</td>
</tr>
<tr>
<td>Household size (mean ± SD)</td>
<td>4.8 ± 1.7</td>
<td>4.7 ± 1.6</td>
</tr>
<tr>
<td>Marital status, n (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Single</td>
<td>46 (25)</td>
<td>47 (26)</td>
</tr>
<tr>
<td>Married</td>
<td>123 (66)</td>
<td>119 (65)</td>
</tr>
<tr>
<td>Widowed</td>
<td>10 (5)</td>
<td>6 (3)</td>
</tr>
<tr>
<td>Separated/divorced</td>
<td>7 (4)</td>
<td>12 (7)</td>
</tr>
<tr>
<td>Completed education, n (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>24 (13)</td>
<td>27 (15)</td>
</tr>
<tr>
<td>Primary (grades 1–5)</td>
<td>94 (51)</td>
<td>104 (57)</td>
</tr>
<tr>
<td>Lower secondary (grades 6–9)</td>
<td>63 (34)</td>
<td>42 (23)</td>
</tr>
<tr>
<td>Upper secondary (grades 10–12)</td>
<td>5 (3)</td>
<td>10 (5)</td>
</tr>
<tr>
<td>Higher education/university</td>
<td>0 (0)</td>
<td>1 (&lt;1)</td>
</tr>
<tr>
<td>Parity, n (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>51 (27)</td>
<td>54 (29)</td>
</tr>
<tr>
<td>1–2</td>
<td>67 (36)</td>
<td>70 (38)</td>
</tr>
<tr>
<td>3–4</td>
<td>51 (27)</td>
<td>42 (23)</td>
</tr>
<tr>
<td>≥5</td>
<td>17 (9)</td>
<td>18 (10)</td>
</tr>
<tr>
<td>Women with parity ≥ 1 who reported to receive, n (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFA during last pregnancy</td>
<td>105/135 (78)</td>
<td>100/130 (77)</td>
</tr>
<tr>
<td>Deworming during last pregnancy</td>
<td>72/135 (53)</td>
<td>72/130 (55)</td>
</tr>
<tr>
<td>Lactating, n (%)</td>
<td>28 (15)</td>
<td>35 (19)</td>
</tr>
<tr>
<td>Taking birth control, n (%)</td>
<td>51 (27)</td>
<td>62 (34)</td>
</tr>
</tbody>
</table>

Total n = 370. Values are mean ± SD or n (%). IFA, iron and folic acid supplementation.

Table 3-7 presents baseline hematological, nutrition and inflammation indicators. Not presented in the table are the median (IQR) endline values for serum ferritin concentration and TSAT. At endline, after 12 weeks of the intervention, the median (IQR) TSAT was 27.93% (20.40, 36.99) in the iron supplementation group and 20.09% (11.42, 28.96) in the placebo group. Further, the median (IQR) endline inflammation-adjusted serum ferritin concentration was 86.22 μg/L (50.86, 123.05) in the iron supplementation group and 33.92 μg/L (14.39, 63.51) in the placebo group.
Table 3-7. Baseline hematological, nutrition and inflammation indicators of women included in the mtDNA content analyses by supplement group.

<table>
<thead>
<tr>
<th></th>
<th>Iron</th>
<th>Placebo</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total enrolled, n (%)</td>
<td>186 (50)</td>
<td>184 (50)</td>
</tr>
<tr>
<td>Hematological indicators</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hb, g/L</td>
<td>116 ± 14</td>
<td>117 ± 13</td>
</tr>
<tr>
<td>Hepcidin, nmol/L</td>
<td>6.0 (1.9, 11.2)</td>
<td>5.5 (1.5, 10.8)</td>
</tr>
<tr>
<td>Hematocrit, %</td>
<td>36.0 ± 3.7</td>
<td>36.4 ± 3.2</td>
</tr>
<tr>
<td>MCV, fL</td>
<td>75.9 ± 10.5</td>
<td>77.7 ± 9.3</td>
</tr>
<tr>
<td>MCH, μg</td>
<td>24.4 ± 3.9</td>
<td>25.0 ± 3.5</td>
</tr>
<tr>
<td>RDW, %</td>
<td>14.2 (13.2, 15.9)</td>
<td>13.8 (13.0, 15.6)</td>
</tr>
<tr>
<td>Nutrition indicators</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Storage iron, ferritin*, μg/L</td>
<td>39.5 (18.8, 81.4)</td>
<td>36.8 (14.8, 62.4)</td>
</tr>
<tr>
<td>Tissue iron, sTfR, mg/L</td>
<td>5.9 (4.8, 8.0)</td>
<td>5.9 (4.6, 7.8)</td>
</tr>
<tr>
<td>TSAT, %</td>
<td>22.5 (14.4, 29.3)</td>
<td>21.3 (12.1, 29.0)</td>
</tr>
<tr>
<td>Inflammation markers</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CRP, mg/L</td>
<td>0.37 (0.18, 0.87)</td>
<td>0.42 (0.22, 1.05)</td>
</tr>
<tr>
<td>AGP, mg/L</td>
<td>0.55 (0.45, 0.72)</td>
<td>0.56 (0.46, 0.70)</td>
</tr>
</tbody>
</table>

Total n = 370. Values are mean ± SD, median (IQR), or n (%). Hb, hemoglobin; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; RDW, red cell distribution width; sTfR, soluble transferrin receptor; CRP, C-reactive protein; AGP, α-1-acid glycoprotein.

