THE EFFECTS OF IRON-FORTIFIED LENTILS ON THE IRON (FE) STATUS OF ADOLESCENT GIRLS IN BANGLADESH: A DOUBLE-BLIND, COMMUNITY-BASED, CLUSTER-RANDOMIZED CONTROLLED TRIAL

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ABSTRACT

This research aimed to investigate if consuming iron-fortified lentils could improve the body iron status of non-pregnant adolescents in rural Bangladesh. A four-month experimental study was carried out with n=1195 Bangladeshi adolescent girls aged 10–17 years, who were randomly assigned to one of three groups. One group consumed cooked iron-fortified lentils (~ 200 g/day, five days per week for 85 feeding days), the second group consumed a similar amount of non-iron-fortified lentils, and the third group was provided no lentils. The lentil recipe (daal) was selected based on cultural preferences. Body iron stores before and after the intervention were measured to determine the differences between and within groups over the four month study. In the group that consumed the iron-fortified lentils, there was a positive effect on body iron status. In this group, serum ferritin was maintained, whereas it declined in both the non-iron-fortified lentil group and the no-intervention group. Girls in the iron-fortified lentil group had higher iron stores compared to girls in the non-iron-fortified lentil and no-intervention groups. No difference in body iron stores was observed between or within these last two groups. Girls who consumed iron-fortified lentils were also less likely to have low hemoglobin levels compared to those in the other groups. However, there was little difference between or within groups in hemoglobin levels, as opposed to body iron stores. This is likely because increased iron stores in non-iron-depleted individuals would have little influence on hemoglobin, but iron stores in iron-depleted individuals who move to the non-depleted population could elevate hemoglobin. As for anemia prevalence (%), we observed no noticeable increase in anemia prevalence in the iron-fortified lentil group compared to the other two groups, in which there was a significant increase in anemia prevalence during the study. Thus, although there were clear differences in iron stores, these did not impact hemoglobin and anemia status. Further analysis of iron-depleted populations across the three groups revealed that iron stores increased substantially in the iron-fortified group between the beginning and end of the study. These results suggest that the iron-depleted population had the most potential to benefit from consuming iron-fortified lentils to resolve their iron deficiency, both clinically and sub-clinically.

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DEDICATION

I would like to dedicate my PhD thesis to my late father Mr. Shah Alam (1950-2006) who dreamt big about me and gave all motivation since my childhood.

REST IN PEACE

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LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
AC	Adolescent clubs
BRAC	No abbreviation
BMI	Body mass index
CDC	Centers for Disease Control and Prevention
CRP	C- reactive protein
CDC:	Crop Development Centre
DPS	Department of Plant Sciences
DDS	Dietary Diversity Score
Fe	Iron
FFQs	Food Frequency Questionnaires
GoB	Government of Bangladesh
Hb	Hemoglobin
IFL	Iron-fortified lentils
ID	Iron deficiency
IDA	Iron deficiency anemia
icddr,b	International Centre for Diarrheal Disease Research, Bangladesh
NaFeEDTA	Sodium iron ethylenediaminetetraacetic acid
NIFL	Non-iron-fortified lentils
ODK	Open data kit
RCT	Randomized controlled trial
RDA	Recommended dietary allowance
RDW	Red blood cell distribution width
RBC	Red blood cell
sFer	Serum ferritin
sTfR	Serum transferrin receptor
TBI	Total body iron
USask	University of Saskatchewan
UID	Unique identifier number
VAS	Visual Analog Scales
WBC	White blood cell
WHO	World health organization
MCV	Mean corpuscular volume
LSBE	Life Skill Based Education

CHAPTER 1 INTRODUCTION

1.1 BACKGROUND

Iron deficiency is the most prevalent micronutrient deficiency in the world. Body iron is considered deficient when iron stores indicated as serum ferritin (sFer) reach <15 μg/L (Daru et al., 2017; Lynch et al., 2018; World Health Organization, 2011a). Poor body iron status hampers red blood cell (RBC) production, leading to low hemoglobin (a protein in the RBC that carries oxygen) concentration in the blood (Pavord et al., 2012; World Health Organization, 2011a). Anemia occurs when body hemoglobin (Hb) levels fall to <12 g/dL. A low Hb concentration due to iron deficiency is known as iron deficiency anemia (Hb <12.0 g/dL plus sFer <15.0 μg/L) (World Health Organization, 2017), and is considered one of the most significant public health problems around the globe (Shaka & Wondimagegne, 2018). About 25% of the global population suffers from anemia, with pre-school children and reproductive-aged women the most vulnerable (World Health Organization, 2015). In Bangladesh, the most recent, reliable nationally-representative anemia statistics were published in 2013, and reported a national 26% and 17.1% anemia prevalence among non-pregnant, non-lactating (NPNL) women and children of 12–14 years, respectively (ICDDRB et al., 2013). Global studies have reported that 25% and 37% of iron deficiency is associated with anemia among pre-school children and non-pregnant reproductive-aged women, respectively (Chaparro & Suchdev, 2019; Petry et al., 2016). Studies report that adolescent girls and non-pregnant, non-lactating women are even more vulnerable because of an increased demand for iron due to poor dietary intake, as well as menstrual blood loss, even though iron absorption is higher in iron-depleted populations (Coad & Conlon, 2011b; E. M. Miller, 2016; Abbaspour et al., 2014; Byrnes et al., 2002; Zijp et al., 2000).

1.2 RATIONALE FOR THE STUDY

This study aimed to measure the effects of iron-fortified lentils on adolescent girls living in rural areas of Bangladesh. The study is important for several reasons.

First, the study captured the iron status of the adolescent girls, who are at high risk for iron deficits due to their substantial body growth (Marangoni et al., 2016; Mesías et al., 2013). Additionally, adolescent girls are nutritionally vulnerable because of their lifestyle and food habits (Lassi et al., 2017). Moreover, adolescent girls experience gender discrimination in Bangladesh regarding nutritious food, and the low education of their parents make them even more vulnerable to malnutrition (Rashid et al., 2011; Sethuraman & Duvvury, 2007). Another study has pointed out that Bangladeshi girls have to sacrifice portions of their meals so that male family members can consume more, and this inferior social position predisposes adolescent girls to undernutrition, particularly those who live in households with food insecurity (Blum et al., 2019). Several risk factors for undernutrition are associated with living in a low-economic setting, such as poor hygiene and infections (Rashid et al., 2011). The World Bank has reported that investing in nutrition for adolescent girls is critical for economic growth and improved health in Bangladesh (Kanti et al., 2019). Although teenage pregnancies have declined by about 3% (33.0% to 30.8% from 1994 to 2014), they remain a major concern in Bangladesh (30.8% of teenage girls in 2014 had children) (Islam et al., 2017). Pregnant women have high iron demands due to pregnancy (0.8 mg/day to 7.5 mg/day from the 1st to 3rd trimester), and girls even more so due to their adolescent body growth (Balarajan et al., 2011; Bothwell, 2000; Milman, 2006). This high iron demand makes it more likely that these girls will suffer from iron deficiency-induced adverse pregnancy outcomes, such as premature labor, maternal and fetal morbidity, and increased mortality risk, low birth weight, fetal cognitive impairment, and growth retardation (Figueiredo et al., 2018; Stangret et al., 2017; Yi et al., 2013).

Second, the study chose lentils as a vehicle for intervention for a variety of reasons. Lentils are nutritious, plant-based food that are rich in macro and micronutrients, and are used as a staple food in many countries, including Bangladesh (Faris et al., 2019; Joshi et al., 2017; Migliozzi et al., 2015; Rathod & Annapure, 2016, 2017). Lentils are also known for their high iron content (Khazaei et al., 2017; Rathod & Annapure, 2016). The University of Saskatchewan (USask) has been engaged in lentil research and its potential for iron fortification research over the past decade. USask research has shown that lentils grown in the province of Saskatchewan in Canada are rich in iron (73–90 mg/kg), zinc (44–54 mg/kg), and selenium (425–673µg/kg) (DellaValle, Thavarajah, et al., 2013; D. Thavarajah et al., 2009; D. Thavarajah, Thavarajah, Sarker, et al., 2011). Additionally, Saskatchewan-grown lentils have been found be low in anti-

iron nutritional factors (phytic acid 2.5–4.4 mg/g), meaning that these lentils have higher iron bioavailability (D. Thavarajah et al., 2009; D. Thavarajah, Thavarajah, Wejesuriya, et al., 2011; P. Thavarajah et al., 2009). Other studies have suggested that iron has a very high binding stability with EDTA (Ethylene diamine tetra-acetic acid), and iron absorption substantially increased when it is formed as NaFeEDTA (Bothwell & MacPhail, 2004; Dueik et al., 2017; Ginanjar et al., 2018). Furthermore, studies have found that lentils fortified with NaFeEDTA have improved the relative iron bioavailability of Caco2 cells, and remain bioavailable even with the natural presence of anti-iron absorbents (Podder et al., 2017; Podder, Dellavalle, et al., 2018; Podder, Khan, et al., 2018).

The addition of a nutrient(s) to food is known as food fortification. The World Health Organization (WHO) has recognized food fortification as a valid approach for reducing global micronutrient deficiencies compared to other food alternative strategies (Allen et al., 2006; Codex Alimentarius Commission (CAC), 2013; Dwyer et al., 2015; Tontisirin et al., 2002; World Health Organization (WHO) & Food and Agriculture Organization of the United Nations (FAO), 2006). Several studies have been conducted on iron fortification using various food vehicles; they have found that both complementary and condiments foods are efficacious (Andang'o et al., 2007; Beinner et al., 2010; Biebinger et al., 2009; Moretti et al., 2006; Van Stuijvenberg et al., 2008; Zimmermann et al., 2010). However, studies of the iron fortification of lentils are very recent. Following The United States Food and Drug Administration (USFDA) food fortification policy and principles, researchers from Usask used spray technology for the first time to fortify lentils and found promising results in bioavailability (Podder et al., 2017). Considering the natural nutritional content of lentils themselves, their potential for higher bioavailability after iron fortification makes iron-fortified lentils an ideal whole, food-based, sustainable solution to combat the global iron deficiency burden.

Third, there is a policy for fortification in place in Bangladesh. The government of Bangladesh has adopted a 'National Strategy on the Prevention and Control of Micronutrient Deficiencies, Bangladesh (NSPCMD) (2015-2024), which is designed to improve the population's access to and affordability of micronutrients (Institute of Public Health Nutrition, 2015). The strategy includes the fortification of oil with Vitamin A, rice fortification with micronutrients, and Vitamin D-fortified foods; however, there are no guidelines for iron fortification. By introducing a novel approach to the iron fortification of lentils (a staple food)

and targeting adolescent girls vulnerable to iron deficiency, this study has the potential to reduce the iron deficiency burden in Bangladesh and, by extension, iron deficiency anemia.

A high prevalence of anemia has been a significant public health problem in Bangladesh since the country gained its independence in 1971. Nearly 75% of school-age children and adolescents were found to be anemic in the 1975/76 and 1981/82 national surveys (Ahmed, 2000). In 1995/96 and 1997/98, anemia was reported in 78% and 43% of adolescent girls, respectively (Ahmed, 2000). One of the reasons for this large variation may be that different surveys used different test methods. The most recent and reliable national representative survey using WHO cut-offs and test parameters reported anemia prevalence of 17.1% in girls aged 12-14 years and 26% in girls and women aged 15-59 years (ICDDRB et al., 2013). This report was published seven years ago, and since then, there has been no national-level data, suggesting that Bangladesh is still experiencing a high prevalence of anemia.

1.3 RESEARCH QUESTION AND OBJECTIVES

1.3.1 Research question

- How efficacious are iron-fortified lentils in improving the iron status (Fe) of non-pregnant adolescent girls in rural Bangladesh?

1.3.2 General objective

- This study aims to establish novel evidence of the efficacy of iron-fortified lentils in improving the body Fe status of non-pregnant adolescent girls in rural Bangladesh.

1.3.3 Specific objectives

1) To create an iron profile of non-pregnant adolescent girls in Bangladesh.
2) To determine the effect size (changes in body iron stores) due to an iron-fortified lentil-based dietary intervention among non-pregnant adolescent girls in Bangladesh.
3) To measure the effect of iron-fortified lentils on iron deficiency (using clinical and sub-clinical cut-offs) among non-pregnant adolescent girls in Bangladesh.
4) To measure the effect of iron-fortified lentils on total body iron (TBI) among non-pregnant adolescent girls in Bangladesh.

5) To measure the effect of iron-fortified lentils on anemia (using mild, moderate, and severe cut-offs) among non-pregnant adolescent girls in Bangladesh.

6) To measure the effect of iron-fortified lentils on iron deficiency anemia (using mild, moderate and severe cut-offs) among non-pregnant adolescent girls in Bangladesh.

It was hypothesized that:

1) Iron deficiency, both clinical and sub-clinical, is highly prevalent among adolescent girls in Bangladesh.
2) Anemia is highly prevalent among adolescent girls in Bangladesh.
3) The prevalence of iron deficiency anemia is not as high as that of iron deficiency.
4) Iron-fortified lentils have a substantial positive influence on the iron status of adolescent girls and increase body iron stores.
5) Iron-fortified lentils benefit iron-depleted adolescent girls.
6) Iron-fortified lentils have a positive effect on body iron stores (serum ferritin), total body iron (TBI), and hemoglobin status.

Earlier evidence suggests that iron deficiency (9.5% among adolescent girls aged 12-14 years) in Bangladesh could be considered a mild public health problem based on WHO public health problem categories (Stevens et al., 2013; World Health Organization, 2015). However, studies suggest that anemia prevalence (26%) in Bangladesh should be considered a moderate to severe public health problem (Stevens et al., 2013; World Health Organization, 2015). A food-based approach could represent a promising attempt to reduce such high prevalence in low-income countries like Bangladesh. Research carried out at the University of Saskatchewan found that lentils have the potential to be part of a food-based fortification approach (D. Thavarajah et al., 2009; D. Thavarajah, Thavarajah, Wejesuriya, et al., 2011; P. Thavarajah et al., 2009), given that lentils are a staple food in many countries. Thus, in the current study lentils were fortified with iron as a part of WHO recommendation, which suggests that fortification can be a sustainable food-based solution (Huma et al., 2007; Hurrell, 1997b).

The current study sought to establish scientific evidence regarding iron-fortified lentils' impact on body iron stores among adolescent girls in Bangladesh. The importance of the study is multi-faceted. First, iron fortification of lentils represents a unique approach used to improve body iron stores. Second, the study results are likely to add new knowledge to existing

fortification-based food items. Third, findings from this study will provide scientific evidence to support the commercialization of iron-fortified lentils, especially for populations with a high potential to benefit from iron fortification, including those with micronutrient-poor diets.

The trial described in this thesis adopted a clustered randomized controlled design, which is thought to be the most robust for making causal observations and conclusions. Cluster-randomized controlled trials eliminate selection bias, and minimize bias from confounding variables, allowing for reliable assessment of the causal effect of, in this case, an iron-fortified lentils-based dietary intervention on body iron status (Hayes & Moulton, 2017; Lorenz et al., 2018; Spieth et al., 2016; Travison et al., 2016). Cluster-randomized controlled trials also ensure better representativeness and minimize contamination between treatment groups, and that community-based cluster sampling is preferable for anemia studies; a precise estimation of anemia prevalence in this design is able to be captured if the sample size is set to 30 individuals drawn from each of 30 clusters in cluster-based sampling (Pasricha et al., 2013). Since the current study was an efficacy trial, the results indicate the impact of iron-fortified lentils on iron status under the ideal and controlled environment; however, the study is unable to reproduce the "real-world" context, which could only have been captured through an effectiveness trial (Kim, 2013; Singal et al., 2014).

Another reason for choosing the efficacy trial design was that an ideal, controlled environment is unlikely to cause harm to the participants, whereas effectiveness trials conducted in the real, natural environment may cause more harm than good if efficacy is unknown (Haynes, 1999; Selker et al., 2019). Since the study was a randomized controlled trial (RCT), it began with a baseline survey followed by the iron-fortified lentil intervention, and an end line survey. Venous blood samples (6 ml) were collected at baseline (0 months), midline (2 months), and end line (4 months). The study had three intervention groups: one group consumed iron-fortified lentils, one consumed non-iron-fortified lentils, and one group maintained a regular diet (usual intake group with no lentils provided). The girls in the two lentil groups were served ~ 200 g of cooked lentils (the equivalent of 37.5 g of raw lentils) per day, on five days of the week, for 85 feeding days. Survey data (socio-demographics, seven-day dietary recall, food security, and anthropometrics) and venous blood samples were collected from the sample of n=1195 adolescent girls from September 2018 to April 2019.

1.4 CONTINUATION FROM MSC TO PHD PROJECT:

Before carrying out a human efficacy trial, an acceptability and implementation feasibility study was warranted among the target population. Therefore, a cross-over trial was carried out as an MSc project to determine the acceptable amount of iron-fortified lentil meals for adolescent girls' daily consumption (Yunus, 2018). Different preparations of a standardized, local lentil recipe (e.g. thick and thin) using the iron-fortified lentils were served to adolescent girls in three different portion sizes (raw amounts): of 25 g, 37.5 g and 50 g for 12 weeks. We observed that the 37.5 g portion size presented as the thick cooking preparation would be feasible for the target population to consume during an efficacy trial to examine the effect of iron-fortified lentils on body iron status. Based on those results, this PhD project was designed and implemented as a double-blind, community-based, cluster-randomized controlled trial among adolescent girls in Bangladesh.

1.5 ORGANIZATION OF THE THESIS

This dissertation followed the manuscript-based thesis format, which includes manuscripts, chapters, and appendices. Chapter 1 describes the introduction and rationale for the study, the problem statement, study's objectives and the hypothesis followed. Chapter 2 describes the literature review, covers relevant aspects of iron physiology and sustainable food-based solutions to resolve iron deficiency. Chapter 3 describes the general methods and materials used for the study. The next three chapters (4-6) are organized in manuscript format, beginning with Chapter 4 that describes the study's protocol for the randomized controlled trial (Yunus, Jalal, et al., 2019). Chapters 5 and 6 present the baseline iron profile study and the efficacy feeding trial, respectively. Finally, Chapter 7 presents a detailed general discussion of the study's findings, strengths and limitations of the study, and our conclusion. Assent and consent forms, questionnaires, and copyright agreements are presented in the Appendices.

CHAPTER 2 LITERATURE REVIEW

This chapter presents an overview of iron deficiency and anemia, both in Bangladesh and within the global context. The review focuses on iron physiology, including an assessment of iron biomarkers and iron fortification trials. The chapter further discusses the potential for food fortification with iron to reduce iron deficiency, as well as the successes and challenges iron fortification research and programs face.