*Thurnham et al. correction factors were used to correct values for inflammation.

The baseline prevalence of anemia, iron deficiency, genetic hemoglobin disorders and inflammation are outlined in

Table 3-8. Baseline anemia prevalence was 55% (n = 205/370). Baseline prevalence of iron deficiency, based on a serum ferritin concentration of <15 μg/L and sTfR concentration of >8.3 mg/L, was 22% (n = 83/369) and 24% (n = 87/369). A total of 75% (n = 276/370) of women had some form of genetic hemoglobin disorder (either a hemoglobin variant and/or α-thalassemia).

The prevalence of an abnormal hemoglobin variant and α-thalassemia was 56% (n = 209/370) and 43% (n = 158/368), respectively. The most common hemoglobin variant was hemoglobin E, with a prevalence of 52% (n = 192/370). Of the women with a hemoglobin E variant, 80% (n = 154/192) were heterozygous (i.e. had hemoglobin E trait) and 20% (n = 38/192) were
homozygous (i.e. had hemoglobin E disease). Among the women with α-thalassemia, the most common trait was α$^{3.7}$-thalassemia, which accounted for 56% ($n = 89/158$) of α-thalassemia cases.

<table>
<thead>
<tr>
<th>Table 3-8. Prevalence rates of anemia, iron deficiency, genetic hemoglobin disorders and inflammation among women included in the mtDNA content analyses by supplement group.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total enrolled, $n$ (%)</td>
</tr>
<tr>
<td>Anemia prevalence</td>
</tr>
<tr>
<td>Anemia, Hb $&lt; 120$ g/L</td>
</tr>
<tr>
<td>IDA, ferritin$^a$ $&lt; 15$ μg/L and Hb $&lt; 120$ g/L</td>
</tr>
<tr>
<td>IDA, sTfR $&gt; 8.3$ mg/L and Hb $&lt; 120$ g/L</td>
</tr>
<tr>
<td>Iron deficiency prevalence</td>
</tr>
<tr>
<td>Ferritin$^a$ $&lt; 15$ μg/L</td>
</tr>
<tr>
<td>sTfR $&gt; 8.3$ mg/L</td>
</tr>
<tr>
<td>Genetic hemoglobin disorders</td>
</tr>
<tr>
<td>Any (Hb variant or α-thalassemia)</td>
</tr>
<tr>
<td>Hb variant (E, CS, H, Bart, or F)</td>
</tr>
<tr>
<td>α-thalassemia variant</td>
</tr>
<tr>
<td>Inflammation</td>
</tr>
<tr>
<td>Acute inflammation, CRP $&gt; 5$ mg/L</td>
</tr>
<tr>
<td>Chronic inflammation, AGP $&gt; 1$ g/L</td>
</tr>
</tbody>
</table>

Total $n = 370$. Values are $n$ (%). Hb, hemoglobin; IDA, iron deficiency anemia; sTfR, soluble transferrin receptor; CRP, C-reactive protein; AGP, α-1-acid glycoprotein.

$^a$Thurnham et al.$^{,2}$ correction factors were used to correct values for inflammation.

### 3.4.2 Variability in the mtDNA Content Assay

The inter-assay CV was calculated as described in Section 3.3.2. Among all accepted runs ($n = 25$), the inter-assay variability was 5.7% for the high mtDNA positive control and 9.8% for the low mtDNA positive control. The intra-assay CV has been reported as 4.3–7.9%.$^{102}$
3.4.3 Unadjusted Absolute and Percent Change in mtDNA Content After 12 Weeks

At baseline, the unadjusted median (IQR) unadjusted mtDNA content was 95 (73, 120) in the iron supplementation group and 90 (65, 115) in the placebo group. The median (IQR) unadjusted mtDNA content at endline was 103 (83, 122) in the iron supplementation group and 105 (78, 135) in the placebo group. Figure 3-6 and Figure 3-7 represent the unadjusted absolute and percent change in mtDNA content after 12 weeks. The median (IQR) unadjusted absolute change in mtDNA content after 12 weeks was 3 (−18, 28) in the iron supplementation group and 11 (−7, 37) in the placebo group. This translates to a median (IQR) unadjusted percent change of 6% (−18, 35) in the iron supplementation group and 12% (−11, 47) in the placebo group, indicating an increase in mtDNA content in both treatment groups.
Figure 3-6. The median (IQR) unadjusted absolute change in mtDNA content.