2.1 INTRODUCTION

Iron deficiency (ID) is one of the world's most prevalent micronutrient problems, affecting millions of infants, children, women of childbearing age, and pregnant women (S. Gautam et al., 2019; Ma et al., 2017; Petry et al., 2019; Seyoum et al., 2019; World Health Organization, 2018c). It is a prominent cause of iron deficiency anemia (IDA), one of the most prevalent nutritional deficiencies, which affects most countries (World Health Organization, 2015). IDA is considered a significant public health problem; however, the prevalence tends to be country-specific (Shaka & Wondimagegne, 2018). Several studies have reported that IDA adversely impacts health-related quality of life (HRQoL) (Farag et al., 2011; Ferrari et al., 2015; Kraai et al., 2012; Wasada et al., 2013). Although there is insufficient data on the global economic burden of ID and IDA, one study reported that productivity losses of $4 per capita or 0.9% of GDP are linked to iron deficiency (Darnton-Hill et al., 2005). In high income countries, losses are at a much greater scale, even though ID is not widespread. In 2005, it was estimated that South Asia had losses of $5 billion annual GDP because of high anemia prevalence (Darnton-Hill et al., 2005). A study carried out among Swiss women reported an estimated CHF 33 million (US $33 million) annual indirect costs due to ID (Blank et al., 2019). In 2014, The Food and Agriculture Organization of the United Nations estimated that an investment of US $1.2 billion annually on micronutrient supplements, food fortification, and biofortification of staple crops for five years would generate US $15.3 billion annual benefits (13:1 cost-benefit ratio) (Food and Agriculture Organization of the United Nations, 2014).

This chapter begins with an overview of ID and IDA and their global and national prevalence in Bangladesh, followed by a description of iron physiology, including the assessment of iron biomarkers. The chapter then discusses the potential for fortifying food with iron to reduce iron deficiency and its successes and challenges in earlier studies.

2.2 ANEMIA, IRON DEFICIENCY, AND IRON DEFICIENCY ANEMIA

Iron (Fe) is a mineral required for maintaining human wellbeing. ID occurs when the body's stored iron is insufficient to support the body's erythrocyte (red blood cell, RBC) production, resulting in a low hemoglobin (Hb) concentration in the blood (Pavord et al., 2012; World Health Organization, 2011a). An absolute reduction of circulating RBC (or Hb) can be defined as anemia (Chaparro & Suchdev, 2019). Thus, anemia appears as a characteristic trait of ID (Andrews, 2008; J. L. Miller, 2013). Among all forms of anemia, ID is the most widespread cause (J. L. Miller, 2013). Anemia caused by ID is known as Iron Deficiency Anemia (IDA). Clinical features of IDA may be non-specific, and depend on the severity of the anemia, age, comorbidities and chronicity, and speed of onset (Lopez et al., 2016).

It is essential to note that Hb levels biologically vary by age, sex, pregnancy status, genetic and environmental factors, and racial status (Chaparro & Suchdev, 2019). Cut-offs of Hb levels to define anemia and its severity were first established by the World Health Organization (WHO) in 1968. Since then, children aged >=12 years and non-pregnant women (>=15 years) who have Hb levels <12 g/dL have been considered anemic, whereas cut-offs were set for men of >=15 years at >13 g/dL (World Health Organization, 2011a)]. Since body serum ferritin (sFer) levels can conclusively detect body iron status, body iron levels are considered deficient when sFer levels are <15 µg/L (Daru et al., 2017; Lynch et al., 2018; World Health Organization, 2011a).

2.2.1 Global statistics

2.2.1.1 Burden of anemia

Approximately 1.6 billion people in the world (25%) suffer from anemia, with children under five years (41.7%), pregnant women (40.1%), and women of reproductive age (32.8%) at highest risk (World Health Organization, 2015, 2016a, 2016b). Further estimations suggest that about 800 million pre-school children and women of reproductive age are anemic; of this population, more than 60% of the pre-school children are from the African region and more than

40% of the women of reproductive age are from South-East Asia (Kassebaum et al., 2014). High prevalence of anemia is found in central Asia (64.7%), Andean Latin America (62.3%), and South Asia (54.8%), and anemia accounts for more than 89% of the disease burden in developing countries (Kassebaum, 2016; Kassebaum et al., 2014). Anemia prevalence varies from country to country (Stevens et al., 2013; World Health Organization, 2015). The World Health Association (WHO) in 2015 has declared anemia as a moderate to severe public health problem in those countries with a high prevalence of anemia (> 20% of their population as stated by WHO). Despite this declaration, reports disagree on the global prevalence of anemia. One report indicated that between 1995 and 2011, global anemia prevalence decreased by about 4–5% in children aged 0–5 years, non-pregnant women, and pregnant women aged 15–49 years (Stevens et al., 2013). Another report published in 2016 indicated that anemia cases increased by about 0.10 billion between 1990 and 2013 (1.83 billion vs. 1.93 billion respectively), but noted that during this same period, those living with disability (YLD) decreased slightly (Kassebaum et al., 2016). Irrespective of the exact numbers, undoubtedly the global burden of anemia is high. To address this burden, the World Health Assembly Resolution (#65.6) included a 50% reduction of global anemia prevalence among women of reproductive age as one of the six global nutrition targets to be achieved by 2025 (World Health Organization, 2012, 2014).

2.2.1.2 Attributes of anemia (global)

A 2016 WHO report on global health estimated that 23,918 global deaths are directly linked to IDA, and the mortality rate is high, with 18,115 deaths since 2000 (World Health Organization, 2018a). Although it has been long assumed that 50% of the global anemia is attributable to ID (Chaparro & Suchdev, 2019), a recent random effect meta-analysis carried out in 23 countries revealed that 25% and 37% of ID was associated with anemia among pre-school children and non-pregnant women of reproductive age, respectively (Petry et al., 2016). The meta-analysis further reported that the level of association is lower in countries with > 40% anemia prevalence and a very high inflammation rate (Petry et al., 2016). The study's results amended earlier evidence of the assumed 50% of anemia due to ID, suggesting that ID should not always be considered as a significant determinant of anemia (Petry et al., 2016). However, Petry et al. (2010) did not clarify why the association between anemia and ID in their study was weaker than previously thought (Petry et al., 2016). In another study, Thurnham et al. (2010) suggested that the association could be underestimated by more than 14% if inflammation was not adjusted,

since active body inflammation may increase serum ferritin (sFer) levels during body's active phase response (Thurnham et al., 2010).

2.2.2. Bangladesh statistics

Bangladesh is a densely-populated country in South Asia, and this region alone contributes to more than half (54.8%) of the global anemia prevalence (Kassebaum et al., 2014). Anemia in Bangladesh is not new: evidence suggests that the country has experienced a high prevalence for more than four decades. Using data from surveys of school-age children and adolescents, a review article on anemia prevalence and etiology in Bangladesh reported prevalence to be about 75% in 1975/76 and 1981/82, 78% in 1995/96, and 38.4% in 1997/98 (Ahmed, 2000). The vast discrepancy between the 1995/96 and 1997/1998 surveys may be attributed to different approaches and methodology used to collect and assess the data. While age- and location-specific data have been collected in Bangladesh, no studies have provided nationally-representative statistics on anemia, ID, and IDA. For instance, the aforementioned 1995-96 survey was conducted in nine urban and thirty-two rural sites among children and adolescents 6-14 years old (Ahmed, 2000). Using laboratory venous blood tests, this survey reported 70.5% anemia prevalence in the urban sites and 80.4% in the rural sites (Khursheed & Mosharaff, 1998). The aforementioned 1997/98 survey used a Hemocue, which measures Hb in a capillary blood sample, and reported anemia prevalence of 38.4% among children 6-11 years (Ahmed, 2000). Another 1996 survey used laboratory venous blood tests of children 11–16 years old in five peri-urban sub-districts and found 27% anemia prevalence (Ahmed et al., 2000; HKI/IPHN, 1999). A 2002 survey using laboratory venous blood samples reported 28% anemia prevalence among pregnant women, while a 2009 survey using Hemocue reported 24.8% anemia prevalence among adolescent girls (Harun-Or-Rashid et al., 2009; Lindström et al., 2011). These results show that there is lack of consistent national level data (due to varying methodology) on anemia, ID, and IDA in Bangladesh.

Although anemia prevalence was assumed to be higher, the Bangladesh National Micronutrient Survey (NMS, 2013), a nationally-representative study, reported 26% anemia prevalence among non-pregnant non-lactating (NPNL) women, and 17.1% among children of 12–14 years (ICDDRB et al., 2013). The NMS (2013) is the only reliable nationally-representative survey in Bangladesh that uses WHO iron biomarkers and cut-offs. It reported that

rural residents contributed more than urban residents to the national prevalence of anemia, i.e., 27.4% among rural NPNL women and 18.1% among rural children of 12–14 years (ICDDRB et al., 2013). Another national report, Bangladesh Demographic and Health Survey (BDHS 2011), reported 40% anemia prevalence among NPNL women (National Institute of Population Research and Training (NIPORT) and Mitra and Associates and ICF International, 2013). Although the NMS survey (2013) and BDHS (2011) were carried out during the same time period, they reported striking differences in anemia prevalence, perhaps due to the use of different iron biomarker parameters, as well as methodological differences. For instance, BDHS 2011 used capillary blood samples, which are not reliable since capillary blood draws require extreme care and training. The NMS (2013), on the other hand, collected venous blood samples, which are globally accepted as the most reliable from which to measure iron biomarkers to calculate anemia prevalence (ICDDRB et al., 2013; National Institute of Population Research and Training (NIPORT) and Mitra and Associates and ICF International, 2013).

It has been almost seven years since the NMS 2013 report was published, and the anemia prevalence in Bangladesh since then seems to have remained static. A recent 2019 small-scale study reported 26.6% anemia prevalence among adolescent girls 10-17 years of age, whereas the NMS study reported 26% prevalence among women of 15 years of age and older using the same iron biomarkers. (ICDDRB et al., 2013; Yunus, Das, et al., 2019). Regardless of the differences in the prevalence of anemia reported in Bangladesh, anemia prevalence is still high and contributes a major burden to the health system.

2.2.2.1 Review matrix of Bangladesh statistics

Table 2.1 presents a review matrix listing all the ID and IDA prevalence studies conducted in Bangladesh over the last 10 years (from the year 2010). Both journal articles and institutional reports were included for a comprehensive understanding of ID and IDA trends in Bangladesh, regardless of study design. The review matrix shows a wide range of ID prevalence reported in small-scale urban and rural studies among specific female age groups. The reliable and nationally-representative survey, the NMS 2013 survey, reported age-specific ID and IDA statistics in Bangladesh. As mentioned above, the NMS survey analyzed venous blood samples and used the most reliable ID and IDA indicators (sFer and Hb, respectively), adjusted for active infection while reporting the iron statistics, used WHO cut-offs for ID (preschool-age children

<12 μg/L and school-age children and non-pregnant non-lactating (NPNL) women <15 μg/L), and IDA (Hb levels <12.0 g/dl in NPNL women, and <11.0 g/dl in preschool-age children) (ICDDRB et al., 2013). NMS reported that children 6-59 months had the highest prevalence of IDA (10.7%), followed by adolescents 12-14 years (9.5%), and NPNL 15-49 years (7.1%). IDA was found to be lower in children of 12–14 years (1.8%) and NPNL women (4.8%). In 2019, baseline data from a large-scale iron fortification study reported 9.2% ID among adolescents of 10-17 years (Yunus, Das, et al., 2019). This study used WHO cut-offs for ID and IDA, and reported statistics were based on the most reliable ID and IDA indicators. Although this study's findings cannot be generalized to all Bangladeshi adolescents, it presents the most recent ID and IDA statistics among adolescent girls in Bangladesh. The majority of the reports and research articles have reported anemia, since they use test machines that screen for Hb, which are unable to differentiate IDA from other forms of anemia.

Table 2.1: Review of iron deficiency and iron deficiency anemia prevalence (%) over the past 10 years in Bangladesh infants, children and women

Authors	**Reports on**	**Study design**	**Study population (All females)**	**Population size (*n*)**	**Prevalence**
(ICDDRB et al., 2013)	- Anemia	National Survey *(National Micronutrients Status Survey, 2011-12)*	- 6–59 months - 6–11 years - 12–14 years - 15–49 years	3150	- 33.1% - 19.1% - 17.1% - 26.0%
	- ID		- 6–59 months - 6–11 years - 12–14 years - 15–49 years	3150	- 10.7% - 3.9% - 9.5% - 7.1%
	- IDA		- 6–59 months - 6–11 years - 12–14 years - 15–49 years	3150	- 7.2% - 1.3% - 1.8% - 4.8%
(National Institute of Population Research and Training (NIPORT) and Mitra and Associates and ICF International, 2013)	- Anemia	National Survey *(Bangladesh Demographic and Health survey, 2011)*	- 6–59 months - 15–49 years	- 2353 - 5676	- 51.0% - 42%
(Mistry et al., 2017)	- Anemia	National survey	- 10–19 years	- 1314	- 51.6%
(Ahmed et al., 2018)	- Anemia - ID - IDA	Baseline survey	- Pregnant women (gestational age ≤20 wk)	- 522	- 34.7% - 27% - 13.4%
(Yunus, Das, et al., 2019)	- Anemia - ID	Baseline survey	- 10–17 years	- 1195	- 26.6% - 9.2%

2.2.2.2 Attributes of anemia (Bangladesh)

Although the prevalence of ID and IDA has been identified in several Bangladesh surveys, variations in iron biomarkers and test methods have resulted in differences in prevalence. Therefore, drawing a logical trend of iron status statistics over time may be difficult. Some surveys used tests that screen only for Hb levels, and reported test results from capillary blood samples, while other surveys used venous blood samples and more complete iron status test panels. Different results can be attributed to differences in the type of blood samples analyzed because Hb levels is higher in capillary blood than in venous blood. Additionally, the screening testing device (HemoCue) has shown limitations compared with automated hematology analysers (Hinnouho et al., 2018; Patel et al., 2013). These are perhaps the major reasons why BDHS (2011) reported higher anemia prevalence than NMS (2013) and reported differences in anemia prevalence. Although NMS (2013) indicated that the prevalence of ID is likely much lower than it was previously assumed to be (ICDDRB et al., 2013), no studies have offered conclusive evidence that ID and IDA prevalence in Bangladesh has declined over time.

A letter to editor suggested that the reason for finding low anemia prevalence in the Bangladeshi population is that the country's drinking water is high in iron (S. Rahman & Ireen, 2019). In 2011, the Bangladesh National Drinking Water Quality Survey reported that the widespread presence of a high level of iron in drinking water affected about 40% of the population (Bangladesh Bureau of Statistics & UNICEF, 2011). Another study reported that 82% of the Bangladeshi population are exposed to a high level of iron because they drink water from deep, shallow tube wells, which exceed the WHO standards for iron in drinking water (>0.3 mg/L Fe) (Akter et al., 2016). In fact, it has been reported that 44.6% of tube wells in Bangladesh exceed Bangladesh drinking water standards for iron (1.0 mg/L Fe) (M. A. Rahman & Hashem, 2019). The iron in the groundwater remains an under-assessed credible source of dietary iron in Bangladesh (Merrill, Shamim, Ali, Jahan, et al., 2012), but a recent study has established a link between the natural presence of iron in drinking water and body iron status (Ahmed et al., 2018).

2.3 IRON PHYSIOLOGY

Iron plays a fundamental biological role in respiration, energy production, DNA synthesis, and cell proliferation (Hentze et al., 2010a).

2.3.1 Iron requirements

Iron is biologically essential for all living beings, plays a vital role in the body's RBC production, and is essential for carrying out various cellular mechanisms, such as enzyme processes, DNA synthesis, and mitochondrial energy generation (Lopez et al., 2016; Pavord et al., 2012; Schneider et al., 2005). The body requires a specific amount of iron to perform its daily biological functions (Wang & Pantopoulos, 2011). The main route through which iron enters the body is diet (Anderson & Frazer, 2017). An adult body contains 3-5 g of iron, of which a small portion (20-25 mg) is required for daily red blood cell (RBC) production and various cellular mechanisms (Steinbicker & Muckenthaler, 2013).

Table 2.2 presents the recommended dietary allowance (RDA) for iron among females by age group. When adolescent girls enter puberty (~14 years), the body's demands for iron almost double due to significant body growth as well as the impact of monthly menstruation (Dallman, 1990; Mesías et al., 2013; World Health Organization (WHO) & Food and Agricultural Organization of the United Nations (FAO), 2004).

Table 2.2: Recommended Dietary Allowance (RDA) for iron for females

Gender	Age	Daily recommended amount
Female	9–13 years	8 mg/day
	14–18 years*	15 mg/day
	19–30 years *	18 mg/day
	31–50 years *	18 mg/day
	51–70 years	8 mg/day
	> 70 years	8 mg/day

Source: (Institute of Medicine, 2003)
**At the time of menstruation, the iron requirement is increased by approximately 2.5 mg/day.*

2.3.2 Iron sources

Heme [Fe^{2+} (ferrous Fe)] and non-heme iron [Fe^{3+} (ferric iron)] are the two forms of dietary iron (Ems & Huecker, 2019; Wessling-Resnick, 2014). Heme iron is found in animal food sources (meat, poultry, and seafood), which supply 10–15% of total iron intake among people who consume animal food (McDermid & Lönnerdal, 2012). Heme iron is tightly sequestered within a protoporphyrin ring, which protects it from inhibitors of Fe absorption, resulting in

higher Fe bioavailability (15-35%); heme iron contributes more than 40% of the body's total iron absorption (Anderson & Frazer, 2017; Hurrell & Egli, 2010; McDermid & Lönnerdal, 2012). A major portion of iron (about 60%) in the body lies in the Hb levels of RBC and in the myoglobin of muscles (Lopez et al., 2016).

On the other hand, non-heme iron is found in both dietary animal and plant sources (black tea, cacao, cereals, vegetables, fruit, etc.). However, plant sources of iron remain in the dormant form (Anderson & Frazer, 2017; McDermid & Lönnerdal, 2012). Absorption of non-heme iron is much lower and more variable (2%–20%) than that of heme iron, and is vulnerable to inhibitors (as well as enhancers) of Fe absorption (Hurrell & Egli, 2010). Non-heme iron is found in various forms such as soluble iron, iron in low molecular-weight complexes, ferritin as storage iron, and iron in the catalytic centers of a wide range of other proteins, and its bioavailability is influenced by dietary constituents and luminal factors (Anderson & Frazer, 2017).

2.3.3 Iron absorption

After iron is consumed in the diet, iron in the form of Ferric iron ($Fe3^{+}$) reaches the lumen of the small intestine (duodenum and upper jejunum) and enters into the intestinal absorptive cells (enterocytes) with the support of the Divalent Metal Transporter 1 (DMT-1) — a group membrane transport protein (Anderson & Vulpe, 2009; Hentze et al., 2010a). The acidity (pH) level in the proximal duodenum influences the transformation of iron from Fe 3+ → $Fe2^{+}$, which permits the subsequent transport of $Fe2^{+}$ across the apical membrane of enterocytes. Therefore, changes in gastric acid production and/or acidity level (pH) control non-heme iron absorption at the early stage (Abbaspour et al., 2014). The transferrin receptor (TfR) holds the iron in the intestinal enterocytes or reticuloendothelial macrophages and releases it into the plasma. This action leads to the iron being taken into the cell membrane (endocytosis), and internalized iron reaches mitochondria for iron synthesis (Abbaspour et al., 2014).