MtDNA, mitochondrial DNA.
Figure 3-7. The median (IQR) unadjusted percent change in mtDNA content.

mtDNA, mitochondrial DNA.
For all women included in the mtDNA analyses (n = 370), iron supplementation was associated with a significantly smaller adjusted absolute and percent increase in mtDNA content after 12 weeks, compared to placebo (β-coefficient: −11 [95% CI: −12, −2]; P = 0.02 and β-coefficient: −11 [95% CI: −20, −1]; P = 0.02, respectively). Further, after restricting analyses to only adherent women (n = 321), iron supplementation was associated with a significantly smaller adjusted absolute (β-coefficient: −12 [95% CI: −22, −3]; P = 0.01) and percent (β-coefficient: −12 [95% CI: −22, −2]; P = 0.02) increase in mtDNA content after 12 weeks, compared to placebo. Age was only significantly associated with adjusted absolute change in mtDNA content after 12 weeks in the interaction model including all women (P = 0.03). The coefficients and 95% CIs obtained from the models of adjusted absolute and percent change in mtDNA content after 12 weeks are presented in Figure 3-8 and Figure 3-9.
**Figure 3-8.** The adjusted effect of iron or placebo on absolute change in mtDNA content among women.

**Figure 3-9.** The adjusted effect of iron or placebo on percent change in mtDNA content among women.

mtDNA, mitochondrial DNA.
3.4.5 The Potential Interaction Effect of Baseline Iron Status on Absolute and Percent Change in mtDNA Content After 12 Weeks

For women included in the mtDNA content analyses (n = 369), baseline inflammation-adjusted serum ferritin concentration did not significantly modify the effect of iron supplementation on the adjusted absolute or percent change in mtDNA content after 12 weeks (β-coefficient: 0 [95% CI: 0, 0]; P = 0.84 and β-coefficient: 0 [95% CI: 0, 0]; P = 0.71, respectively). This remained true for the adjusted absolute or percent change in relative mtDNA content after further restricting analyses to only adherent women included in the relative mtDNA content analyses (n = 320) (β-coefficient: 0 [95% CI: 0, 0]; P = 0.72 and β-coefficient: 0 [95% CI: 0, 0]; P = 0.68, respectively).

Due to a missing value for baseline serum ferritin concentration, n = 1 specimen was excluded from the above interaction models. Age was adjusted for, however, it was not significantly associated with either measure.

3.4.6 The Potential Interaction Effect of Having a Genetic Hemoglobin Disorder on Absolute and Percent Change in mtDNA Content After 12 Weeks

For all women included in the mtDNA content analyses (n = 370), the presence of any genetic hemoglobin disorder variant did not significantly modify the effect of iron supplementation on the adjusted absolute or percent change in mtDNA content after 12 weeks (β-coefficient: 18 [95% CI: −3, 39]; P = 0.09 and β-coefficient: −15 [95% CI: −7, 37]; P = 0.19, respectively). Further, after restricting analyses to only adherent women (n = 321), the presence of any genetic hemoglobin disorder variant did not significantly modify the effect of iron supplementation on
the adjusted absolute or percent change in mtDNA content after 12 weeks (β-coefficient: 22 [95% CI: −1, 45]; \( P = 0.07 \) and β-coefficient: 20 [95% CI: −5, 44]; \( P = 0.11 \), respectively). Age was adjusted for; however, it was only significantly associated with adjusted absolute change in mtDNA content after 12 weeks in the interaction model including all women \((n = 370)\) \( P = 0.02 \).

3.5 Specimen Quality and Assay Performance

The quality of the specimens and the performance of the assays was confirmed by the following:

In theory, the average albumin copy number (i.e. the copy number of the nuclear gene) for each specimen should be similar between the relative LTL and mtDNA content assays, as the dilutions used in the assays were sourced from the same DNA extract. Indeed, a positive correlation was observed between the average albumin copy number from the two assays for both baseline \((r = 0.89, \text{spearman’s correlation, } P < 0.001)\) and endline \((r = 0.89, \text{spearman’s correlation, } P < 0.001)\) measures. Since the relative LTL assays were run in October 2017, and the mtDNA assays were run between May–December 2018, this also positively confirms that minimal nDNA degradation occurred.

If the blood was always processed in exactly the same manner, the WBC (white blood cell) count of each participant should be correlated with the average albumin copy number of the buffy coat sample for that participant, as albumin is a surrogate indicator for cell count. For the relative LTL assays, average albumin copy number and WBC count were weakly correlated at baseline \((r = 0.19, \text{spearman’s correlation, } P < 0.001)\) and at endline \((r = 0.27, \text{spearman’s correlation } P < \)
For the mtDNA content assays, albumin concentrations and WBC count were also weakly correlated at baseline ($r=0.21$, spearman’s correlation, $P<0.001$) and at endline ($r=0.29$, spearman’s correlation, $P<0.001$). One reason the correlation may have been weaker than expected in my study is because of differences in processing of the buffy coat. Another potential reason may be inter-blood tube differences, as the albumin concentration was measured in extracted DNA that was obtained from a different blood specimen than the WBC count (different vacutainers were used to collect blood for the buffy coat and for the CBC count). Some inherent variation between albumin concentration and WBC count in these two blood specimens is expected.

I also investigated the correlation between WBC count and both the relative LTL (T/S ratio) and the mtDNA content (mtDNA/nDNA ratio). For the relative LTL assays, WBC count and relative LTL (T/S ratio) were weakly correlated at baseline ($r=0.13$, pearson’s correlation, $P<0.007$) and at endline ($r=0.17$, pearson’s correlation, $P<0.001$). Similarly, for the mtDNA content assays, WBC count and mtDNA content (mtDNA/nDNA ratio) were weakly correlated at baseline ($r=-0.21$, pearson’s correlation, $P<0.001$) and also at endline ($r=-0.31$, pearson’s correlation, $P<0.001$). These findings suggest that the WBC count of a specimen did not affect the ratio of a specimen.

Overall, I am confident in the specimen quality and the performance of the assays, as all included specimens and assays passed QC criteria and had relatively low inter- and intra-assay variability. Further, according to the calculated correlations involving average albumin copy number and WBC count, no issues with specimen quality or assay performance were revealed.
Chapter 4: Discussion

In this chapter, I discuss the key findings of my research. I will compare my key findings with the published literature, as well as discuss the strengths and limitations of the research methods and design. Following these sections is a summary of the significance of this research and its contribution to the published literature, finishing with potential future research directions.

4.1 Effect of Iron Supplementation on Relative LTL

The first objective of this research was to assess relative LTL change in Cambodian women who received 60 mg of elemental iron as ferrous sulphate \( (n = 191) \) or a placebo \( (n = 186) \) for 12 weeks. I hypothesized that women in the iron supplementation group would have a greater decrease in relative LTL after 12 weeks, compared to women in the placebo group. This hypothesis was based on numerous observational studies that reported inverse associations between iron status or iron intake and relative LTL.\(^{81-85}\) However, my findings did not show any association as there was almost no change in relative LTL among the iron and placebo treatment groups after 12 weeks. There are two possible interpretations of this finding: relative LTL is not affected by or not a suitable biomarker for this iron supplementation regime.

There are multiple reasons that can explain discrepancy between my results and the published literature, including the fact that the baseline and endline iron levels of the women in my study were low compared to those of the participants in previously published observational studies that investigated the association between iron status and relative LTL.\(^{81-84}\) Thus, it is plausible that 60
mg/d of elemental iron as ferrous sulphate for 12 weeks is not a high enough dose of iron to elucidate an effect on relative LTL. Past research has demonstrated that iron supplementation of similar doses and forms has the potential to elicit NTBI production and biomarkers of oxidative stress among iron-replete and iron-deplete women with unsaturated TSAT at durations shorter than 12 weeks. Additional, previous work in animal models has indicated that iron exposure leads to an increase in oxidative stress in a dose-dependent manner. Therefore, it is also plausible that relative LTL is not a suitable biomarker for this study.

There are no known published studies which have investigated the effect of 60 mg of elemental iron for 12 weeks on the change in relative LTL over a defined time period; therefore, the duration of exposure to iron supplements which may lead to the damage of telomeric DNA is not known. The previous observational studies were observational and cross-sectional in design, therefore they were subject to the typical biases of such studies. In contrast, specimens in my study were obtained from a prospective randomized controlled trial, with both baseline and endline specimens available for analysis. The study design of this randomized controlled trial is more rigorous to inform a change in relative LTL that is a result of iron supplementation, as compared to the design of a cross-sectional study that can only infer association (not causation). Thus, it is most likely that 60 mg/d of elemental iron as ferrous sulphate does not affect relative LTL. However, it is well documented that telomere attrition typically occurs gradually in adults (20-30 base pairs per year), although there is high inter-individual variability. It is possible that supplementation protocols that are repeated or are longer in duration than what was undertaken in my study could elucidate a decrease in relative LTL, given that they may cause increased iron exposure and oxidative stress.
The null results of the effect of iron on relative LTL observed in my study are also in opposition with one published observational study that reported an inverse association between iron intake and relative LTL in a cohort of American women aged 35–74 years who were sisters of subjects with breast cancer. Xu et al. found that women who were iron supplement users \( (n = 41) \) had significantly shorter relative LTL compared to nonusers \( (n = 527) \) \( (P = 0.007) \); however, no information was provided on the duration of the exposure to iron supplements among women in the study. Further, no information regarding the dose of iron, adherence to iron, or type of iron supplements was provided, which are major limitations of the study, as higher doses and higher adherence may cause increased iron exposure and more oxidative stress. Further, different types of iron supplements vary in bioavailability and in the magnitude of oxidative stress produced.

On another note, the findings of a study in an experimental animal model by Brown et al. suggest that telomerase activity increases in hepatocytes in response to iron exposure. However, it is unlikely that increased telomerase activity was the reason no change was observed in relative LTL among women in the iron supplementation group. This is because telomerase activity in leukocytes is typically non-existent or only detectable at low levels.

It is important to note that there are numerous other factors that could lead to the discrepancy in findings between the published literature and my study. One factor is an individual’s diet, as certain dietary macronutrients and micronutrients have been shown to be associated with relative LTL. For example, higher vitamin intakes (e.g. vitamin C and folate) are reported to be
positively associated with relative LTL.\textsuperscript{86,88} Unfortunately, no dietary data was collected in the randomized controlled trial, so I could not assess or control for dietary intake. Although, as previously noted, the Cambodian diet is limited in fruit and vegetable intake,\textsuperscript{32} so it is unlikely that high vitamin intake would be a confounding factor among the women included in my study. Other factors include body mass index (BMI), which has been reported to be inversely associated with telomere length, and cigarette smoking, which has been reported to be inversely associated with telomere length in a dose-dependent manner.\textsuperscript{119} Cambodian women in my study were not suspected to have high BMIs or smoke cigarettes,\textsuperscript{120,121} however, this data was not collected. Notwithstanding this, due to the randomized controlled design of the trial, it is expected that any of these potentially confounding factors would be equally weighted among both the iron and placebo treatment groups. Thus, it is expected that the confounding effect of these other variables on the effect of the intervention on change in relative LTL would be minimal.