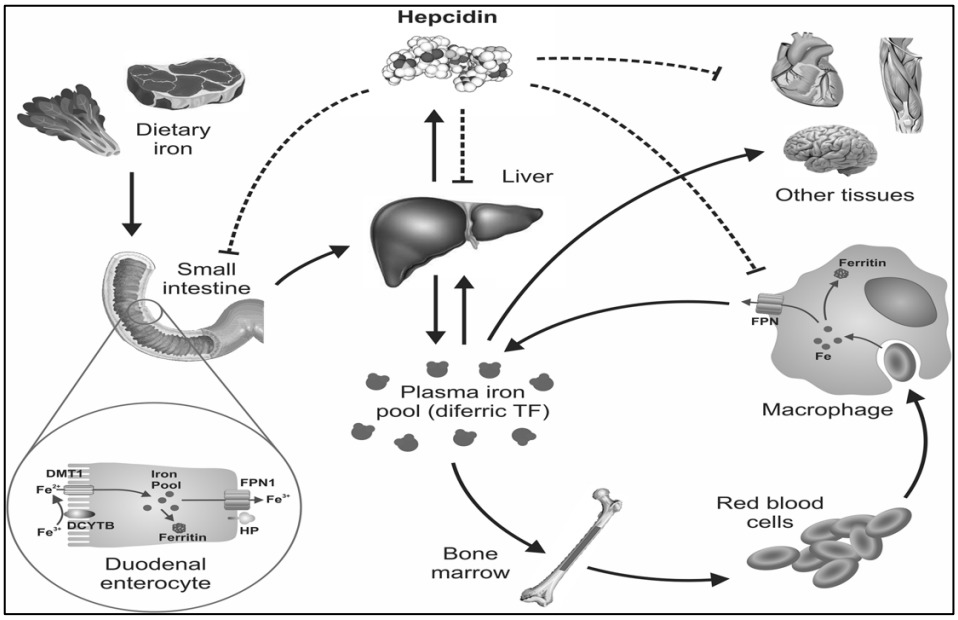
A small portion of iron is stored as ferritin in the ferrous iron ($Fe2^{+}$) in the small intestine. When this ferrous iron leaves the enterocyte, it is then ready to move into circulation. Most of this iron is used for erythropoiesis (RBC production), and small portions move to the liver (Frazer & Anderson, 2005; Hurrell, 1997a; Nadadur et al., 2008), where the amount of absorption is tightly regulated by the liver's existing feedback mechanism (Abbaspour et al., 2014). The mechanism of heme iron transportation across the apical membrane is slightly different from that

of non-heme iron, and the mechanism is not completely understood. The heme iron is digested by heme oxygenase 1 (HO-1) in the enterocyte regardless of gastric acid production and/or acidity level (pH); therefore, it initiates uninterrupted absorption at the early stage of absorption (Wang & Pantopoulos, 2011).

Certain elements interact with iron (mostly non-heme), and thus influence uptake. Phytate and polyphenols are the major inhibitors for iron absorption, and they are widely available in non-heme iron (Hurrell & Egli, 2010). Vitamin C (ascorbic acid) can reduce the effect of inhibitors on iron absorption (e.g. phytate, polyphenols, and calcium), and therefore, enhance absorption of non-heme Fe (Hurrell & Egli, 2010; Zijp et al., 2000). Thus, citric acid and ascorbic acid help non-heme iron to become more soluble (Anderson & Frazer, 2017).

2.3.4 Iron homeostasis

Iron homeostasis is maintained by the standard balance among iron uptake, transport, storage, and utilization (Lieu et al., 2001). Figure 2.1 illustrates iron homeostasis. Dietary iron intake, intestinal absorption, and recycling have to be well-regulated since iron does not have an excretion pathway (Lopez et al., 2016). Iron is primarily regulated at the point of absorption. However, apart from during menstruation, other bleeding, or pregnancy, only a tiny amount (~1mg/day) of iron is excreted through the skin, stool, sweat, the genitourinary tract, and the gastrointestinal tract (Abbaspour et al., 2014; Fairbanks, 1999; Finberg, 2011; Hunt et al., 2009; Hurrell & Egli, 2010; McDowell, 2003; Steinbicker & Muckenthaler, 2013). Hepcidin—a circulating liver enzyme—plays a primary role in balancing the use and storage of iron (Nemeth & Ganz, 2006). It binds with an iron transporter ferroportin, which is present in all major iron absorption areas, such as the cells of the intestinal duodenum, macrophages, and placenta (Nemeth et al., 2004). Ferroportin internalizes and degrades Fe by binding with hepcidin, therefore preventing iron from entering the cell (De Domenico et al., 2007; Nemeth et al., 2004). As mentioned, iron is stored in the body in the form of ferritin, which is primarily stored in the liver, spleen, and bone marrow (Wood & Ronnenberg, 2005). The level of serum ferritin (sFer) reflects both reticulo-endothelial and parenchymal iron stores in the body (Cazzola et al., 2002; Queiroz-Andrade et al., 2009). Thus, sFer is a convenient laboratory test that can estimate body iron status (Abbaspour et al., 2014; Camaschella, 2015; Mei et al., 2005; Zimmermann & Hurrell, 2007).



Source: The American Journal of Clinical Nutrition, Volume 106, Issue suppl_6, December 2017, Pages 1559S–1566S, https://doi.org/10.3945/ajcn.117.155804 (Anderson & Frazer, 2017)
Abbreviation: Duodenal cytochrome B (Dcytb); divalent metal-ion transporter 1 (DMT1); ferroportin (FPN); hephaestin (HP); transferrin (TF).
[Copyright permission granted (Appendices C)].

Figure 2.1: Body iron homeostasis. Iron is present in the diet in both heme and non-heme forms. Although the mechanisms underlying heme absorption are poorly understood, it is known that non-heme iron enters the circulation after traversing the enterocyte apical membrane via Divalent Metal-ion Transporter 1 (DMT1) and the basolateral membrane via ferroportin 1 (FPN1). Iron binds to plasma transferrin (TF) and is distributed to tissues throughout the body. Quantitatively, most iron is used by immature Red Blood Cell (RBC) in the bone marrow for Hb production. Senescent RBCs are phagocytosed by macrophages, and the iron is released from catabolized Hb and re-enters the circulation. The liver-derived peptide hepcidin plays a critical role in the regulation of body iron intake and distribution by binding to plasma membrane FPN1 on enterocytes, macrophages, and most body cells and facilitating its internalization and degradation. Hepcidin, in turn, is regulated by body iron demand. Thus, when the body is iron deficient, hepcidin concentrations are low, thereby favoring iron absorption and delivery to the plasma from storage sites, but when the body is iron replete, a higher hepcidin concentration reduces iron absorption and impairs iron release from stores.

2.3.5 Causes of iron deficiency

The primary focus of ID research is its etiology (DeLoughery, 2017). A range of physiological, environmental, and pathological factors responsible for ID are listed in Table 2.3. Inadequate dietary iron intake is among the major causes of ID, and this particularly affects infants and women of childbearing age living in developing countries (Aspuru et al., 2011).

Substantial iron demands occur during the rapid growth phases of life (e.g. infancy, adolescence, and pregnancy), leaving the body physiologically vulnerable to ID (Balarajan et al., 2011). Iron demands increase after birth. In the first six weeks of life, Hb levels decrease by about 170 mg/L to 120 mg/L as the body naturally expands its blood volume, leading to self-regulated iron hemostasis that occurs when the infant doubles its weight at about 4-6 months of age (Domellöf et al., 2014). Further, breastmilk and most weening foods are not good sources of dietary Fe.

Children, adolescents, and women of reproductive age, particularly pregnant women, are at high risk for iron deficiency due to their increased iron demand, as well as their poor dietary intakes of Fe (Domellöf et al., 2014; Marangoni et al., 2016; Mesías et al., 2013). Menstruating girls and women are further vulnerable to ID due to menstrual blood loss (accounting for roughly 0.5 mg iron per day) (Coad & Conlon, 2011a; Cook, 1990; E. M. Miller, 2016). During pregnancy, iron requirements increase from 0.8 mg per day in the first trimester to 7.5 mg per day in the third trimester (Bothwell, 2000; Milman, 2006). Several studies have reported that newborn babies are vulnerable to ID and IDA if mothers were iron deficient during pregnancy (Colomer et al., 1990; De Pee et al., 2002; Kalaivani, 2009; Meinzen-Derr et al., 2006). Furthermore, low-birth weight babies, including those born pre-term, are at higher risk of ID since they were born with low iron stores, which adversely affects early childhood development (Balarajan et al., 2011; Chaparro, 2008). For post-menopausal women, gut lesions are considered to be the main reason for developing ID (Firquet et al., 2017).

Table 2.3: Causes of iron deficiency

- Inadequate intake due to:
  - Habitual/discretionary inadequate intake of bioavailable iron
  - Nutritional iron "insecurity," i.e., inadequate access to or availability of bioavailable dietary iron (e.g., poor dietary diversity)
- Iron malabsorption due to:
  - Celiac disease (gluten enteropathy)
  - Chronic Helicobacter pylori gastritis
  - Autoimmune atrophic gastritis
  - Some surgical procedures involving the stomach or the upper small bowel

- Iron-refractory iron-deficiency anemia (IRIDA)
- Accelerated physiological requirements
- Increased requirements for growth:
  - Premature infants
  - Early childhood
  - Adolescent growth spurt
- Menstruation
- Pregnancy
- Pathological blood loss:
  - Bleeding from the gastrointestinal and genitourinary tracts
  - Parasitic infections, notably helminth infections such as hookworm and schistosomiasis
  - Menorrhagia
  - Intravascular hemolysis
- Blood donation

Adopted from (Lynch et al., 2018)

2.3.6 Consequences of iron deficiency

The most common consequence of ID is the development of IDA (Johnson-Wimbley & Graham, 2011). There are, however, other non-Fe-related nutritional causes of anemia (low-Hg), which include inadequate intakes of vitamins A, B_6, B_{12}, C, E, folate, copper, and zinc(Chaparro & Suchdev, 2019). Malaria, worm infestation, chronic infections, and genetic disorders are major causes of non-nutritional anemias. Of these, malaria has a particularly complex relationship with iron status. Several studies have suggested that iron deficiency actually plays a protective role against malaria (Adam, 2016; Gwamaka et al., 2012; Jonker et al., 2012) because *Plasmodium*, a malarial parasite, requires the host's iron to survive. While a body with low iron store impedes *Plasmodium* growth (Clark et al., 2014), also develops anemia when infected with a malarial parasite. The parasite raptures the body's RBC early in the disease process, leading to decreased RBC in the circulation resulting in anemia (N. J. White, 2018). A Cochrane review of 68 trials reported that the incidence of clinical malaria or death was not increased among children living in

malaria-endemic areas who received iron supplements; however, higher levels of malarial parasitemia was found among children receiving iron. Regardless, the intensity of malarial transmission, local immunity, ID prevalence, and the general health of the population determines the balance between the benefits and harm of malarial infection (Pasricha et al., 2013). In fact, WHO has advised iron supplementation for children in malaria-endemic areas, in addition to malarial preventive, diagnostic, and treatment measures (World Health Organization, 2011c). As for ID and IDA in Bangladesh, malaria prevalence is low (3.97% in endemic areas); however there is 28% prevalence of thalassemia, which was found in a small scale study (there are no national data) and reported as an associated risk factor for non-nutritional anemia (Haque et al., 2009; Merrill, Shamim, Ali, Labrique, et al., 2012).

Conclusive evidence suggests that ID adversely affects several body mechanisms, particularly in infancy and childhood, and among females. Several studies have reported that ID hampers neural development among infants (Beard, 2003; Georgieff, 2008; Lozoff, 2007, 2011; Lozoff et al., 2008; Madan et al., 2011; Monga et al., 2010; Walter, 2003). However, WHO has stated that ID-induced cognitive impairment could be significantly reduced by iron therapy (Jáuregui-Lobera, 2014; Zhukovskaya et al., 2019). ID during pregnancy is linked to adverse maternal and fetal morbidity, including intrauterine growth retardation, premature labor, low birth weight, and even increased mortality risk (Figueiredo et al., 2018; Stangret et al., 2017; Yi et al., 2013). Among pregnant women, ID increases the risk of perinatal infection, pre-eclampsia, bleeding, post-partum cognitive impairment, and behavioral difficulties in their children (Beard, 2003; Milman, 2012). ID during the first trimester has an even more negative effect on fetal growth than at the later stage of pregnancy (Allen, 2000; C. S. Gautam et al., 2008). Additionally, ID is linked to increased infectious disease morbidity due to its adverse effects on the immune system (Oppenheimer, 2001). Several studies have reported that ID leads to decreased work capacity among construction, agricultural and industrial workers regardless of their gender (DellaValle, 2013; Haas & Brownlie, 2001), because ID affects the function of the Fe-containing enzymes required for aerobic metabolism (Stugiewicz et al., 2016).

2.3.7 Assessment of iron biomarkers

Unlike most other micronutrient deficiencies, specific laboratory-based biochemical markers can identify ID. Table 2.4 presents the conventional biomarkers of iron status, their

reference values, indications, and challenges. The table also includes the meaning of each biomarker to better understand the tests used to assess iron status. Serum iron, sFer, and serum transferrin saturation are widely used for identifying ID (Camaschella, 2015). However, the body's active inflammation status or rare inherited defective iron metabolism alter levels of some biomarkers, making it difficult to form a conclusive diagnosis in some cases, in the absence of additional information, such as biomarkers of inflammation (Archer & Brugnara, 2015). While bone marrow iron content remains the most precise measurement to assess body iron status, its measurement requires an invasive surgical procedure (Baird-Gunning & Bromley, 2016; García-Casal et al., 2015; Phiri et al., 2009; Rocha et al., 2009). sFer remains the most efficient and cost-effective test to assess body iron status, but requires correction for inflammation (Mei et al., 2005; Zimmermann & Hurrell, 2007).

Table 2.4: Biomarkers of iron in the human body[a]

Test Name (Unit)	Unit	Ref. Value (female)[b]	Represents	Indicates	Challenges/remarks
Bone marrow					
1. Bone marrow Fe stain (hemosiderin)	µg/L	20–200	Fe content	↓ Depleted or absent body Fe stores	Invasive and traumatic sample collection procedure
Complete blood counts (CBC)					
1. Hemoglobin (Hb)	g/l	12.0–16.0	Fe-containing oxygen-transport protein	↓ Anemia ↑ Lung, health, kidney disease, etc.	Anemia occurs without ID
2. Red blood cells (RBC)	10^12/L	3.80–5.20	Number of RBC	↓ IDA, bone marrow failure. erythropoietin deficiency, etc. ↑ Indicates symptom of a disease or disorder	Typically carried out as a part of complete blood count (CBC).
3. Hematocrit or packed cell value (PCV)	%	36–46	Proportional volume of RBCs in whole blood	↓ Conditions such as cell destruction, blood loss, and failure of bone marrow production ↑ Dehydration or an abnormal increase in RBC production	Depends on factors affecting centrifuge, e.g., stable power supply
4. Mean cell volume (MCV)	Femtolitre [fl (10^{-12})]	78–100	Average size of RBCs	↓ Microcytic (small average RBC size) ↑ Macrocytic (large average RBC size)	Requires expensive machine and a late finding of ID.[c]

5. Mean cell hemoglobin (MCH)	Petagram (pg)	28.4–30.7	Hb in an average RBC	↓ Due to malnutrition or nutritional deficiencies ↑ Sign of macrocytic anemia	Requires expensive machine; and slow to respond to ID.
6. Mean corpuscular hemoglobin concentration (MCHC)	%	31.0–36.0	Avg. concentration of Hb inside a single RBC	↓ Due to ID ↑ Macrocytic anemia and autoimmune hemolytic anemia	-
7. Red cell distribution width coefficient of variation (RDW) – CV	%	11.5–14.5	Abnormal range in size of RBCs	↓ Thalassemia and inflammation ↑ Nutrient deficiency, such as a deficiency of Fe, folate, or vitamin B-12	Requires expensive machine
8. Reticulocyte hemoglobin concentration (RET-He or CHr)	Pg	28–36	Concentration of Hb in new RBCs	↓ Aplastic anemia, IDA, pernicious anemia ↑ Much bleeding, a move to a high altitude, or certain types of anemia	Requires expensive machine
Iron regulators					
1. Serum or plasma Fe	µg/dl µmol/l	26–170 µg/dl	Fe bound to transferrin in blood.	↓ ID ↑ Hemolytic anemia, Fe overload or Fe poisoning	Major variation in day-to-day levels. [c]
2. Erythrocyte protoporphyrin (EP)	µmol/mol whole blood or RBCs	< 70 µmol/mol haem [e]	Restricted supply of Fe to developing RBCs	↓ Fe supply to the bone marrow ↑ Fe-deficient erythropoiesis	Lead poisoning raise free protoporphyrin. [c]
3. Zinc protoporphyrin (ZPP)	µg/dl	35 µg/dl	Lack of Fe for developing RBCs	↑ ID, inflammatory disorders, exposure to lead	Lead poisoning raise free protoporphyrin. [c]

4. Serum Ferritin (sFer)	µg/L	20–200	Size of Fe stores	↓ ID ↑ Fe storage disorder	Ferritin increased in inflammation; need to correct for inflammation
5. Total Fe binding capacity (TIBC)	µg/dl µmol/l	240 to 450 µg/dl	Total capacity of circulating transferrin bound to Fe	↓ Inflammatory disorders ↑ ID, low in inflammatory disorders	Sensitive but not specific. [c]
6. Transferrin saturation	%	25–35	Value of serum Fe divided by the TIBC	↓ IDA ↑ Fe overload or hemochromatosis (45%>) [d]	-
7. Transferrin receptor (sTfR)	µg/ml	1.9–5.0	Fe transporter	↓ Infection ↑ Likely to be IDA if anemia presents	Affected by the rate of erythropoiesis
8. Total Body Fe (TBI): Ratio of transferrin receptor to ferritin– [log (TfR/ferritin ratio) –2.8229] /0.1207 [e]	mg/kg	Average total body Fe content 35-45 mg/kg	Measure of body Fe status	↓ ID ↑ Hemochromatosis	-
9. Hepcidin	ng/ml	1–55	Fe regulator	↓ Hemochromatosis and Fe overload ↑ Anemia of inflammation, chronic kidney disease and Fe-refractory IDA [f]	Assay methods and interpretation of results is under development

[a] Partially adopted from (World Health Organization (WHO) & Centers for Disease Control and Prevention (CDC), 2007)
[b] Laboratory reference value used in International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B)
[c] (Worwood et al., 2017)
[d] (Langini et al., 1993)
[e] (DeLoughery, 2017)
[f] (Nemeth & Ganz, 2006)

2.4 POTENTIAL SUSTAINABLE FOOD-BASED SOLUTIONS FOR IRON DEFICIENCY

ID can be prevented by adopting one of the four following strategies, either alone or in combination: (1) dietary diversification, (2) selective breeding and genetic modification to increase iron content, (3) iron fortification of manufactured foods, and/or (4) iron supplementation with pharmaceutical doses (Hurrell et al., 2010). Nutrition education combined with dietary diversification and food fortification should be included in public health nutrition programs to increase micronutrient intake among the population as part of WHO-recommended food-based approaches (Allen et al., 2006). Although supplementation provides a faster solution to micronutrient deficiencies, food fortification provides a much broader and more sustainable solution (Allen et al., 2006).

2.4.1 Dietary diversity

Dietary diversity generally refers to the consumption of foods from a variety of food groups over a given period time. For many people, a diverse diet means increasing the quantity, as well as the range, of micronutrient-rich foods they consume daily (Allen et al., 2006). This ideally looks like a balanced combination of increased consumption of iron rich food, especially animal products, as well as colorful fruits and vegetables containing ascorbic acid (which enhances non-heme iron absorption), and reduced intake of tea and coffee (which contain tannins, inhibitors of non-heme iron absorption) (Hurrell, 2002; World Health Organization (WHO) & Food and Agricultural Organization of the United Nations (FAO), 2004). However, diversifying the diet may bring several challenges to those in poorly-resourced settings. For instance, producing and purchasing “nutritious” foods may hinder the achievement of broader diversity goals (Allen et al., 2006). However, culturally-appropriate and low-cost micronutrient-rich foods are being explored to feed those in poorly-resourced communities (Allen, 2003; De Pee et al., 2000; Gibson et al., 2000; Ruel, 2001).