4.2 Effect of Iron Supplementation on mtDNA Content

The second objective of this research was to assess mtDNA content change in the same study participants. I hypothesized that women in the iron supplementation group would have a greater change (decrease or increase) in mtDNA content after 12 weeks, compared to women in the placebo group. This hypothesis was based on the theory that mtDNA damage, which can be caused by oxidative stress, can lead to mitophagy or mitochondrial biogenesis, due to the nature of mitochondrial homeostasis.\textsuperscript{100} In contrast to my hypothesis, I found that iron supplementation was associated with a significantly smaller adjusted absolute and percent increase in mtDNA after 12 weeks, compared to placebo. Therefore, there was a significantly greater increase in
mtDNA content among women in the placebo group. This was an unexpected finding. I speculate that the increase in mtDNA content among women in the placebo group may reflect an increase in mitochondrial biogenesis, likely in response to mtDNA damage.\textsuperscript{100} Damage to mtDNA, and thus alterations in mtDNA content, may occur among the women included in my study, due to factors such as poor air quality. The WHO reports that Cambodia has poor air quality, with fine particulate matter (PM2.5) exceeding a yearly average of 10 μg/m³.\textsuperscript{122} Levels of PM2.5 have been associated with alterations in mtDNA content among non-smoking Chinese women, and has also been recognized as a potential mitochondrial toxicant.\textsuperscript{123,124} Given that the only difference between the treatment groups was the iron supplementation, it can be assumed that women in the iron group would also exhibit a similar increase in mtDNA content if no iron supplementation was provided. However, women in the iron supplementation group exhibited only a small increase in mtDNA content after 12 weeks. The speculation that iron supplementation caused altered mitochondrial homeostasis is difficult to ascertain. It is plausible that oxidative stress caused by iron supplementation resulted in mitophagy (characterized by decreased mtDNA content), which balanced out a baseline state of mitochondrial biogenesis (characterized by increased mtDNA content) caused by other factors (i.e. PM2.5 levels), resulting in what appears as a smaller change in mtDNA content, compared to placebo, after 12 weeks.\textsuperscript{100} It is also plausible that oxidative stress caused by iron supplementation halted the baseline state of mitochondrial biogenesis (characterized by increased mtDNA content), resulting in a smaller change in mtDNA content, compared to placebo, after 12 weeks.\textsuperscript{100} Of important note, it is unlikely that iron was providing a protective effect against some external environmental factor, given its pro-oxidant nature.\textsuperscript{20}
My study findings are in contrast to three of the reported observational studies that assessed the association between iron status or iron exposure and mtDNA content. This may be due to a number of reasons. In one observational study, Liu et al. investigated the association between factors that are associated with oxidative stress (plasma ferritin) and mtDNA copy number (mtDNA content) in leukocytes from healthy adults aged 25–80 years, who were recruited from a health clinic in Taiwan. A significant association between plasma ferritin concentration and mtDNA content \( (P = 0.049) \) was observed, suggesting that higher biochemical iron status is associated with an increase in mtDNA content. Another observational study by Lal et al., compared the mtDNA content in leukocytes between subjects with thalassemia aged 4–53 years, recruited from an American thalassemia clinic, and healthy adult controls aged 19–46 years. They found that subjects with thalassemia who received a blood-transfusion had a higher mtDNA content compared to the healthy control group \( (P = 0.026) \). The findings from these aforementioned observational studies suggests that increased iron status or iron exposure is associated with an increased mtDNA content. Baseline and endline iron status was much lower among the women included in the mtDNA content analyses of my study, compared to the baseline iron status of the Liu et al. study, in which mean ± SD plasma ferritin concentrations were 163.1 ± 49.7 μg/L, and to the Lal et al. study, in which 76% of subjects with thalassemia had a serum ferritin concentration > 1000 μg/L. Perhaps higher iron status affects mitochondrial homeostasis in such a way that causes an increase in mtDNA content, whereas a comparably lower iron status results in mitophagy or the halting of mitochondrial biogenesis.

It is important to note that the Lal et al. study included subjects with thalassemia, a genetic disorder associated with altered iron metabolism, iron overload, and other serious complications that limits the ability to compare findings with my studied population.
Further, the previously published observational studies were cross-sectional in design.\textsuperscript{103,104} Thus, the prospective trial design undertaken in my study is more rigorous to capture the change in mtDNA content over time, as it captures the rapid and dynamic nature of changes in mtDNA.\textsuperscript{100} It is possible that iron-induced mtDNA damage could, at first, result in mitophagy or halting of mitochondrial biogenesis, but then over time could lead to mitochondrial biogenesis as the mitochondria try to compensate for the loss of healthy mitochondria.\textsuperscript{100}

The findings from the Lal et al. study may also differ from the findings of my study due to confounding factors. Lal et al. did not have an age-matched control group, nor did they adjust for age when comparing the mean mtDNA content between the thalassemic subject group and the control group.\textsuperscript{104} The mean mtDNA content could have been higher in the thalassemic patient group, compared to the control group, as the thalassemic patient group contained younger individuals and mtDNA content is reported to be inversely associated with age.\textsuperscript{105} A two-sample \( t \) test determined that there was no significant difference in mean age between women in the iron \((n = 186)\) and placebo \((n = 184)\) groups included in the mtDNA analysis in my study. In addition, I adjusted for age in all models. This could explain why Kim et al. found no significant association between serum ferritin concentration and mtDNA content (\(\beta\)-coefficient: \(-0.056, P = 0.06)\) among \(n = 129\) healthy Korean women aged > 60 years, as the model was adjusted for multiple factors (e.g. age) that could affect mtDNA content.\textsuperscript{106}

A number of studies in experimental animal models have suggested that exposure to iron results in a decrease in mtDNA content in cardiac tissue.\textsuperscript{107,109} It is difficult to relate results from the
animal models to results from the human studies, particularly since the iron exposure was much higher in the animal models, compared to the dose of elemental iron provided in my study. For example, Gao et al. used 10 mg/d of elemental iron for mice weighing approximately 0.02 kg, which translates to a dose of 500 mg/kg/d of elemental iron. Assuming that the average weight of a Cambodian woman is approximately 52 kg, 60 mg/d of elemental iron only translates to a dose of 1.15 mg/kg/d. Thus, iron-induced changes in mtDNA content may have a more rapid-onset or greater magnitude in animal models, as compared to a longer-onset or lower magnitude in human populations. It is plausible that oxidative stress caused by iron supplementation could result in mitophagy (characterized by decreased mtDNA content), yet the 12 week duration of my study may not have been sufficient enough time to observe a decrease in mtDNA content of great enough magnitude to outweigh the baseline state of mitochondrial biogenesis (characterized by increased mtDNA content).

In summary, assuming that mtDNA content was increasing at baseline in both treatment groups, there are two most likely scenarios to explain the findings of mtDNA content in my study. First, iron supplementation resulted in mitophagy (characterized by decreased mtDNA content), which balanced out a baseline state of mitochondrial biogenesis (characterized by increased mtDNA content) caused by other factors, resulting in what appears smaller change in mtDNA content, compared to the placebo group, after 12 weeks. Second, the alternative explanation is that iron supplementation halted the baseline state of mitochondrial biogenesis (characterized by increased mtDNA content), resulting in smaller change in mtDNA content, compared to the placebo group, after 12 weeks. More research is needed to understand the effect of iron supplementation on mitochondrial homeostasis.
4.3 Interaction of Baseline Iron Status or Genetic Hemoglobin Disorders and Iron Supplementation on Relative LTL and mtDNA Content After 12 Weeks

I also assessed the potential interaction between baseline iron status (as baseline inflammation-adjusted serum ferritin concentration) and iron supplementation, or the presence of a genetic hemoglobin disorder and iron supplementation on the change in relative LTL or the change in mtDNA content after 12 weeks.

I hypothesized that among women in the iron supplementation group, those with higher baseline iron status would have a greater decrease in relative LTL or a greater change (decrease or increase) in mtDNA content after 12 weeks, as compared to women with lower baseline iron status. However, I found that no significant interaction effects were observed in any of the models, indicating that baseline iron status did not modify the effect of iron supplementation on change in relative LTL or change in mtDNA content. This finding is in contrast to the published literature that iron status is inversely associated with relative LTL,82,83,86 as well as the findings from Liu et al.,103 who reported a positive association between iron status and mtDNA content. The discrepancies in findings may be due to the differences in iron status of the participants included in the aforementioned studies and this study, as explained in Section 4.1 and 4.2. Thus, the baseline iron status’ among women in the iron supplementation group may not have been high enough to modify the effect of iron supplementation on change in relative LTL or change in mtDNA content.
Second, I hypothesized that among women in the iron supplementation group, those with a genetic hemoglobin disorder would have a greater decrease in relative LTL or a greater change (decrease or increase) in mtDNA content after 12 weeks, as compared to women with no genetic hemoglobin disorder. However, no significant interaction effects were observed in any of the models, indicating that the presence of a genetic hemoglobin disorder did not modify the effect of iron supplementation on change in relative LTL or change in mtDNA content. While studies among Cambodian and Thai women support that HbE homozygosity (i.e. HbE disease) and α-thalassemia heterozygosity are associated with higher concentrations of serum ferritin (i.e. saturated iron stores),9,18 the concentrations among the women included in my study were still well below the levels reported in individuals in the previously mentioned cross-sectional studies that investigated the association between iron status and relative LTL.81–83 Thus, the serum ferritin concentrations associated with the genetic hemoglobin disorders common among the women included in my study (i.e. HbE disease, α-thalassemia) may not have been high enough to modify the effect of iron supplementation on change in relative LTL or change in mtDNA content.