2.4.2 Supplementation

Supplementation comes in many forms, generally as pills, capsules, or syrups, to provide an optimal dose of a specific nutrient(s). Developing countries have widely used nationwide supplementation programs to supply a variety of nutrients to at-risk populations. For instance, iron and folic acid are broadly distributed to pregnant women, and vitamin A to infants, children

under five years of age, and post-partum women (Allen et al., 2006). However, different micronutrients require different doses, frequencies, and vehicles. For example, a single high dose of Vitamin A supplement in oral drop form is sufficient to maintain the body's vitamin A hemostasis for four to six months (Allen et al., 2006). A bi-weekly combination of elemental iron (60mg), along with Folic acid (400µg) is recommended to treat both IDA and prevent neural tube defects among Bangladeshi adolescent girls and NPNL women (National Institute of Population Research and Training (NIPORT) and Mitra and Associates and ICF International, 2013).

WHO recommends 60 mg of elementary iron tablets daily for menstruating adult women and adolescents for three consecutive months in a year where anemia prevalence among these groups is more than 40% (World Health Organization, 2020). It further suggests that 60 mg elementary iron is equal to 300 mg of ferrous sulfate, 180 mg of ferrous fumarate, or 500 mg of ferrous gluconate. Among forms of oral iron supplementation, ferrous sulfate and ferrous gluconate are preferred because of their high availability and low cost (DellaValle, 2013).

Oral iron supplements are commonly prescribed to pregnant women in developing countries since they enter the pregnancy stage with low body iron status (C. S. Gautam et al., 2008). However, oral iron supplements do not immediately result in increased Hb; they take about three to four weeks to impact iron status (DeLoughery, 2017). Reasons for a lack of response to oral therapy include active blood loss, malabsorption (either anatomical or inhibiting factors), incorrect diagnosis (e.g. anemia not due to ID), and non-compliance (Beutler et al., 2003). Supplementation program management is critical, requiring regular purchasing of supplements, an effective distribution system, and high-level population compliance. Lack of supplies of micronutrient supplements and poor compliance are noted as major barriers to successful, sustainable nutrient supplementation programs (Allen et al., 2006).

2.4.3 Biofortification

Since traditional approaches to combatting micronutrient deficiencies such as supplementation and dietary diversification have unique challenges, a long-term sustainable complementary solution known as "biofortification" has been proposed by researchers and nutritionists (Combs et al., 1997; W. H. Pfeiffer & McClafferty, 2007; R. M. Welch, 2002; P. J. White & Broadley, 2005). Biological fortification or biofortification is a procedure by which crops are "*nutritionally enhanced using agronomic practices, conventional plant breeding*

practices, and/or genetic modification" (Codex Alimentarius Commission (CAC), 2007). Biofortification brings together agriculture and nutritional science to deal with persistent global micronutrient deficiencies (García-Casal et al., 2016). Several micronutrients have the potential for biofortification, such as iron, zinc, provitamin A carotenoid, and amino acid and protein, and common biofortification vehicles include rice, beans, sweet potato, cassava, maize, sorghums, and legumes (World Health Organization, 2019). Biofortification can increase the iron content of vegetables, fruits, and grain, whose natural iron content is typically low and variable. Depending on the variety, wheat contains about 25-56 mg/kg per grain, whereas rice contains only about 2-23 mg/kg (Abbaspour et al., 2014). Cereals and legumes have low bioavailability due to their higher phytic acid (Gupta et al., 2013). Plant breeding can increase the iron content of beans and millets (Abbaspour et al., 2014). Biofortification has the potential to be a sustainable solution, as once a biofortified line or cultivar is produced, that cultivar (as seed) could be transferred and grown over and over again. As long as research on biofortified crops includes surveillance (e,g for Fe concentration and bioavailability) to ensure stability of the desired trait(s), this is a sustainable solution and has the potential to end micronutrient deficiencies on a global scale.

A systemic review of three randomized trials on iron-biofortified rice, pearl millet, and beans carried out in Philippines, India, and Rwanda, respectively, reported no significant reduction of ID and anemia; however, the consumption of biofortified foods improved cognitive performance (attention and memory) (Finkelstein et al., 2019). In the double-blind RCT study on iron-biofortified rice among non-anemic Filipino women found increased sFer and body iron (Haas et al., 2005). In the Rwandan bean study, while Hb and sFer levels of iron-depleted women increased due to the consumption of iron-biofortified beans, but the increase of iron in their diets did not improve their work efficiency (Haas et al., 2016; Luna et al., 2020).

2.4.4 Fortification

The World Health Organization (WHO) has documented food fortification as a valid technology for reducing global micronutrient deficiencies, since it could benefit broader populations and has the potential to be a more sustainable solution compared to supplementation or dietary diversification (Allen et al., 2006; Tontisirin et al., 2002; World Health Organization (WHO) & Food and Agriculture Organization of the United Nations (FAO), 2006). Food fortification is defined as *"the addition of one or more essential nutrients to a food whether or*

not it is normally contained in the food, for the purpose of preventing or correcting a demonstrated deficiency of one or more nutrients in the population or specific population groups" (Codex Alimentarius Commission (CAC), 2013). The common micronutrient fortificants are iodine, iron, vitamin A, vitamin D, vitamin E, vitamin C, and B-complex vitamins (Allen et al., 2006; Chadare et al., 2019; Fulgoni et al., 2011; Hombali et al., 2019). Several high-income, middle-income, and low-income countries have adopted fortification since it can reduce large-scale micronutrient deficiencies (Darnton-Hill & Nalubola, 2002; Dwyer et al., 2015).

Allen and colleagues (2006) documented the major advantages and disadvantages of food fortification. Among the advantages are (1) regular consumption of well-balanced fortified food maintains the body's optimal nutrient store; (2) the fortification of most consumed foods meets large population needs; (3) fortification does not require alteration of food items/patterns since commonly consumed foods are fortified; (4) fortification requires less focus on the delivery system than other interventions since it can use the existing food chain and multiple nutrients can be fortified together to maximize nutritional achievement at a low cost; and (5) there is a minimal risk of toxicity compared to other food-based options (Allen et al., 2006). As for the disadvantages of fortification, Allen et al. (2006) cite (1) it is not a substitute for a good diet since it is mainly limited to single or few micronutrients; (2) it delivers higher micronutrients to those who may not require them; (3) fortified foods may not meet the micronutrient demands of infants and young children since they consume small amounts of actual foods; (4) fortified foods may not reach the poorest segment of the population due to its low purchasing power or an underdeveloped distribution channel; (5) there are challenges with finding appropriate staple food vehicles for single or multiple fortification; (6) fortification technology, interactions, alterations of food properties (such as colour flavour, and stability) and acceptability have yet to be fully explored; (7) fortified foods may be costly compared to regular foods.

2.5 IRON FORTIFICATION

Although ID can be treated with multiple food-based approaches, fortification remains the primary approach to treat and prevent IDA (J. L. Miller, 2013). It is recommended that staple foods be fortified to maximize the fortification benefit (Allen et al., 2006). Of these, wheat flour, pasta, rice, milk, cocoa products, salt, sugar, sauce (soy and fish), juice and soft drinks, and

cereal-based complementary foods are the common food vehicles used for iron fortification (Allen et al., 2006). Regarding iron fortificants, ferrous sulfate and ferrous fumarate are commonly used because of their higher bioavailability and low cost (Allen et al., 2006). Additionally, several studies have reported that Fe-EDTA (Ethylene diamine tetra-acetic acid) remains bioavailable even after a complex interaction with anti-iron absorbents (Heimbach et al., 2000; Hurrell et al., 2000; Yeung et al., 2004). EDTA is a chelating agent affected by pH levels, and ferric iron has the highest stability to bind with EDTA. Furthermore, EDTA protects iron from binding with inhibitors like phytate and polyphenols, thus, increasing Fe absorption (Dueik et al., 2017; Ginanjar et al., 2018). Iron absorption increases when it is formed as NaFeEDTA, compared to iron added as ferrous sulfate (Bothwell & MacPhail, 2004). The International Nutritional Anemia Consultative Group (INACG) strongly recommends NaFeEDTA as a potential iron fortificant for use in developing countries (Dueik et al., 2017).

Iron fortification as NaFeEDTA is found to be most efficacious when cereal- and legume-based products such as whole-wheat flour, cornmeal, and soybean are used as food vehicles (Bothwell & MacPhail, 2004). Other studies have argued that NaFeEDTA is not equally effective for all food vehicles (Bothwell & MacPhail, 2004; Hurrell et al., 2004). Additionally, the effectiveness of NaFeEDTA as a potential Fe fortificant has been confirmed, with no direct toxic effects associated with fortifying foods with 5-10 mg of NaFeEDTA. One study assessed the acceptability level of NaFeEDTA-fortified noodles and reported no change in consumer acceptability (Le et al., 2007).

A meta-analysis of 60 iron-fortified trials reported that 42% of iron fortified foods are cereal based, 14% are salt based, 11% are based on fish and soy sauces, and 11% are based on milk products (Gera et al., 2012). The review article further reported that 28% of iron fortificants use ferrous sulfate, 20% NaFeEDTA, 13% electrolytic iron, 8% ferric pyrophosphate, 7% ferrous fumarate, 3% hydrogen-reduced iron, 3% heme or ferric (III) phosphate, 2% amino acid chelates, and 1% iron gluconate or ammonium citrate. Others reported that the total iron content of fortified foods (natural iron content of the host food vehicle + iron fortificant) should not exceed the Upper Intake Level (UL) of iron (FAO et al., 2010). A cost-effectiveness study reported a high cost-benefit ratio of iron fortification (cost US $0.12 vs benefit US $4.04) and concluded that 24% of IDA can be reduced by spending only $0.10 USD (Allen et al., 2006).

2.6 IRON FORTIFICATION: SUCCESSES AND CHALLENGES

Research on the effectiveness of iron fortification using various food vehicles and fortificants has been conducted in many countries around the globe. These studies have concluded that iron-fortified foods can potentially increase body iron levels, reducing the global burden of IDA. The aforementioned meta-analysis that included 60-iron fortification trials reported that iron-fortified foods significantly increased Hb and sFer levels in the experimental groups, and noted a reduced risk of developing ID and anemia (Gera et al., 2012).

Table 5 presents a review matrix summarizing evidence from rigorous studies that evaluated the causal effects of iron-fortified foods on iron status in studies conducted between 2000 to 2019, which used different fortificants on different food vehicles. In developing this matrix, the researcher searched the PubMed database using the search term 'iron fortification' and the article types: 'clinical trial,' 'controlled clinical trial,' 'evaluation studies,' 'meta-analysis,' 'randomized controlled trial,' and 'systematic reviews.' Only human studies were included in the review matrix. The studies were given grades based on their evidence's degree of certainty and according to whether the results reflected the intervention's impact: high, moderate, low, and very low (Balshem et al., 2011). Grading was downgraded under the following conditions: if there was potential selection bias, if the study lacked a control group, or if no detailed information was provided on the study design. The details about the grading are provided at the end of the matrix.

Table 2.5 is sub-divided into staple food trials (Table 2.5a) and complementary and condiments food vehicle trials (Table 2.5b). Table 2.5a shows that wheat flour, rice, and corn flour were the most common staple food vehicles used with various iron fortificants in the trials. The most rigorous studies (double-blind RCTs) found that iron-fortified staple foods significantly reduced anemia and increased iron stores during six month trials; however, iron-fortified foods were the most effective for those who were iron depleted or anemic, regardless of age. It also suggests that staple foods should be the first choice for fortification after considering the cultural aspects of food (Allen et al., 2006). Table 2.5b shows that iron-fortified complementary and condiment foods increase iron stores and reduce anemia; however, the period between consumption of these foods and improvements was slightly longer than for iron-fortified staple foods, likely because insufficient amounts of these foods were consumed at a lower frequency.

Table 2.5a Review matrix of iron-fortified staple food trials

Author, year ***journal***	**Country**	**Fe Fortificants**	**Fe dose**	**Food vehicle**	**Frequency**	**Study duration**	**Study population and Sample size**	**Results**	**Certainty of the evidence (Grade)***
(García-Casal & Layrisse, 2002) *Nutr Rev*	Venezuela	Ferrous fumarate	50 mg of Fe 20 mg of Fe	Precooked maize flour Wheat flour	No data	7 years	Children 7, 11 and 15 years N= no information	↑ sFer	⊗OOO Very low certainty No control group
(Moretti et al., 2006) *Am J Clin Nutr.*	India	Micronized ground ferric pyrophosphate (MGFP)	10 mg Fe/g	Rice-based lunch meal	Daily	7 months	6-13 year (iron depleted) N=184	- ↑ sFer - ↓ Iron deficiency anemia	⊗⊗⊗⊗ High certainty Double-blind RCT
(J. Sun et al., 2007) *Asia Pac J Clin Nutr*	China	Electrolytic Fe, ferrous sulfate and NaFeEDTA	Electrolytic Fe 60 mg Fe/kg, FeSO4 30 mg Fe/kg NaFeEDTA 20 mg Fe/kg	Wheat flour	Daily	6 months	Children 11–18 years N=400	- ↑ Hb electrolytic Fe, FeSO4 and NaFeEDTA fortified wheat flour group. - ↑ Hb in NaFeEDTA in electrolytic Fe and FeSO4 Fe groups.	⊗OOO Very low certainty Study design not clear
(Andang'o et al., 2007) *Lancet*	Kenya	NaFeEDTA and electrolytic Fe	NaFeEDTA (56 mg/kg and 28 mg/kg) Electrolytic Fe (56 mg/kg)	Maize flour	5 times/wk	5 months	Children 3–8 years N=516 (260 girls and 256 boys)	- ↑ Fe-status indicators in NaFeEDTA (56 mg/kg) group. - ↓ ID in NaFeEDTA (28 mg/kg) - No improvement in Electrolytic Fe group	⊗⊗⊗⊗ High certainty Randomly assigned control group
(Van Stuijvenberg et al., 2008) *J Nutr*	South Africa	NaFeEDTA, electrolytic Fe and ferrous fumarate	NaFeEDTA 2.35 mg Ferrous fumarate 4.70 mg Electrolytic Fe 8.30 mg	Brown bread	5 d/wk	8 months	Children 6–11 years N=361	- No changes in Hb, transferrin saturation, sFer, Fe, and sTfR levels.	⊗⊗⊗⊗ High certainty RCT

(Seal et al., 2008) *Public Health Nutr.*	Zambia	No data	14 mg/400 g	Maize meal.	No data	12 months	Children 6–59 months Children and adolescents 10-19 years Adults 20–49 years N=157, N=212, and N=118 (respectively)	- ↓ Anemia in children by 23.4% but no changes in adolescents or women.	⊗OOO Very low certainty No control group and design not clear
(Angeles-Agdeppa et al., 2008) *Int J Vitam Nutr Res.*	Philippines	Extruded ferrous sulfate (ExFeSO4) Extruded micronized dispersible ferric pyrophosphate (ExFeP80)	-	Rice meal	5 d/wk	6 months	Children 6–9 years (anemic) N=180	- ↑ sFer - ↓ Anemia - ExFeSO4 and ExFeP80 had similar effect	⊗⊗⊗⊗ High certainty Double-blind RCT
(Beinner et al., 2010) *J Nutr*	Brazil	Micronized ground ferric pyrophosphate (MGFP)	10.4 mg Fe/g MFP	Rice meal	Daily	5 months	Children 6–24 months (mild anemia group) N=175	- ↑ sFer - ↓ Anemia	⊗⊗⊗⊗ High certainty Double-blind RCT
(Aguirre Arenas et al., 2013) *Gaceta Sanitaria*	Mexico	Ferrous fumarate	No data	Corn flour	No data	9 months	Children 6–24 months N=194	- ↓ Anemia	⊗OOO Very low certainty Non-experimental study

***GRADE Working Group grades of evidence** (Balshem et al., 2011)
High certainty: Very confident that the true effect lies close to that of the estimate of the effect.
Moderate certainty: Moderately confident in the effect estimate: the true effect is likely to be close to the estimate of the effect, but there is a possibility that it is substantially different.
Low certainty: Confidence in the effect estimate is limited: the true effect may be substantially different from the estimate of the effect
Very low certainty: Very little confidence in the effect estimate: the true effect is likely to be substantially different from the estimate of effect

Table 2.5b Review matrix of iron-fortified complementary and condiment food trials

Author, year ***journal***	**Country**	**Fe Fortificants**	**Fe dose**	**Food vehicle**	**Frequency**	**Study duration**	**Study population and Sample size**	**Results**	**Certainty of the evidence (Grade)***
(Van Thuy et al., 2003) *Am J Clin Nutr*	Vietnam	FeNaEDTA	10 mg Fe/d	Fish sauce	6 d/wk	6 months	Women 17–49 years N=152	- ↑ Hb - ↑ sFer - ↓ sTfR - ↓ ID - ↓ Anemia in Fe fortified groups.	⊗⊗⊗⊗ High certainty Double-blind RCT
(Zimmermann et al., 2003) *Am J Clin Nutr*	Morocco	Ferrous sulfate hydrate encapsulated	1 mg Fe/g	Salt (bread, fava beans)	3-d weighed food records	9 months	Children 6–15 years N=377	- ↓ IDA by 27% - ↑ Hb, sFer, sTfR, and ZPP in Fe fortified group.	⊗⊗⊗⊗ High certainty Double-blind RCT
(Van Thuy et al., 2005) *J Nutr*	Vietnam	NaFeEDTA	9 mmol (500 mg) Fe/L	Fish sauce	Daily	18 months	Women 16–49 years N=576	- ↓ IDA by 18.3% - ↓ Anemia by 16.2%	⊗⊗⊗⊗ High certainty Double-blind RCT
(Zimmermann et al., 2005) *Am J Clin Nutr*	Thailand	Ferrous sulfate, electrolytic Fe, or hydrogen-reduced Fe	12 mg Fe/d	Wheat flour baked snacks	6 d/wk	8 months	Women 18–50 years N=330	- ↑ SFer and Fe stores in Fe fortificants groups.	⊗⊗⊗⊗ High certainty Double-blind RCT
(Chen et al., 2005) *Food Nutr Bull.*	China	NaFeEDTA	29.6 mg Fe/100 ml	Soy sauce	No data	18 months	Children >= 3 years N=14000	- ↑ Hb and sFer in Fe-fortified group. - ↓ Anemia (~10% to 25%)	⊗⊗⊗⊗ High certainty Double-blind RCT
(Ziegler et al., 2009)	United States	Ferrous sulfate	7.0–7.5 mg	- Medicinal Fe - Fruit-cereal	Daily	23 months	Infants 4–9 months (followed up at 2 years)	- ↑ sFer level and ↓ sTfR	⊗OOO Very low certainty

Am J Clin Nutr							N=159		Control group was contaminated.
(Biebinger et al., 2009) *Br J Nutr*	Kuwait	Ferrous sulfate (encapsulated) and H-reduced Fe powder	Ferrous sulfate (encapsulated) 10 mg Fe H-reduced Fe powder 20 mg Fe	Wheat flour biscuits	5 d/wk	5 months	Women 18–35 years (low body Fe stores) N=279	- ↑ sFer by 88% - ↑ Body Fe by 1.28 mg/kg in Ferrous sulfate group - No changes in H-reduced Fe group.	⊗⊗⊗⊗ High certainty RCT
(Miglioranza et al., 2009) *Public Health Nutr*	Brazil	Elemental Fe in the form of H2-reduced Fe	9.8 mg Fe/100 g	Corn flour-derived biscuits, cakes and pies	No Data	6 months	Children 7–14 years N=162	- ↓ ID by12.4% - ↓ IDA by 13.7%	⊗OOO Very low certainty No control groups and design not clear
(Zimmermann et al., 2010) *Am J Clin Nutr*	Cote d'Ivoire	Electrolytic Fe	20 mg Fe/d	Biscuits	4 times/wk	6 months	Children 6–14 years N=139	- No differences in Fe status, anemia, or hookworm prevalence	⊗⊗⊗⊗ High certainty Double-blind RCT
(Lozoff, 2012) *Arch Pediatr Adolesc Med*	Chile	Nothing reported	High dose: 12.7 mg/L Low dose: 2.3 mg/L	Infant formula	Daily	6 months but followed up at 10 years	Infants 6–12 months N=473	- ↓ IQ, arithmetic achievement, visual perception, and motor coordination in high dose Fe group	⊗⊗OO Low certainty RCT (attrition rate 56.6%)

***GRADE Working Group grades of evidence** (Balshem et al., 2011)
High certainty: Very confident that the true effect lies close to that of the estimate of the effect.
Moderate certainty: Moderately confident in the effect estimate: the true effect is likely to be close to the estimate of the effect, but there is a possibility that it is substantially different.
Low certainty: Confidence in the effect estimate is limited: the true effect may be substantially different from the estimate of the effect
Very low certainty: Very little confidence in the effect estimate: the true effect is likely to be substantially different from the estimate of effect

The challenges of fortifying foods with iron include: (1) providers face challenges in properly implementing food fortification programs, and (2) consumers are challenged by limited knowledge, awareness, and mixed perceptions. However, proper planning and active policy implementation can reduce these barriers. An earlier study gathered both technical and practical barriers of food fortification at different stages, such as at design, formulation, production, evaluation, and implementation (Uauy et al., 2002). Key technical barriers are insufficient clinical and epidemiological research, inappropriate selection of food vehicles, unsuitable choices of fortificants, unknown bioavailability, actions of enhancers/inhibitors, lack of stability, low acceptability, and safety issues, while practical barriers include problems with program management and insufficient advocacy/legislation (Uauy et al., 2002). A recent article shares the conclusions of earlier studies by emphasizing the challenges faced by all countries: the need for an ideal food vehicle for fortification, a supply chain reaching targeted populations, control of overconsumption among the non-target group, and regular monitoring of nutritional status (Dwyer et al., 2015). WHO has advised each member state to follow four major steps before carrying out iron fortification programs: (1) quantify the daily iron intake of the risk group for ID, (2) evaluate the bioavailability of the diet, (3) estimate iron intake in comparison with bioavailability (based on dietary iron bioavailability), and (4) calculate the iron deficit and include this calculated amount in iron-fortified foods. However, in following these steps, countries are faced with major challenges, such as those mentioned above (Allen et al., 2006). Mehansho (2002) lays out the specifics of these challenges, indicating that to implement a successful iron fortification program, two groups of tasks must be done simultaneously: (1) production and distribution; and (2) education and social engagement and to have marketing under the two groups (Mehansho, 2002). Apart from implementing the iron fortification program, finding suitable iron fortificants and food vehicles remains a big challenge. Furthermore, discolouration of the fortified foods due to the use of NaFeEDTA and ferrous fumarate as iron fortificants adds an extra burden on iron fortification (Van Stuijvenberg et al., 2008). Another study recommends evaluating iron-fortified foods in realistic scenarios using studies with a rigorous design before a country-wide scale-up (Van Thuy et al., 2003). At the consumer level, nutritional knowledge has been found to be vital for a successful iron fortification program (X. Sun et al., 2006). And earlier study reported that providing nutritional knowledge with focused

nutritional education increases (20%) the frequency of consuming iron fortified foods (Pounis et al., 2011).

2.7 GENERAL DISCUSSION OF THE LITERATURE REVIEW

Iron fortification has the potential to reduce the global ID and IDA burdens, and to meet the body's iron demands among the most ID. Since iron has a high upper tolerable intake level (UL, 45 mg/day) well beyond the recommended dietary allowance (RDA), iron-fortification is likely not to pose adverse toxic effects to the target population of the fortification intervention. While there are challenges in implementing successful iron-fortification programs, active engagement of all stakeholders, including the target community, will help result in a successful iron fortification program.

As seen in the review matrix in Table 5, numerous studies across the globe have been conducted to investigate the effects of iron-fortified foods using various fortificants and vehicles. All studies reported increased iron status biomarkers (e.g. sFer, Hb) in participants that had consumed iron-fortified foods. However, different magnitudes of effects were observed in anemia, ID, and IDA prevalence, and there were clear variations in the effects of iron-fortified foods on iron status. This variation occurred because of differences in the bioavailability of iron-fortified food, and differences in baseline body iron status.

Because the bioavailability of food vehicles vary, the effects of consuming iron-fortified foods are also variable. Therefore, before iron fortificants and food vehicles are selected for a study or a program, extensive bioavailability research is required. Fortificants with high Fe bioavailability paired with the appropriate food vehicles do not necessarily result in high iron absorption. The level of iron absorption depends on the body's baseline iron status. Iron absorption is greater in an iron depleted individual compared to and iron replete individual, all other factors being equal (Abbaspour et al., 2014; Byrnes et al., 2002; Zijp et al., 2000). Apart from Fe bioavailability and body iron status, other reasons for variable effects could be an ineffective study design, food vehicles unsuitable for fortification or inappropriate for the selected age groups, and/or failing to control for potentially confounding factors.

Another problem in the iron research could be considering anemia as an outcome variable. The body develops IDA when body iron stores fall to a very low level, which hinders normal RBC production, the last stage of ID (Camaschella, 2015; Herbert, 1987; Johnson-

Wimbley & Graham, 2011). Hb levels of non-iron depleted individuals will not increase. It is also possible that researchers are biased towards positive results, which may lead to the publication of fewer negative results. This publication bias is a common phenomenon among researchers who wish to publish positive results in journal articles (Dwan et al., 2008), but researchers need to publish evidence-based results, whether they be positive or negative, to support the direction of future studies and programs. Problems may also exist with the evaluation of iron-fortified public health programs, hence also limiting knowledge (SUSTAIN, 2011). The group SUSTAIN (2011) argues that there is an urgent need for iron fortification to be efficacious and effective to reduce gaps in knowledge. Despite these issues, recently-adopted guidelines and regulations have improved the state of research. For instance, all RCTs must be registered in various trial registry databases if researchers wish to publish their results in scientific journals (DeAngelis et al., 2004).

Although the success of iron-fortified food programs depends on biological dimensions, socio-demographic characteristics and policy support play an equally major role. Culture, and not just the food culture of a society, strongly determines success. For instance, gender inequality could hinder the expected outcome even of a well-designed food fortification program. Gender discrimination against women is widespread, and they have less access to power and resources (Food and Agriculture Organization of the United Nations, 2012). Gender discrimination could have a critical impact on iron fortification because women are vulnerable to ID, and if their livelihood, meals, and nutrition are threatened, so is their iron status. One study reported that malnutrition related to gender discrimination is widespread in South Asia (Sethuraman & Duvvury, 2007). Another study reported that household protein consumption was higher when the mother was educated and that the father's education mattered less than the mother's when it came to the family's healthy eating (Rashid et al., 2011).

In Bangladesh, women play a major role in household-level food consumption and decisions about the daily food consumption (Yunus, 2018); however, it is the men who actually buy the food (Rashid et al., 2011). It has been suggested that in Bangladesh, education, occupation, the gender of the head of household, household size, food prices, and household age-sex composition are the main determinants of micronutrient intake (Rashid et al., 2011). Therefore, an iron fortification program needs to consider the socio-demographic perspectives of the target population.

Another dimension to consider is the fortification policy of the target country. One study highlighted the reluctance of policymakers to fortify foods even if the evidence advises otherwise (Dwyer et al., 2015). While it has been reported that short-term results, such as with iron supplementation, are preferred by policymakers who often fail to capture and understand the underlying causes of micronutrient deficiencies, stakeholders must consider all sustainable options (Rashid et al., 2011). As for anemia and ID control programs, supplementation and micronutrient powders are considered to be short-term strategies; fortification (staple, complementary, and condiments) as medium-term; and dietary diversity, nutritional education, biofortification and socioeconomic development as long-term (Pasricha et al., 2013).

Fortification programs can be implemented on various scales: mass fortification (generally mandatory for the general public), universal fortification (animals and humans), and targeted fortification (age-specific groups, either mandatory or voluntary); however, the context for mandatory and voluntary programs depends on the significance of the problem (Allen et al., 2006; Nutrition International, 2020). Mass iron fortification may be a suitable public health solution for iron deficiency, particularly for the high ID and anemia prevalence countries because the body has high tolerance for higher intake levels of iron (UL of 45 mg/day) compared to its RDA (8-18 mg/day, depending on age and gender) (Institute of Medicine, 2003). In 2015, Bangladesh adopted its 'National strategy on prevention and control of micronutrient deficiencies, Bangladesh (NSPCMD) (2015-2024),' in which it established fortified foods as one of its means for the population to improve access and affordability to micronutrients (Institute of Public Health Nutrition, 2015). This NSPCMD strategy includes fortification of oil with vitamin A, rice fortification with micronutrients, and fortification of foods with vitamin D; however, there are no specifics for iron fortification. To minimize anemia, ID, and IDA prevalence, the NSPCMD has recommended targeted interventions–iron and folic acid supplementation and micronutrient powder–for pregnant women, NPNL women, children 6–59 months, and young children (Institute of Public Health Nutrition, 2015).

National and international stakeholders should focus on policy advocacy and consider medium- and long-term strategies to reduce micronutrient deficiency. As for the costs and benefits of iron intervention, one study has reported that targeted iron supplementation for pregnant women costs much less than iron fortification for the general population ($800 vs $2000

USD per life saved, respectively); however, iron fortification is considered more sustainable (Darnton-Hill et al., 2005).

CHAPTER 3: GENERAL METHODS AND MATERIALS

The chapter describes the general aspect of the methods used for the cross-sectional study and the randomized controlled trial. The chapter includes details of data collection tools and techniques including blood sample collection, randomization, variables assessed, sample size calculation, data quality protocol and analysis plan. The chapter provides a detailed understanding of the methods and materials used in the study.

3.1 STUDY SITE AND STEPS

The study was conducted over a 4 month period among adolescent girls residing in the Mymensingh district of Bangladesh (Figure 3.1). Prior to any data collection, informed assents were received from n=1260 eligible adolescent girls and informed consents were collected from their respective parents. Next, all consented and assented participants were invited to gather at their respective assigned BRAC adolescent club for blood sample collection at baseline for measurement of body Fe status, and other surveys. This baseline measurement of iron status and all other baseline measurements was analyzed as its own cross-sectional survey. For those electing to continue to participate in the RCT, an 85 day double-blind community-based cluster-randomized controlled trial was carried out to respond to the objectives, and blood samples were collected at the mid- and end-points of the trial. Similar data surveys as baseline were carried out at the end of the study.

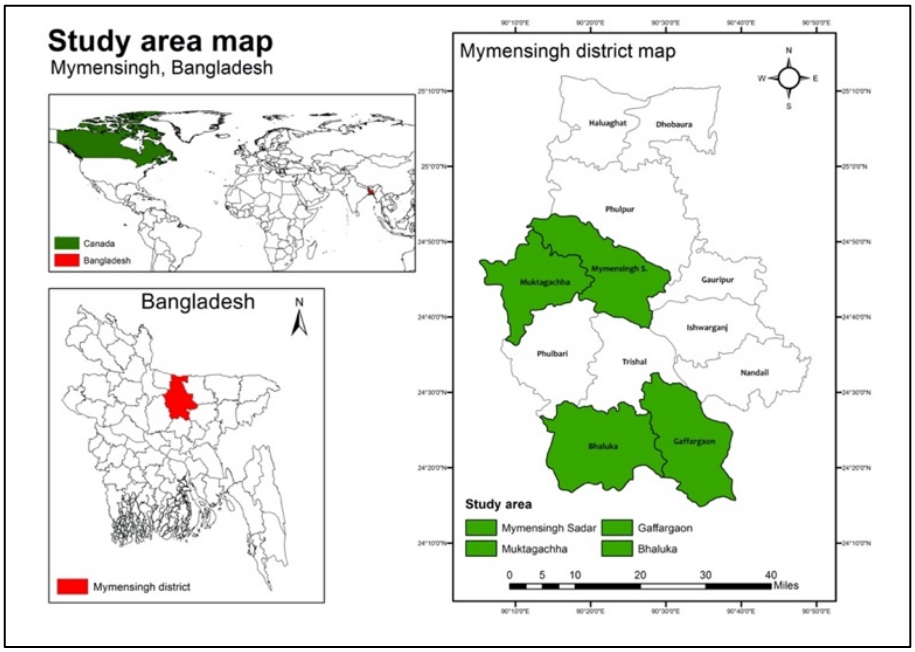


Figure 3.1: Map of study site

The study hypothesized that consuming iron-fortified lentils would increase Fe status of Bangladeshi adolescent girls (Figure 3.2). Furthermore, the study hypothesized that it would increase hemoglobin levels of the girls.

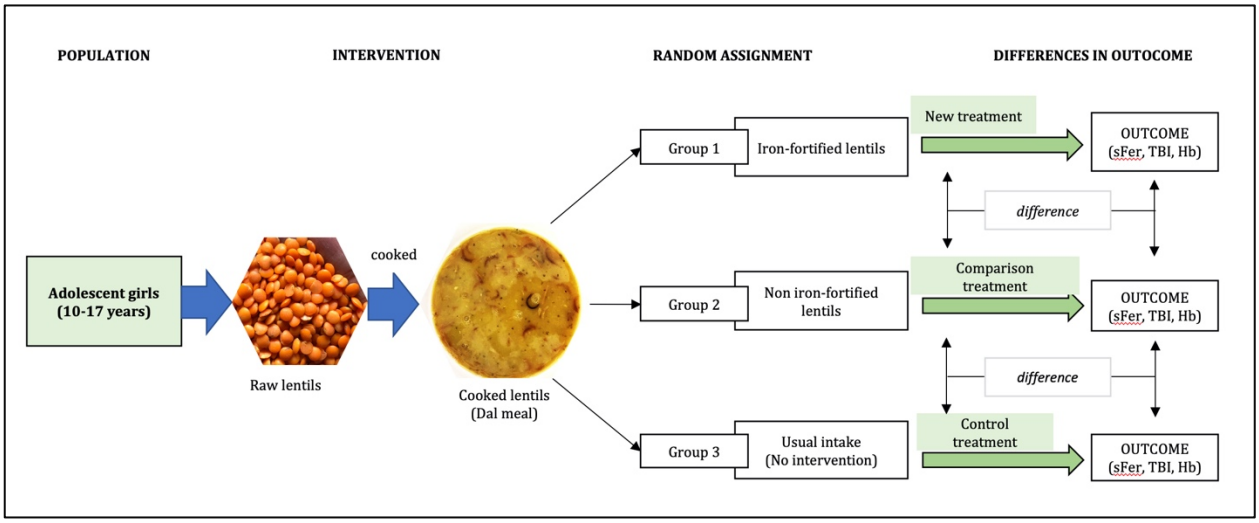


Figure 3.2: Iron-fortified lentil study randomized controlled trial design

There was no data on iron-fortified lentils; however, iron fortification is common in various foods such as wheat flour, pasta, rice, milk, cocoa products, salt, sugar, sauce (soy and fish), juice and soft drinks, and cereal-based complementary foods (Allen et al., 2006). To the

best of the researcher's knowledge, this study was the first attempt to investigate the efficacy of iron-fortified lentils on the Fe status of adolescent girls. The study followed one of the WHO food-based approaches, fortification, as it has potential to be more sustainable to combat the global micronutrient deficiency compared to supplementation, because supplementation has low compliance in resource-poor settings (Huma et al., 2007; Hurrell, 1997b). This study sheds light on staple food-based solutions if it reduces iron deficiency through a plant-based protein, that is also a staple food in South Asia and other parts of the world.

3.2 ETHICAL APPROVAL OF THE STUDY

The study was approved by the following human subjects review boards: University of Saskatchewan Research Ethics Board (Bio#17–177), Canada; Marywood University Institutional Review Board (IRB#1139116–2), USA; and Bangladesh Medical Research Council (BMRC/NREC/2016–2019/455), Bangladesh. A copy of the consent and assent forms were provided to both the parents and girls (Appendices A). Participants were given the complete address of the study investigator for future communications or questions. Details of the study, including its objective, data collection points, deworming, and venous blood sample collection, and the risks and benefits of participation in the study were described explicitly to each of the participants and their parents both in writing and verbally by the researcher. It was mentioned that participation in the study was voluntary and that participants could withdraw from the study at any time without further consequences.

3.3 DATA COLLECTION SUMMARY

Three types of data were collected in four consecutive steps. First, (baseline data point), cross-sectional survey data and venous blood samples were collected. In the second step, the feeding trial was conducted, where data was collected on the amount of cooked lentils consumed and the residual amount following consumption. A research assistant completed the feeding trial data, as it was a measurement-related task. It helped to minimize the burden on the participants and ensured reduction of erroneous data. Midpoint data points were determined at the midpoint of the feeding trial, and venous blood samples were collected at this time. In the fourth step, end line survey and venous blood samples were collected. All survey and feeding trial data were collected digitally by the Open data kit (ODK) application under an android-based platform using a 7"

tablet. Each tablet was connected to the internet, and the data were sent to BRAC's secure server at the end of each interview. The data manager saw the data in real time to ensure data quality.

The study was carried out among n=1195 adolescent girls aged between 10–17 years. A detailed flowchart of the study is shown in Figure 3.3. This figure was published in the earlier protocol manuscript (Yunus, Jalal, et al., 2019). BRAC adolescent clubs situated in the Mymensingh district were randomly selected to participate in the study. Initially, research assistants visited the clubs, where they briefed potential participants who then were sent an invitation to participate in the study. The research assistants personally visited each of the girls and their parents at their homes. Prior to their agreement to participate in the study, they read the consent and assent forms. Each of the potential participants was given time to understand the study and discuss it with their parents. The study research assistants gave the girls one day to decide if they were willing to participate in the study. Following the receipt of the signed consent and assent forms, a formal invitation with date and location for the draw of the baseline blood samples (6 ml of venous blood) was sent to participants. Next, participants were invited to take part in the survey data collection. All participants were dewormed at baseline prior to the survey.

Adolescent girls aged 10-17 years were randomized to one of the three groups; one group consumed Fe-fortified lentils, the second group consumed non-Fe-fortified lentils, and group three (the usual intake group) received no lentils. The two groups who consumed cooked lentils (either iron-fortified or non-fortified lentils) were served approximately 200 g of cooked lentils (the equivalent of 37.5 g raw lentils) five days a week for 85 feeding days (about four months) using a locally acceptable recipe. The recipe was the same for both groups. The third group was not served any cooked or raw lentils and received no restrictions on lentil consumption. Additionally, the study did not restrict any group's consumption of other foods. Upon completion of 85 feeding days, endpoint survey data and venous blood samples were collected.