### 4.4 Strengths and Limitations of the Study

#### 4.4.1 Strengths

Strengths of this study include the rigorous design of the double-blind randomized controlled trial from which the specimens were obtained. Further, adherence and retention in the trial was high. Women in the iron supplementation group received 60 mg/d of elemental iron as ferrous
sulphate, a dose of iron that is in accordance with the WHO global policy on untargeted iron supplementation. For this reason, the findings in my study are relevant to global policy among countries that are currently or considering implementing this supplementation policy.

In addition, the MMqPCR assay used in my study for the relative LTL and mtDNA content analyses is a cost-effective and high-throughput method, that has been optimized in the Côté Lab in the Department of Pathology and Laboratory Medicine at the University of British Columbia. This method has been validated against other methods used to measure telomere length. A study conducted by Hsieh et al. in the Côté Lab found that telomere lengths measured in lymphocyte and granulocyte subsets from cord blood specimens by flow-FISH and by MMqPCR were highly correlated ($R^2 = 0.81$ and $R^2 = 0.71$, $P < 0.001$).\textsuperscript{112} Telomere lengths measured in placenta specimens and cultured HT-29 cells by TRF and MMqPCR were also highly correlated ($R^2 = 0.88$, $P < 0.001$).\textsuperscript{112} The MMqPCR assay has also been validated against the conventional monoplex qPCR assay. In addition, a moderate correlation has been reported between relative telomere length ($R^2 = 0.67$, $P < 0.001$) measured by monoplex qPCR and MMqPCR in dried blood spot and cord blood specimens.\textsuperscript{112} Similarly, a high correlation has been reported between mtDNA content ($r > 0.98$, $P < 0.001$) measured by monoplex qPCR and MMqPCR.\textsuperscript{102}

### 4.4.2 Limitations

Due to the exploratory nature of my study, certain well-established biomarkers of potential risk (e.g. TBARS, MDA, fecal ROS, gut pathogens) could not be assessed as different specimen types or specimen preparations would have been needed at the time of collection. Thus, my study
was limited to the assessment of biomarkers of potential risk that could be assessed using the specimens we had available (i.e. buffy coat). Another limitation of my study was the inherent lack of information regarding certain variables of interest that have been reported to affect LTL and leukocyte mtDNA content, such as BMI and cigarette smoking.119,125

The trial included a short supplementation period of 12 weeks, as the primary objective of the trial was to assess the effect of iron on hemoglobin. While 12 weeks is the approximate time required for erythrocyte turnover, it is likely not a sufficient time period to observe a change in relative LTL. It is difficult to determine how long a study may need to be in order to observe a change in relative LTL, as the published literature currently only consists of cross-sectional studies among humans. However, it is likely that a supplementation duration of one year or 12 weeks of supplementation over a number of years, may be needed to assess the effect of oral iron supplementation on change in relative LTL. Another limitation is the lack of published literature on the effect of iron supplementation on mtDNA homeostasis in humans. More studies are needed to understand the biological pathways and potential consequences of daily oral iron supplementation.

4.5 Research Significance

This is the first study to explore the potential risk of daily oral iron supplementation with 60 mg of elemental iron for 12 weeks by measuring biomarkers of cellular damage (i.e. relative LTL and mtDNA content) among non-pregnant Cambodian women. Recent surveys have reported a low prevalence of iron deficiency among non-pregnant Cambodian women, suggesting that iron
deficiency is not a major cause of the high prevalence of anemia in the region.\textsuperscript{7–10} Both efficacy and potential risk of untargeted daily oral iron supplementation programs should be established before recommendations should be made in Cambodia, or globally. This is particularly the case for iron supplementation, as excess exposure to iron may cause harm.

### 4.6 Future Research Directions

The current WHO guideline recommends daily oral iron supplementation for 12 consecutive weeks in a year during a woman’s reproductive years (15–49 years). Therefore, future studies could assess the change in relative LTL and the change in mtDNA content over the course of multiple, repeated exposures to oral iron supplementation following these guidelines. This would allow for better understanding of the long-term impacts of repeated oral iron supplementation on these biomarkers. Future studies could also assess the change in mtDNA content over shorter intervals of an oral iron supplementation period, in order to better understand the effect of oral iron supplementation on mitochondrial homeostasis.

Future studies could be designed to directly measure biomarkers of oxidative stress, as opposed to cellular damage, in response daily oral iron supplementation among Cambodian women. For example, markers of lipid peroxidation (e.g. MDA, TBARS) have been significantly positively associated with oral iron supplementation and plasma ferritin concentration.\textsuperscript{71,103} These biomarkers are typically measured in plasma, serum, or urine specimens. Although plasma and serum specimens were available for this study, their storage time made TBARS and MDA measurement unrealistic as increased storage time can falsely increase these biomarkers.\textsuperscript{126}
While these biomarkers of lipid peroxidation are accepted as indicators of oxidative stress, there is a general consensus in the literature that a panel of multiple biomarkers of oxidative stress is preferred. Measurement of multiple biomarkers would allow for a more comprehensive analysis of oxidative stress, as well as aid in a better understanding of the current published literature.