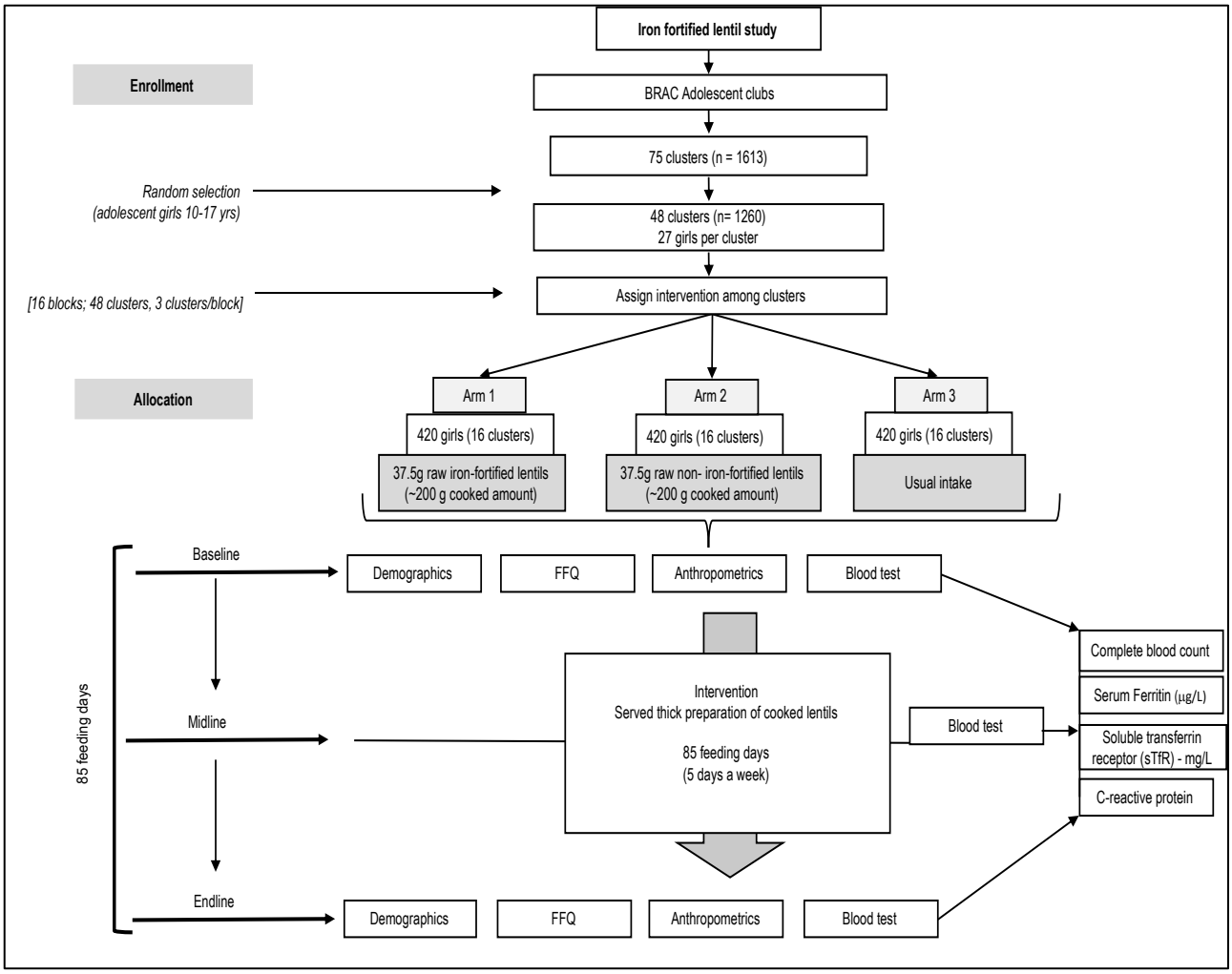


Figure 3.3: Study profile
Source: (Yunus, Jalal, et al., 2019)

3.4 DATA QUALITY MEASUREMENT

3.4.1 General precautions for ensuring data quality

The research assistants implemented the close-ended questionnaire via face-to-face interviews using a 7" Tablet-Android-based ODK app. All tablets were connected to the Internet. A research assistant (RA) sent the data to BRAC's secure server, allowing us to check the data coming to the server in real-time. The study took the following steps to ensure the data quality:

- All recruited research assistants were free of any form of conflict of interest concerning the study participants. This helped to minimize "acquiescence bias."

- All enumerators and supervisors were directly trained on the questionnaire by the same researchers, field managers, and data manager for the same duration. It helped the research assistant to understand the format of the questionnaire and to adhere to it with the same degree of questioning and probing for each participant. First, a paper-based hard copy questionnaire was used to train the research assistant. The questionnaires were standardized following several revisions. Second, a revised questionnaire was installed in the ODK app. Data collectors were trained using role play.
- All interviews were carried out separately for each participant in a private setting with the presence of a witness to minimize the social desirability bias.
- Each of the questions was conditionally set within the app meaning that RA will not be able to put unusual numbers/responses.
- The date popped up as a monthly view calendar so that date was entered uniformly, and no mistake takes place.
- Age was entered as a condition set as 'days' – 2 digit 1-31 days; 'month'- 1-12; 'year' after 1999.
- Different symbols set for single and multiple responses and directions on how to complete each question were provided.
- Data managers checked the data daily to see if there are any unusual number and contacted with the respective RA for clarification. All data were secured in the BRAC server.
- Data manager were also assessed, including whether there was a need for additional training for the RAs.
- All participants were identified with their assigned ID. The RA who supervised the data collection kept a master-list (only for the baseline survey).
- All questions and response options were in Bengali; however, data codes i.e., code numbers entered in the server, were in English.

3.4.2 Variable management to ensure data quality (Appendices B)

Screening questions:

- The questionnaire app (each question) was strictly conditionally set logically according to the given response options. Any wrong/unusual information would not let the RA proceed to the next questions.

- All response options were pre-coded within the system.

Demographic information:

- Date of birth: It was enlisted after the following documents sequentially:

    I. Birth certificate

    II. Vaccination card

    III. School record

Food frequency data:

- Each RA was provided a set of standards serving tools for collecting the food consumption data. For example, for rice- a weighed cup was used to serve the 250 g portion of cooked rice. The study replicated the serving size tool samples used previously by BRAC Research Division. The study provided all relevant standard measurement tools (e.g., cup, spoon, bowls, hand-made measurement size) to each RA to calculate the total amount consumed by each participant during the data collection process.

Deworming data:

- Cautions were taken to preserve data on the deworming tablet that was provided to participants. Each parent of the adolescent girls verified the history of deworming (in the last 4 months). The study team also did not push anyone to take the deworming tablet; however, deworming categorical information was collected, such as taken immediately; no, will take later; Refused to take; will consider taking.

Anthropometric measurement:

- Standard techniques and tools were used, such as a digital body weight scale, stadiometer, and measuring tape for participants' weight, height and waist and hip measurements, respectively.
- Interviews and anthropometric measurements were carried out individually in a private setting with a witness present.
- The study measured and entered each of the anthropometric measures three times to minimize measurement error.

Blood sample collection, processing, storing and transporting:

- Fresh blood separation was carried out on the day it was collected and stored at local field site.
- The temperature chart was maintained during the transportation of the blood sample. The study used digital cold-chain box, which had a built-in temperature monitor. RA took the temperature every hour and documented on their temperature log sheet during their travel from field to the central laboratory.
- Blood samples were transported early in the morning to avoid extreme heat.

Intervention (Lentil consumption / compliance):

- The data manager built a separate questionnaire app for collecting the daily lentil consumption data.
- Cooked lentil amount to be served to each participant was calculated automatically.
- The questionnaire app automatically created the required number of pages to be filled out for each participant.
- UID conditions were set to eight digits and direction were marked in red colour.
- RA measured each of the servings before serving the cooked lentils for 85 days to the participants using a rechargeable digital weight measuring scale.
- The RA measured residual amounts for each serving. It helped to calculate the actual amount of cooked lentils that the participants ate. A rechargeable digital scale (by +/- 1 g precision) was used to measure the amount served and residual amount.
- Both the serving amounts (before the meal) and residual amounts (after finishing the meal) were entered electronically using the internet-connected ODK app installed in an Android powered 7” tablet by matching their respective pre-assigned UIDs.

3.5 ANALYTIC APPROACH

Data were analyzed using both descriptive and inferential statistics. There were two phases of data analysis; however, both share a common method, including study design, sampling, and data collection tools and are part of a community-based, cluster-randomized

controlled trial. Normality of all variables was examined, and outliers were identified. The following sub-section sketches the analysis of each phase of data. Data were analyzed using SPSS for Windows PAWS version 25. The significance level was set at $p < 0.05$ for all main effects.

3.5.1 Baseline survey as cross-sectional data (Chapter 5)

The baseline survey of the efficacy trial was treated as its own cross-sectional analysis. Baseline characteristics such as socio-demographics, and Fe related biomarkers such as sFer, sTfR, TBI, Hb, MCV, RDW, CRP, and WBC count were analyzed. Normality was examined with the outcome variables (sFer and TBI). sFer was log-transformed (natural) since it was not normally distributed and TBI was calculated using the log-transformed parameters [log (TfR/ferritin ratio)].

At first, descriptive statistics - frequency and cross-tabulation were carried out among socio-demographics, anthropometrics, Fe related biomarkers, iron related knowledge, food security, food frequency table, handwashing practice, and deworming history of the adolescent girls. Anthropometrics were measured, and details explained as stated in earlier paper (Yunus, Jalal, et al., 2019). BMI was also calculated using the CDC Teen BMI calculator (Centers for Disease Control and Prevention (CDC), 2017). Associations and correlations were computed between categorical and continuous variables by performing the Chi square and Pearson correlations, respectively. Independent student 't' test and ANOVA tests were used to determine the differences between and among groups of continuous variables.

Fe related biomarkers were recoded and created as per cut-off point recommended by WHO: cut-off point of anemia (<12.0 g/dL) and iron deficiency at clinical (sFer < 15μg/L and sFer <15 μg/L + sTfR >5.0 μg/ml) and subclinical levels (sFer < 30 μg/L and sFer <30 μg/L + sTfR >5.0 μg/ml), and iron deficiency anemia (Hb <12.0 g/dL + sFer <15.0 μg/L). Total body iron (TBI) was calculated using the ratio of sTfR to sFer (Cook et al., 2003). Each of the Fe-related outcome variables were set to its severity such as mild, moderate and severe. Mixed model linear regression models were examined to determine the association of sFer and TBI and socio-demographics, anthropometrics, knowledge on Fe rich food and handwashing practice. Those variables that were significantly different between groups at baseline were included in the final models. Since sFer was not conformed with normality assumptions, it was natural log

transformed and presented as percent (%) change instead of beta estimates. To calculate the percent (%) change of the naturally log transformed data, beta coefficient values in the regression model were first exponentiated and subtracted 1 from the exponentiated number and then multiplied by 100 (UCLA: Statistical Consulting Group, 2020).

3.5.2 Community-based randomized controlled trial (Chapter 6)

Changes in participant's iron status over 85 feeding days was the main outcome of the trial. sFer, sTfR, Hb, and TBI were the outcome variables, and to determine the changes, venous blood samples were analyzed at baseline- midline- and end line of all three groups (iron-fortified, non-iron-fortified and no intervention). The significance level was set at $p < 0.05$.

First, it was essential to determine whether randomization was effective. ANOVA was used to assess group differences. It concluded that all three groups were identical in-terms of outcome variables and confounders, as all were equally distributed among all three groups at baseline. Descriptive (mean, SD and frequencies) of the key variables and potential confounders were presented in a tabulated form. One-way ANOVA post-hoc Tukey's test was used to assess differences among interventions among the continuous variables and associations were measured through chi-square among the categorical variables. Since outcome variable sFer was not normally distributed, it was log-transformed (natural). The other two outcome variables- Hb met the normality assumptions, and TBI [log (TfR/ferritin ratio) was not log-transformed because it was calculated by log-transformed parameters. Repeated measure ANOVA (RMANOVA) were used to test group-by-time interactions that were assessed to determine the changes of body iron status (interaction model stated below). Finally, multiple linear regression analysis [mixed models (with the cluster as the random effect)] were used to determine the effect of iron-fortified lentils on iron status of the adolescent girls after adjusting for age and baseline serum ferritin for the post-intervention data (reduced model stated below). These linear mixed effects models simply modeled the fixed and random effects variables as having a linear form. Additionally, study's outcome variables (sFer, Hb, and TBI- each used in the separate model) contributed to additive fixed and random effects (as well as an error term). Other potential confounders such as menarche status, iron intake, BMI, and drinking water from tube well were not included in the final model since they were not significantly associated with the outcome variables (stated below as full model).

Repeated measure ANOVA (RMANOVA):

Interaction model:

$Yi= \beta_0+\beta_1 X_{i1}+\beta_2 X_{i2}+\beta_3 X_{i1}X_{i2}+\varepsilon$……. (3.5.2.1)

Y_i = Body iron status, X_{i1}= baseline iron status (time), X_{i2}= intervention, X_{i1} X_{i2}= baseline iron status (time)*intervention

Linear mixed effect model:

Full model (use for sFer, Hb, TBI separately):

$y_{ij} = \beta_0 + \beta_1 X_{1ij} + \beta_2 X_{2ij} + \beta_3 X_{3ij} + \beta_4 X_{4ij} + \beta_5 X_{5ij} + \beta_6 X_{6ij} + \beta_7 X_{7ij} + \beta_8 X_{8ij} + b_{i1} Z_{1ij} + \varepsilon_{ij}$ ……..
(3.5.2.2)

y_{ij} = Body iron status, β_0 = constant, β_1 through β_8 = fixed effect regression coefficients, X_{1ij} = age, X_{2ij} = menarche status, X_{3ij} = iron intake from 7DDR (diff. baseline and end line), X_{4ij} = BMI, X_{5ij} = Drinking water source (tube well), X_{6ij} = baseline iron status, X_{7ij} = iron-fortified lentils (dummy 1), X_{8ij} = non-iron-fortified lentils (dummy 2), b_{i1} = random effect coefficients, Z_{1ij} = upazilla (random effect variables), ε_{ij} = is the error for case j in group i where each group's error

Reduced model (final model used for sFer, Hb, TBI separately):

$y_{ij} = \beta_0 + \beta_1 X_{1ij} + \beta_2 X_{2ij} + \beta_3 X_{3ij} + \beta_4 X_{4ij} + b_{i1} Z_{1ij} + \varepsilon_{ij}$ ……..….. (3.5.2.3)

y_{ij} = Body iron status, β_0 = constant, β_1 through β_8 = fixed effect regression coefficients, X_{1ij} = age, X_{2ij} = baseline iron status, X_{3ij} = iron-fortified lentils (dummy 1), X_{4ij} = non-iron-fortified lentils (dummy 2), b_{i1} = random effect coefficients, Z_{1ij} = upazilla (random effect variables), ε_{ij} = is the error for case j in group i where each group's error.

Results of the sFer level in the mixed model is presented as percent (%) instead of using beta estimates because it was natural log transformed. Coefficient values in the regression were first exponentiated and then subtracted 1 from the exponentiated number and multiplied by 100 (UCLA: Statistical Consulting Group, 2020). However, Hb and TBI are presented as their original regression coefficient estimates. A dose-response relationship was tested among the iron-fortified lentil groups by measuring changes in sFer level and the amount of iron consumed from the iron-fortified lentils by the iron-depleted population over time. Furthermore, a multinomial logistic regression model was used to estimate the likelihood of changes of ID (at different clinical and subclinical cut-offs) due to consuming iron-fortified lentils after adjusting for age

only. Similar logistic models were carried out for IDA and anemia at various level of severity. Inflammation was identified by CRP >5 mg/L and/or WBC >11.5x10^9/L, and was adjusted for in all models. Effect size was calculated by calculating the mean difference between end line – baseline (0-4 months) and midline-baseline (0-2 months) of sFer, TBI, and Hb among study groups.

3.6 SAMPLE SIZE CALCULATION

Universal fortification provides a sufficient amount of micronutrients in food to improve status of the deficient section of the population, yet prevents excess consumption of micronutrients by population groups who are not deficient, including males. Lentils are widely-consumed in varying amounts in Bangladesh across all socio-economic strata. Fortified lentils, once proven efficacious and effective, will be intended to be consumed by all population groups. Therefore, the study expected to see an effect on body iron status that conforms to universal fortification's purpose in general. Unlike expectations as assumed in the case of supplementation to improve Fe status, a smaller effect size was expected. It may not be clinically-significant, yet, it can significantly influence public health at the population level.

Considering the sample size required for an iron supplementation trial, a higher number of samples were required to detect a statistically-significant change in the mean differences in iron status between pre- and- post intervention. While ID prevalence was known from 2013 statistics, we were not certain what portion of the ID population fell immediately below the cut-off of sFer>12 µg/L. Considering ~30% prevalence of ID (sFer <12 µg/L) among rural adolescents girls, the study estimated a conservative effect of sFer at 5 ±20 µg/L by which the iron-fortified lentils could potentially shift the mean sFer from 22.5µg/L to 27.5µg/L over the course of the study, meaning a reduction of 20% of the current prevalence. Table 3.1 presents the estimation of iron-fortified lentil absorption at different Fe doses (Fe concentration x lentil portion size), given estimated bioavailability.

Table 3.1: Estimates of absorbed iron for Bangladeshi teenaged females from fortified compared to control lentils under different levels of lentil consumption observed over 85 days

Lentil Consumption*	**Low**	**Average**	**High**
Cooked weight, g/day	50	75	100
Dry weight, g/day	25	37.5	50

Bean type	Control	High Fe	Control	High Fe	Control	High Fe
*Iron concentration in lentil***						
Fe content (mg/g)	50	210	50	210	50	210
Fe content (ppm)	50	210	50	210	50	210
Iron from lentil (mg/g)	*1250*	*5250*	*1875*	*7875*	*2500*	*10500*
Iron bioavailability #						
Bioavailable iron (mg/d)	62.5	362.25	93.75	543.4	125	724.5
Percent of EAR (800ug/d)##	7.8	45.3	11.7	67.9	15.6	90.6
Difference (High Fe -Control)	5.3	30.8	8.0	46.2	10.6	61.6
Absorbed Fe (µg/d)	299.75		449.625		599.5	
Absorbed Fe (mg/d)	0.29975		0.45		0.5995	
Absorbed Fe (mg/85d)	25.5		38.2		51.0	
Percent of EAR	37.5		56.2		74.9	

Diane M. DellaValle, PhD, RDN, LDN, 2014
** Based on cooked weight measured for each woman and averaged over 85 feeding days;*
Dry weight x 2.0 = Cooked weight
*** Best estimate*
 # Assumes 5% absorption
EAR (Estimated Average Requirement) = 8 mg/d from DRIs for USA, IOM 2003, p339, for adolescent girls (14-18 yrs)
Assumes constant 50 ppm differential Fe content of controls and high Fe lentils (50 vs 100 ppm)

Considering mean serum ferritin (5 ±20 µg/L), 80% power, 95% CI, and inter-cluster correlation (ICC) 0.025, a total of 48 clubs were selected from the sampling frame (out of 75 clubs). All these clubs (treated as 'clusters') fall under 16 blocks (48/3=16). Sixteen blocks were created for the three-intervention arms of the study. Each block had three clubs and three intervention arms (iron-fortified, non-iron-fortified and usual intake) randomly assigned to each three clubs. Twenty-seven eligible adolescent girls were selected from each club. The study further considered a 20% attrition rate (so, an additional 420 girls) for various possible reasons, such as migrated to other areas, wellness, and moving to other schools etc. The final target sample size was n=1256 girls (Table 3.2).

Table 3.2: Sample size calculation for the Efficacy Study

Options	*Expected difference in mean serum ferritin (d)*	*SD*	*Significance level (a)*	*Power (1-b)*	*ICC*	*Samples per group*	*Samples per cluster*	*# of clusters*	*Total sample with lost to follow up (20%)*
1	6 µg/L	20	0.05	90%	0.025	306	13	72	1144 [305*(100/80)*3]
2	6 µg/L	20	0.05	80%	0.025	219	11	60	822 [219*(100/80)*3]
3	6 µg/L	20	0.05	80%	0.03	223	10	69	836 [223*(100/80)*3]
4	**5** µg/L	**20**	**0.05**	**80%**	**0.025**	**335**	**11**	**59**	**1256 [335*(100/80)*3]**

Diane M. DellaValle, PhD, RDN, LDN and Jalal Chowdhury, PhD, 2014

Cluster sample size calculator:
https://www.abdn.ac.uk/hsru/what-we-do/tools/index.php#panel177

CHAPTER 4: PROTOCOL OF DOUBLE-BLIND COMMUNITY-BASED RANDOMIZED CONTROLLED TRIAL

Based on the findings from the feasibility study (Yunus, 2018), this chapter describes the development of study design of the double-blind community-based randomized controlled trial. This protocol paper chapter published in the Trial journal by BioMed Central (BMC) (Yunus, Jalal, et al., 2019).

4.1 ABSTRACT

Background:

Lentils are generally considered to be a nutrient-dense food, and a good source of iron (Fe). This study aims to establish novel evidence of the effectiveness of the consumption of Fe-fortified lentils in improving the body Fe status and thus cognitive performance, of non-pregnant adolescent girls in rural Bangladesh, compared to consumption of ordinary lentils.

Methods:

We have designed a double-blind (both trial participants and outcome assessors), community-based, cluster-randomized controlled trial among n=1260 Bangladeshi adolescent girls between ages 10–17 years who are non-smoking, not married, non-pregnant, not breastfeeding, and generally healthy at the time of enrollment. The intervention will include three arms: 1) Fe-fortified lentils; 2) unfortified lentils; and 3) usual intake group. Participants will be served a thick preparation of cooked Fe-fortified lentils (37.5 g raw lentil, approximately 200 g cooked) 5 days per week for 85 feeding days (around 4 months) using a locally-acceptable recipe. Lentils were fortified with Fe in the laboratory at the Department of Plant Sciences at the University of Saskatchewan in Canada. A subsample of participants (n= 360) will be randomly invited to be included in cognitive testing.

Discussion:

Socio-demographic characteristics, household food security status, adolescent food habits and cognitive testing will be collected at baseline and end line (4 months). Venous blood samples will be collected at baseline, midpoint (2 months) and end line to measure adolescents' Fe status. Computerized cognitive testing will include five common measures of attentional (3 attention) and mnemonic functioning (2 memory) carried-out by DMDX software. The results of this study will be used to garner support for and to substantiate large-scale production and market expansion of Fe-fortified lentils, and will contribute to knowledge about how to enhance Fe status of adolescents worldwide in resource-poor settings using staple food crops.

TRIAL REGISTRATION: ClinicalTrials.gov NCT03516734. Registered May 24, 2018 - https://www.clinicaltrials.gov/ct2/show/NCT03516734?cond=Iron-fortified+Lentils+to+Improve+Iron+%28Fe%29+Status+in+Bangladesh&rank=1

KEYWORDS: Micronutrient deficiency, Iron (Fe), Food-based approach, Food technology, Fortification.

4.2 BACKGROUND

Based on data from 2011-2012, it has been estimated that 26% of non-pregnant non-lactating (NPNL) women and 17.1% of adolescents aged 12-14 years were anaemic (<12.0 g/dl) in Bangladesh, and that prevalence was higher in rural areas (S. Rahman et al., 2016). National levels of iron deficiency anaemia (hemoglobin <11.5 g/dl and ferritin <15.0 μg/L) in children of 6-11 years was 1.3%, and in adolescents 12-14 years was 1.8% (hemoglobin <12.0 g/dL and ferritin <15.0 μg/L) (ICDDRB et al., 2013). Adolescents females are particularly vulnerable to Fe deficiency with and without anemia due to their significant growth and development, lifestyle and food habits, and their regular menstrual losses of Fe (McNulty et al., 1996; Zimmermann & Hurrell, 2007).

Improving dietary Fe intake via Fe supplementation or high-Fe foods is extremely important among adolescent girls living in Bangladesh and other resource-poor settings in order to maintain nutrition status and growth. Food-based approaches such as Fe fortification have the potential to be sustainable in meeting the increasing demand for Fe among adolescent girls, whereas direct Fe supplementation is often a problem due to poor compliance (Huma et al., 2007; Hurrell, 1997b). Lentils (locally known as 'masur dal' in Bangladesh) are a nutrient-dense staple in South Asia, and a good source of Fe. Lentil fortification and biofortification research has made great progress over the past decade (DellaValle, Thavarajah, et al., 2013; D. Thavarajah et al., 2009; D. Thavarajah, Thavarajah, Wejesuriya, et al., 2011). Lentils grown in Saskatchewan, Canada are rich in Fe (73 – 90 mg/kg), zinc (Zn, 44 – 54 mg/kg), and selenium (425 – 673 μg/kg) (D. Thavarajah et al., 2009; D. Thavarajah, Thavarajah, Sarker, et al., 2011). Further research has shown that Saskatchewan-grown lentils are also naturally lower in phytic acid (2.5 – 4.4 mg/g), indicating that Fe and Zn would be more bioavailable [7,10,11,12]. Furthermore, Fe status is associated with improved cognitive performance (Murray-Kolb et al., 2017; Scott et al., 2018). Therefore, a tremendous opportunity exists for lentil to be part of a whole food based, sustainable solution to the global micronutrient deficiency problem. This community-based trial is designed to examine the effectiveness of Saskatchewan-grown Fe-fortified lentils on the Fe status and cognitive performance of adolescent girls in Bangladesh.

4.3 TRIAL OBJECTIVES

The primary objective of the study is to determine the effectiveness of an Fe-fortified lentil-based dietary intervention compared to ordinary lentils and usual intake group in improving the Fe status of non-pregnant adolescent females in Bangladesh. The primary comparison would be iron-fortified lentils vs non-iron-fortified lentils and secondary comparison would be iron-fortified lentils vs usual intake. We hypothesize that the supplemental food-based Fe from the fortified lentils will improve body Fe status of adolescent females, and thus cognitive performance, compared to ordinary lentils and usual intake. Furthermore, we will examine the effect of Fe-fortified and non Fe-fortified Canadian lentil consumption (intervention) on growth (height, body weight, triceps skinfolds, mid-upper arm circumference) of non-pregnant adolescent females in Bangladesh.

4.4 MATERIAL AND METHODS

4.4.1 Feasibility study

Before designing the effectiveness trial, a feasibility study was conducted in Bangladesh to examine logistics and feasibility of the implementation of a human food intake trial of Fe-fortified lentils among adolescent girls (10-17 yrs) for 12 weeks in 2017 (Yunus, 2018). The purpose of the study was to determine the viability of the proposed effectiveness trial. Both thin and thick traditional lentil dishes were prepared based on uncooked/raw lentil amounts of 25 g, 37.5 g and 50 g/person (Yunus, 2018). The study findings suggested that adolescents were keen to eat cooked lentils over the 3 months study period, and the drop-out rate was 0% (with 5.2% of meal missed by participants). Adolescents' hunger, fullness, gastrointestinal discomfort before and after consuming cooked lentil portions were assessed using Visual Analog Scales (VAS). Higher palatability was observed for the thick preparation of cooked lentils compared to the thin preparation for all three intervention raw amounts of 25 g vs 37.5 g vs 50 g (cooked amount ~157.9 g vs 202.7 g vs 256.6 respectively). There were no significant difference between uneaten amounts of the lentil dishes containing 37.5 g vs 50 g raw lentils (cooked amount ~202.7 g vs 256.6 g respectively). Moreover, 37.5 g raw amount would provide approximately 86.3% and 46% of the RDA for Fe for adolescent girls aged 9-13 and 14-18 years respectively (Yunus, 2018). Considering the cultural appropriateness of the amount of dal consumed and its Fe

content, the study adopted 37.5 g raw lentil (~200 g cooked amount) as the intervention portion size for the future human effectiveness trial.

4.4.2 Study design

This manuscript describes the protocol for a double-blind (both trial participants and outcome assessors), community-based, cluster-randomized controlled trial designed to test the effectiveness of consuming Fe-fortified lentils to improve Fe stores and cognitive performance (attention, and memory) of rural adolescent girls in Bangladesh. Given the context of this problem, the 'cluster-randomized controlled trial' allows us to: 1) avoid confounding and ensure representativeness at baseline; 2) minimize risk of contamination between treatment arms; and 3) examine the temporal relationship of our intervention on our outcome.

4.4.3 Study settings and target population

The effectiveness study will be conducted at the BRAC Adolescent Clubs (BRAC, 2018). These clubs target all adolescent boys and girls in the community, regardless of their school attendance, marital status, or socio-economic status. The clubs provide a unique opportunity for adolescents to socialize in both rural and urban settings. Each club has a membership of 25-40 adolescent boys and girls aged 10-19 years. The clubs operate in the afternoons in BRAC school environments. In the absence of such facilities, a room is rented locally by BRAC. The clubs have various activities to encourage the participation of adolescent girls and boys such as Life Skill Based Education (LSBE) sessions, access to mini-library facilities, cultural activities, and sports. BRAC provides all materials related to these activities, e.g., books, magazines, and games equipment. The clubs will be selected from 4 Upazilas i.e., Muktagacha, Nanadail or Mymensingh Sadar (Central), Bhaluka, and Gaffargaon of the Mymensingh district.

The study has been carefully designed to ensure that all of the adolescent girls between the ages of 10-17 years meet the study's inclusion criteria: non-smoking, not married, non-pregnant, not breastfeeding, and generally healthy. Adolescent girls who choose not to participate, those who are ill during recruitment or have known cases of infectious disease will be excluded. SPIRIT (Standard Protocol Items: Recommendations for Intervention Trials) 2013 will be used to report the study (Chan et al., 2013) presented in Figure 4.2. Table 4.1 presents a summary of the trial according to the World Health Organization (WHO) Trial Registration Minimal Data Set as described by Moja et al (Moja et al., 2009).

Table 4.1: Summary of the trial according to WHO Trial Registration Minimal Data Set

Sl no	Data	Information
1	Trial Identification number (Unique trial number)	ClinicalTrials.gov NCT03516734
2	Trial registration date	May 24, 2018
3	Secondary IDs	Bio# 17-177 (ERC, University of Saskatchewan) IRB#1139116-2 (IRB, Marywood University) BMRC/NREC/2016-2019/455 (Bangladesh Medical and Research Council)
4	Funding source(s)	Global Institute for Food Security, University of Saskatchewan, Canada; Nutrition International, Canada
5	Primary sponsor	Albert Vandenberg, PhD (University of Saskatchewan)
6	Secondary sponsor	Carol J Henry, PhD (University of Saskatchewan)
7	Responsible contact person (Contact for public queries)	Carol J Henry, PhD, College of Pharmacy and Nutrition, University of Saskatchewan, 104 Clinic Place, Saskatoon, SK, S7N 2Z4, Saskatchewan, Canada. carol.henry@usask.ca
8	Research contact person (Contact for scientific queries)	Diane M DellaValle, PhD, RDN, LDN, Nutrition, Athletic Training and Exercise Science, Department of Nutrition and Dietetics, Marywood University, 2300 Adams Avenue Scranton, PA 18509, USA. ddellavalle@marywood.edu
9	Title of the study (brief title)	Iron-fortified lentils to improve iron (Fe) status among adolescent girls in Bangladesh
10	Official scientific title of the study	Iron-fortified lentils to improve iron (Fe) status among adolescent girls in Bangladesh- study protocol for a double-blind randomized controlled trial.
11	Research ethics review and Country of recruitment	• Ethical approved received from 1. University of Saskatchewan REB (Ref: Bio# 17-177)

		2. Marywood University IRB (Ref: IRB#1139116-2) 3. Bangladesh Medical and Research Council. (Ref: BMRC/NREC/2016-2019/455) • Country of recruitment: Bangladesh
12	Health condition studied	Iron deficiency
13	Intervention	Fe-fortified lentils
14	Key inclusion and exclusion criteria	Inclusion criteria: Adolescent girls ages of 10 – 17 years, non-smoking, not pregnant, not breastfeeding, and generally healthy. Exclusion criteria: Adolescent girls with active illness during recruitment, or with known infectious disease.
15	Study type	Double-blind, community-based, cluster-randomized control trial
16	Trial start date (anticipated)	Mid-September 2018
17	Target sample size (total)	1260 adolescent girls.
18	Recruitment status	Not yet recruiting
19	Primary outcome	Serum ferritin level and cognitive performance of the adolescent girls.
20	Key secondary outcomes	1. Growth (height, body weight, triceps skinfolds, mid-upper arm circumference) of non-pregnant adolescent females in Bangladesh 2. Blood hemoglobin level of non-pregnant adolescent females in Bangladesh

4.4.4 Lentil fortification with Fe

Lentils were fortified with Fe at the Saskatchewan Food Industry Development Centre Inc. (Food Centre) in collaboration with the Department of Plant Sciences of the Crop Development Centre (CDC) of The University of Saskatchewan, Canada. A small red lentil

variety (CDC Maxim) was used, similarly to the previously developed protocol (Podder et al., 2017). In brief, the fortification method development identified the most suitable product types (decorticated unsplit lentil dal), appropriate methods for fortification, dosage and colorimetric changes and storage ability for Fe-fortified lentil dal among three different Fe fortificants ($FeSO_4 \cdot 7H_2O$, NaFeEDTA and $FeSO_4 \cdot H_2O$). NaFeEDTA was found to be the most suitable Fe fortificant for lentil dal at 1600 µg g^{-1} (fortificant Fe concentration), providing 13-14 mg more Fe 100^{-1} g of dal (Podder et al., 2017). The fortified lentils were bagged in 20 kg airtight polyvinyl bags and stored at room temperature before being shipped to Bangladesh.

4.4.5 Intervention

There will be three arms in this effectiveness trial. Arm-1 will receive Fe-fortified lentils, arm-2 will receive unfortified lentils, and arm-3 will receive no intervention i.e. no additional lentil and will serve as a control group. The usual intake group will serve a control for the lentil-related research questions, but not those pertaining to fortification itself. Participants will not be asked to change anything about their food intakes during the study, including lentil intake. Participating adolescents will be served a thick preparation of cooked lentils (37.5 g raw lentil) 5 days per week for 85 feeding days (around 4 months).

A team of three will manage the distribution of the lentil preparation: (i) a locally recruited cook, (ii) a research assistant who will measure and serve the cooked lentils, and (iii) an adolescent club leader will assist in serving and ensuring that safe drinking water is available. A locally acceptable, standard lentil dal recipe identified during the earlier feasibility study will be used. For the thick cooking preparation of 100 g uncooked lentils, the recipe will include turmeric 5 g, chopped onion 40 g, garlic 8 g, green chili 3 g, water 700 ml, salt 1.5 teaspoon, soybean oil 10 teaspoons, and one small bay leaf (tejpata). The average cooking time would be around 18 min, and the approximate weight (after cooking of 37.5 g raw lentil) would be approximately 200 g (Yunus, 2018). All adolescent boys and girls who attend the BRAC adolescent clubs will be offered the cooked lentils during the period of the intervention; however, the information of those girls not meeting the inclusion criteria will not be analysed.

4.4.6 Sample size, randomization and blinding

Considering the lower estimation of the expected difference in mean serum ferritin (5 ± 20µg/L) with 80% power at $p<0.05$ significance level and inter-cluster correlation (ICC) at

0.025, a total of 48 clusters will be selected falling under 16 blocks. Each block will have 3 clubs resulting in a total of 48 clubs. Each club is considered as cluster. Within each cluster, n=27 eligible adolescent girls will be selected. Clusters will be randomly assigned to the intervention within each block. In this cluster RCT, units of randomization will be adolescent clubs where data will be collected from individual girls. Firstly, a total of 48 clubs will be randomly selected out of 75 clubs. Each cluster will be randomly assigned under 16 blocks. There will be 3 cluster in each block and 3 study arms (either iron fortified lentil or non-iron-fortified lentils or usual intake) will be then randomly allocated within blocks using computer generated random assignments. Equal number of clubs (n=16) will be assigned to each arm and equal number of participants (n=420) will fall in each arm. Finally, n=420 adolescent girls will be included in each arm including an additional 20% to account for loss to follow-up. The total sample size including all three arms will be n=1260 adolescent girls. For the cognitive testing, a subsample of the intervention (n=80 adolescent girls in each group, 5 per cluster, a total of n=240 participants) will be selected assuming a two-tailed, 5% type I error rate with 80% power, and an ICC of 0.025. This is based on a recent biofortified iron study with 70% effect size (Donner et al., 1981; Scott et al., 2018). We further increased the sample size to a total of n=360 adolescent girls (e.g. n=120 adolescent girls in each group, considering a 50% attrition rate). We assumed 20% attrition rate in our first outcome; however, we assume attrition rate may be higher in the cognitive part as it would take longer time plus many of the participants may have not seen or physically touched laptop in their lifetime. We assume these create more burden to them resulting higher attrition rate. Clubs, clusters and participants will be identified by sequential numbers such as 01, 02……48; 1001, 2002….1616 and 01, 02….1260 respectively. We did not make cluster numbering sequential because we wanted to make our UID an 8 digit number: 2 digits for the club ID, 4 digits for cluster, and 2 digits for the participant. This makes the UID more unique and each part of the UID will not be confused among the research assistants. A potential problem with sequential numbering (e.g.1..16) would be different digits among participants that may lead to different sizes of the UID stickers, leading to difficulty using the UID stickers during blood sample collection (e.g. on paperwork, blood sample tubes, etc).

The study will follow the double-blind strategy in distributing the Fe-fortified or non-Fe-fortified lentils to the participants. The double-blind strategy (both trial participants and outcome assessors) will be carried out by a third party under the direct supervision of the principal

investigator of the project. Each lentil packet (hard paper carton box) will contain double-layered colour coded bags. The outer layer will be marked with adhesive coloured sticker. The inner layer (food grade bag) will be representing Fe-fortified or non Fe-fortified lentils. Colour coded packets will be delivered to the intervention adolescent clubs. Blinding will be broken within 24 h availability in any instance of unexpected or untoward conditions, for example, mass hysteria, mass diarrhea linked to served lentils, unexpected death or life-threatening condition of the participants, etc. During the data collection period, the researchers will be in touch with the local research assistants on a daily basis to receive daily updates of the research from the field. If required, an urgent message will be sent by field researchers for any unexpected or untoward conditions to all the study investigators. All IRBs will be informed if such situation occurs.

4.4.7 Data collection tools and technique

Four types of data will be collected in three rounds. These include: survey data, cognitive performance data, venous blood samples, and daily lentil consumption data. Round 1 (baseline) will include all forms of data. Round 2 (midline) will only include blood sample data at 2 months. Round 3 (end line) will be the same as baseline at 4 months (Figure 4.1).