Additionally, future studies could also assess biomarkers of oxidative stress in fecal matter and/or altered gut microbiome in response to oral iron supplementation. For example, Orozco et al. and Lund et al. both demonstrated that the generation of ROS and free radicals in fecal matter were significantly increased after oral iron supplementation. Given that Lund et al. used a dose of 19 mg/d of elemental iron as ferrous sulphate, it is likely that daily oral iron supplementation with 60 mg elemental iron as ferrous sulphate for 12 weeks would also generate free radicals in fecal matter. However, this has not yet been demonstrated in the published literature. Further, iron that is not absorbed into the enterocyte can provide a source of iron to gut pathogens, which may support the virulence and colonization of these pathogens. A review of randomized controlled trials that investigated the effects of micronutrient powders on the gut microbiome in infants and children reported that iron-containing micronutrient powders decreased the number of beneficial bacteria and increased the number of gut pathogens. The typical dose of iron within a micronutrient powder for children is 12.5 mg of elemental iron, which is much lower than the 60 mg of elemental iron recommended to non-pregnant women of reproductive age by the WHO. Thus, there is strong potential for an iron supplement in a dose of 60 mg to elucidate similar adverse effects on the gut. Currently, there is no published literature
regarding the effect of 60 mg of elemental iron for 12 weeks on the gut microbiome. Thus, future research in this area is warranted.

Lastly, more research needs to be conducted to determine the efficacy and safety of alternative doses and forms of iron supplements, such as lower doses of ferrous sulphate and/or other forms of iron (e.g. NaFeEDTA and IPM). In a study by Schumann et al., it was demonstrated that 100 mg of elemental iron as ferrous sulphate resulted in an increase in serum NTBI concentration that was significantly greater than the same dose of NaFeEDTA and IPM ($P < 0.001$).\textsuperscript{63} Although the clinical significance of an elevated NTBI remains unclear, the accumulation of NTBI has the potential to be harmful as it can catalyze the formation of ROS and lead to oxidative stress-induced damage within the body.\textsuperscript{19–23} Thus, future research is needed to assess the bioavailability of different forms of iron, as the bioavailability is one factor that determines the risk of excess iron accumulation within the body.
Chapter 5: Conclusion

The current WHO global policy recommends untargeted daily oral iron supplementation with 60 mg of elemental iron for three consecutive months per year for non-pregnant women of reproductive age in regions where anemia prevalence is ≥40%. One such region is Cambodia, where the estimated prevalence of anemia is 43% among non-pregnant women of reproductive age. However, recent reports have suggested that the prevalence of iron deficiency is ≤10% among non-pregnant Cambodian women of reproductive age, while the prevalence of genetic hemoglobin disorders has been reported to be >50%. As iron deficiency does not seem to be a predominant cause of anemia among non-pregnant Cambodian women, untargeted daily oral iron supplementation may be a waste of resources in the effort to reduce anemia prevalence. Worse, providing iron supplementation to iron-replete women, particularly those with genetic hemoglobin disorders, may increase the risk for accumulating excess iron in the body (i.e. free-iron and NTBI). The accumulation of free-iron and NTBI has the potential to be harmful as they can catalyze the formation of ROS and lead to oxidative stress-induced damage within the body.

To date, there is minimal evidence regarding the potential risk of untargeted daily oral iron supplementation with 60 mg of elemental iron (as per the 2016 WHO policy). Previously, a double-blind randomized controlled trial was conducted to assess the effect of 12 weeks of untargeted daily oral iron supplementation with or MMN, compared to placebo, on hemoglobin concentration and hemoglobin response in \( n = 809 \) non-pregnant Cambodian women of reproductive aged 18–45 years. For this research, secondary analysis of specimens from women
who completed the trial in the iron \((n = 191)\) and placebo \((n = 186)\) treatment groups were conducted. Two biomarkers of cellular damage, relative LTL and mtDNA content, were measured in buffy coats using MMqPCR.

The results of my study suggest that iron supplementation was not significantly associated with a change in relative LTL after 12 weeks. It was hypothesized that 60 mg of elemental iron as ferrous sulphate for 12 weeks would cause an increase in oxidative stress, and thus cause telomere attrition. However, this was not observed.

On the other hand, my results show that iron supplementation was associated with a significantly smaller change in mtDNA content, compared to placebo, after 12 weeks. There are two most likely explanations for this finding. First, iron supplementation resulted in mitophagy (characterized by decreased mtDNA content), which balanced out a baseline state of mitochondrial biogenesis (characterized by increased mtDNA content) caused by other factors, resulting in what appears smaller change in mtDNA content, compared to the placebo group, after 12 weeks. Second, the alternative explanation is that iron supplementation halted the baseline state of mitochondrial biogenesis (characterized by increased mtDNA content), resulting in smaller change in mtDNA content, compared to the placebo group, after 12 weeks. Ultimately, more research is needed to understand the effect of oral iron supplementation on mitochondrial homeostasis in humans.
Finally, neither baseline iron status nor the presence of a genetic hemoglobin disorder modified the association between iron supplementation and change in relative LTL or change in mtDNA content.
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