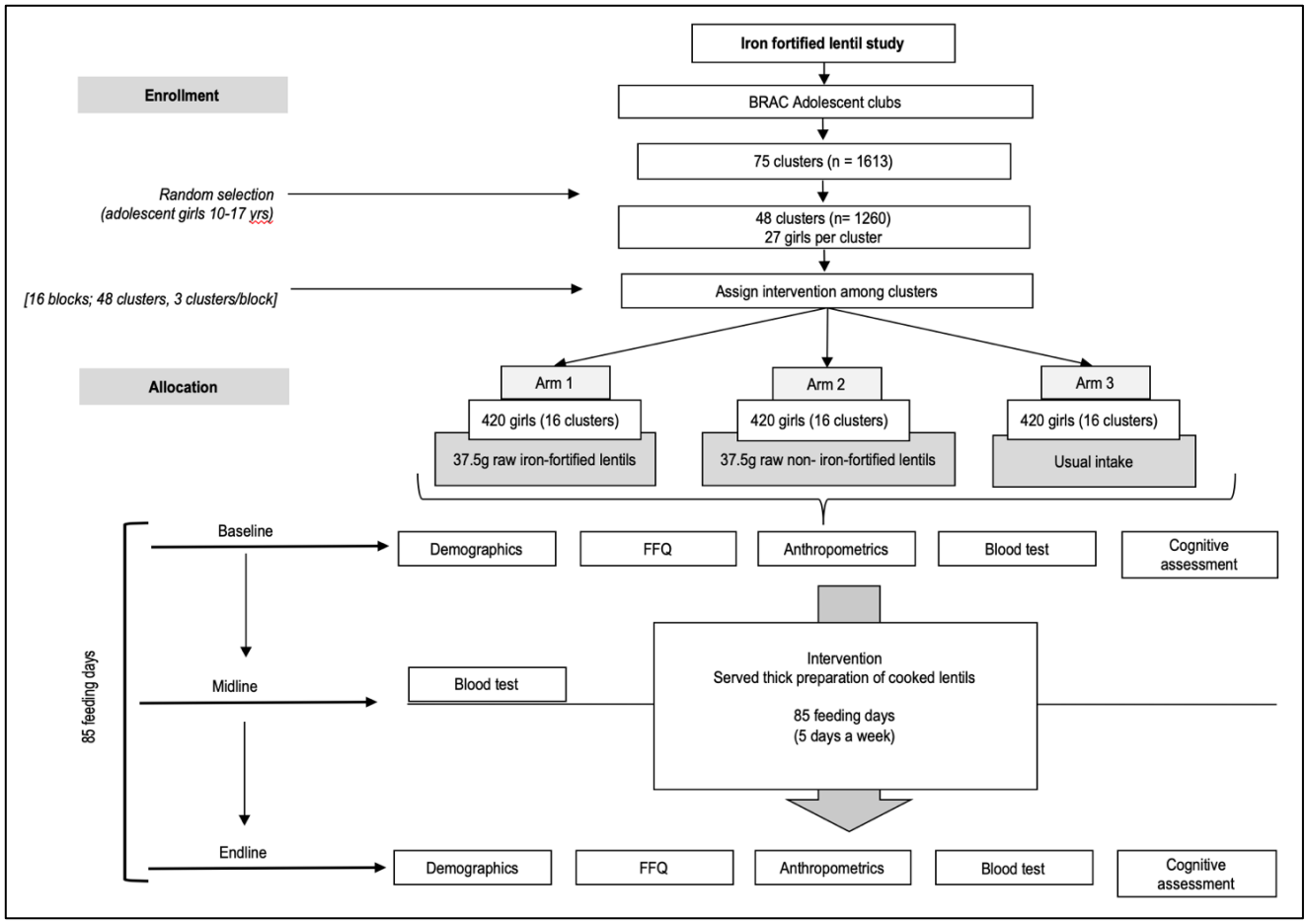


Figure 4.1: Flowchart of randomized controlled trial.

First of all, willing participants who are healthy female adolescents aged 10-17 years, non-smoking, non-pregnant, not breastfeeding, will be included in the study. Those who give consent will be invited to gather in the BRAC adolescent clubs and venous blood samples will be collected in a private setting within the club by a three-person team consisting of (i) a medical technologist, (ii) a female research assistant and (iii) a male research assistant. BRAC local staff will be present to monitor the entire process. Albendazole (400mg in tablet form) will be provided so that participants are dewormed at the time of feeding trial meaning that they are free from parasites, such as roundworm, flukes and tapeworm infestation. Earlier studies suggested that worm infestation is linked with anaemia and deworming improves anaemic status among children (Girum & Wasie, 2018; Watthanakulpanich et al., 2011; V. A. Welch et al., 2017).

All participants will be provided with the contact details of the study coordinator to provide guidance on future questions and concerns. A copy of the consent form will be given to the participants. Publications and scientific presentations of the findings from the study will be

presented in aggregate, and the identity of individual participants will be kept confidential. The study will use study codes for hard copy data documents (e.g., completed questionnaire) instead of recording identifying information and will maintain a separate document that links the study code to subjects' identifying information. It will be locked up in a separate location with restricted access to this document (e.g., only allowing access for primary investigators). Laptop computers used by researchers that collect and manage data will be protected with whole-drive disk encryption that prevents data access in case the laptop is lost or stolen. Face sheets containing identifiers (e.g., names and addresses) from survey instruments containing data will be removed after receiving them from study participants. Sensitive identifiers (e.g., names and addresses) will not be permitted to be stored on memory devices or transmitted over unsecured networks. Each researcher will have password protected online cloud-based storage for sharing of study-related files. Study communications will be conducted via secured institutional email servers.

Survey data will include information on socio-demographic characteristics, household food security status, and adolescent food habits by standardized food frequency questionnaire (FFQ). Survey data will be collected by the trained enumerators.

Venous blood samples will be collected by a trained phlebotomist. (~10 ml) will be collected from each participant using lithium heparinized vacutainers following aseptic procedure and using disposable syringe and needle. Vacutainers will be carried to International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDRB) based in Dhaka within 12 hours of collection. In its state-of-the-art facility laboratory, the serum will be separated and stored at 2-8ºC temperature and then analyzed. The blood collection per participant will take about 10 min. We propose to measure complete blood count (CBC) that includes the following: ESR, Hb, hematocrit, PCV, MCH, MCV, MCHC, RBC, WBC total count, differential count, platelet count; serum ferritin, soluble transferrin receptor (sTfR), C-reactive protein (CRP) and blood ABO typing.

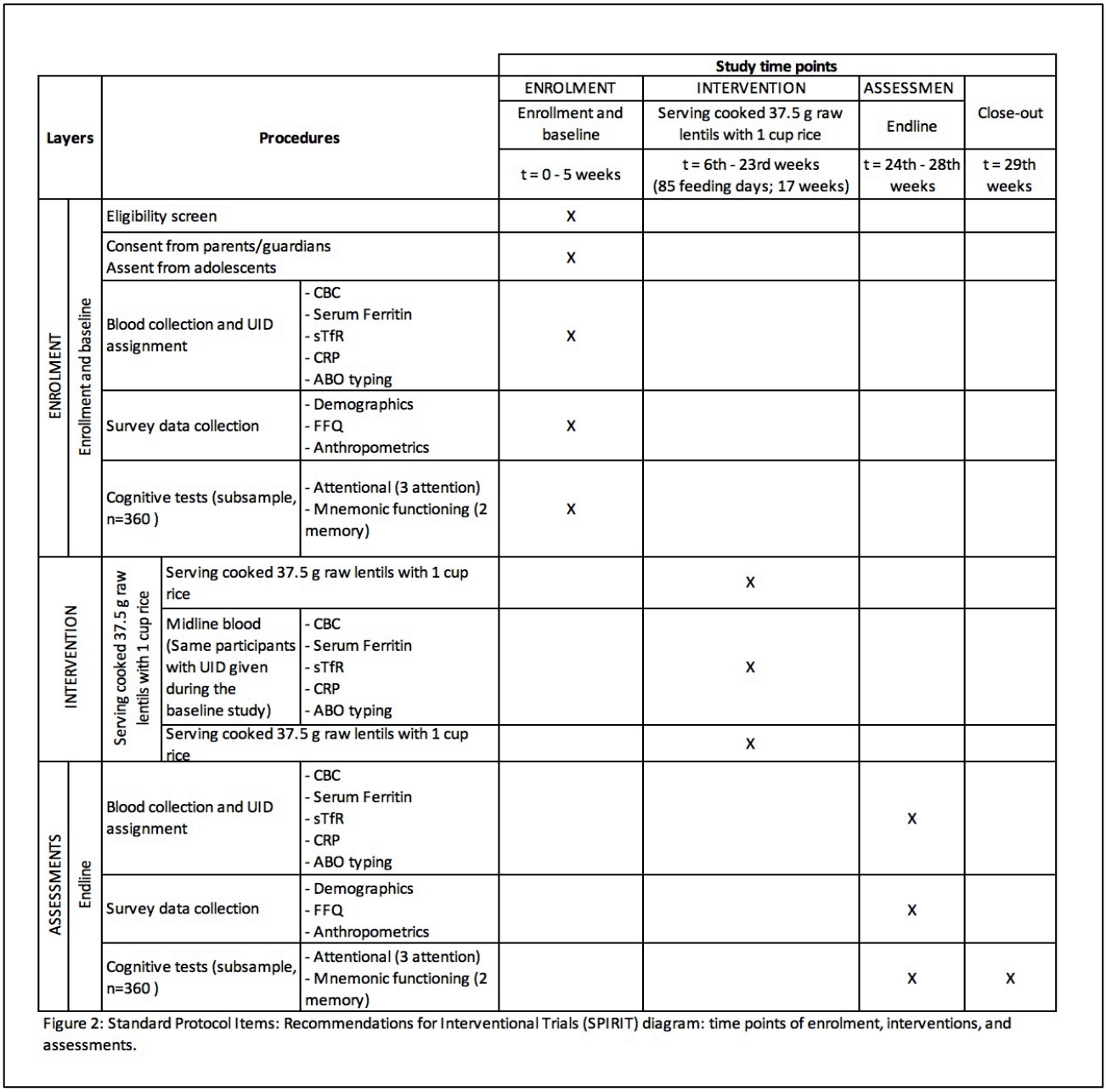
Five common measures of attentional (3 attention tasks) and mnemonic functioning (2 memory tasks) will be used to assess the use of cortical systems and circuits that have been documented to have some dependence on Fe status, based on the literature describing either human or animal studies. These measures and tasks have been used extensively in the literature in

human experiments, and have each been described previously (Murray-Kolb et al., 2017; Scott et al., 2018). These computerized assessments will be used to measure attention and visual memory, as used in previous biofortification feeding trials in similar target populations (Rhoten et al., 2014; M. J. Wenger et al., 2011, 2012, 2014). Briefly, all tasks will be undertaken by pre-installed DMDX software (Developer: University of Arizona) and will include detailed instructions and practice tests (Forster & Forster, 2003). This computer-based application will be administered by trained local research assistants.

The simple reaction time (SRT) task provides an estimate of the speed of the simplest possible behavioral response to a visual stimulus and requires a participant to press a button in response to the onset of the task stimulus (Wickens et al., 2004). The go/no-go (GNG) task provides an estimate of the efficiency of sustained attention and the speed of simple attentional capture without the need to filter information from any immediately competing stimuli (Wickens et al., 2004). It requires a participant to press a button in response to the presentation of an infrequent stimulus (presented on 20% of the trials) and to withhold a response to a frequent stimulus (presented on 80% of the trials). The attentional network task (ANT) is a modified flanker task that provides an estimate of the effectiveness of three distinct components of attention (Fan et al., 2002). In each trial, a participant will be presented with either an informative or an uninformative cue and will be required to press a button to indicate whether an arrow in the center pointed to the left or right while attempting to disregard flanking elements. The cued recognition task (CRT) is a modified version of a classic visual recognition memory task that estimates the speed, accuracy, and efficiency of recognition based on short-duration visual memory (Ebbinghaus, 1885; M. K. Wenger et al., 2010). Participants will be presented with a set of pictures of common, easily named objects, after which an equal number of previously seen and new items will be presented, and participants will be required to indicate whether each item was in the original set or is new. The Sternberg memory search (SMS) task estimates the speed and accuracy with which immediate visual memory can be searched (Sternberg, 1966). In each trial, participants will be first shown a set of 1, 3, or 6 simple graphical symbols to remember, then a test stimulus will be presented, and the participant indicates whether they remember the test stimulus from the previous set of symbols. It would take around 45 min per participants.

Data on how much cooked lentils were served to and how much remained uneaten by each adolescent girl will be collected on a daily basis from the research assistants. This

information will be used to determine the actual amount of cooked lentils consumed by the adolescents.



Layers		Procedures		Study time points			
				ENROLMENT	INTERVENTION	ASSESSMEN	Close-out
				Enrollment and baseline	Serving cooked 37.5 g raw lentils with 1 cup rice	Endline	
				t = 0 - 5 weeks	t = 6th - 23rd weeks (85 feeding days; 17 weeks)	t = 24th - 28th weeks	t = 29th weeks
ENROLMENT	Enrollment and baseline	Eligibility screen		X			
		Consent from parents/guardians Assent from adolescents		X			
		Blood collection and UID assignment	- CBC - Serum Ferritin - sTfR - CRP - ABO typing	X			
		Survey data collection	- Demographics - FFQ - Anthropometrics	X			
		Cognitive tests (subsample, n=360)	- Attentional (3 attention) - Mnemonic functioning (2 memory)	X			
INTERVENTION	Serving cooked 37.5 g raw lentils with 1 cup rice	Serving cooked 37.5 g raw lentils with 1 cup rice			X		
		Midline blood (Same participants with UID given during the baseline study)	- CBC - Serum Ferritin - sTfR - CRP - ABO typing		X		
		Serving cooked 37.5 g raw lentils with 1 cup rice			X		
ASSESSMENTS	Endline	Blood collection and UID assignment	- CBC - Serum Ferritin - sTfR - CRP - ABO typing			X	
		Survey data collection	- Demographics - FFQ - Anthropometrics			X	
		Cognitive tests (subsample, n=360)	- Attentional (3 attention) - Mnemonic functioning (2 memory)			X	X

Figure 2: Standard Protocol Items: Recommendations for Interventional Trials (SPIRIT) diagram: time points of enrolment, interventions, and assessments.

Figure 4.2: Standard Protocol Items: Recommendations for Interventional Trials (SPIRIT) diagram: time points of enrolment, interventions, and assessments

4.4.8 Outcome measurement

Assessment of Fe status and anthropometrics: Fe status measurement will include hemoglobin, hematocrit (volume of red blood cells), serum ferritin, (sTfR), C-reactive protein

and imputed total body Fe. Total body Fe (TBI, mg/kg) will be calculated using the ratio of sTfR to sFer (Cook et al., 2003).

Participants' height, weight, waist, hip and mid-upper arm circumference (MUAC) of adolescents will be measured to determine change in growth (Bacopoulou et al., 2015; National Health and Nutrition Examination Survey (NHANES), 2007; World Health Organization, 1995). Participants will be kept bare-footed, minimal clothing and we will be avoiding carpets, sloping, rough, and uneven surface before the anthropometrics measurements. For height, Frankfurt horizontal plane will be ensured, and they will be requested to put their heels together. In addition, their backward curved body parts (buttocks and shoulder blades) and head will be placed against the plane. For weight, participants will be requested to stand still putting face forward and placing palm on their respective side and digital body weight bathroom scale will be used after removing any sort of shoes and socks. Participants' waist circumference will be measured under the midline of armpit and at the midpoint between the inferior part of the last rib and the top tip of the hip bone by using a constant tension tape. Hip circumferences will be measured at the point of maximum diameter of buttocks using the same constant tension tape. Same tape will be used to measure the MUAC. First, participants will be requested to put their left arm at 90° angles, and midpoint will be marked between the distance of proximal and distal point. Tape will be then wrapped around the point and measured ensuring that tape will be not be either too tight or too loose. Measurement unit of all anthropometrics will be in centimeters (cm) except weight will be captured in kilograms (kg). Participants' mean Body Mass Index (BMI) will be calculated by using BMI Percentile Calculator for Children and Teens (Centers for Disease Control and Prevention (CDC), 2017).

Dietary intake will be assessed by a field research assistant in the baseline and end line survey using culturally-appropriate FFQs.

Cognitive performance: Participants' attention, and memory [# of correct responses and time (seconds and milliseconds)] will be measured using DMDX software.

4.4.9 Statistical analysis

Baseline characteristics, i.e., all covariates, will be examined for group comparability to determine if randomization was successful. An intent-to-treat approach will be followed in the analyses of outcome data at the end of the intervention. Outcome variables such as serum ferritin,

and Hb will be set using different cut-offs using National Micronutrients Status Survey 2011-12 (ICDDRB et al., 2013). Data will be analysis with and without adjustments for baseline characteristics. Group means for changes in biochemical variables, anthropometric measures, and post-intervention morbidity rates will be compared by using mixed models (cluster as random effect), with the respective baseline values serving as the primary covariate. After these preliminary analyses, key outcome variables (Serum ferritin level and cognitive performance of the adolescent girls) will be re-examined with additional covariates will be included in the model such as factors that differed by intervention arms i.e age, socio-economics, anthropometrics, food security, drinking safe water, hygiene practice etc). Additional continuous outcome variables will also be examined using GLM, adjusted for confounding factors such as age, socio-economics, anthropometrics, food security, drinking safe water, hygiene practice etc. Categorical variables will be compared by chi-square tests or logistic regression adjusted for possible confounding factors. With the assumption that data will be missing at random (MAR), multiple imputation or likelihood-based mixed models will be used for analysis, after complete case analysis and examination of missing data within and between treatment groups. All outcome variables will be examined for normality, and outliers will be identified. Data collected from adolescent girls suffering from severe Fe deficiency and/or acute infection will participate in all aspects of the study but results of supplementation in this group will be analyzed separately. All analyses will be performed using SPSS for WINDOWS PAWS version 25. $P < 0.05$ will be considered statistically significant for all main effects.

4.5 DISCUSSION

The study aims to measure the effectiveness of consuming Fe-fortified lentils on the body Fe status and cognitive performance of adolescent girls. Cooked Fe-fortified lentil (Dal) is the only intervention considered in this study. To measure its effectiveness, we controlled other covariates that may have influence over the study outcomes: we are providing non-Fe-fortified lentils; cooked rice is being provided to increase compliance (based on the previous pilot study findings); and participants are being dewormed by Albendazole at the time of feeding trial (Girum & Wasie, 2018; Watthanakulpanich et al., 2011; V. A. Welch et al., 2017).

Adolescent girls are prone to Fe deficiency, particularly in resource-poor settings due to a variety of factors. Although this study is limiting inclusion to adolescent girls, we will serve the

Fe-fortified lentil to all children and teens who are participants in the BRAC adolescent club during the intervention period for the purposes of equity. Adolescents who do not meet the inclusion criteria, however, will not be included in the analyses. Spill-over effect is less likely to occur as the study does not provide knowledge or any other information that may affect the study outcomes, which are biological in nature.

Another aspect that may affect our outcomes is that as the study proposes to serve the same recipe for ~ 4 months, it is possible that consumption of a single recipe daily for this length of time could lead to boredom among participants and may reduce consumption or may result in drop-outs after several weeks. We predicted this situation would occur and attempted to address it in our pilot study which ran for 3 months and tested two different recipes (thick and thin preparation) in 3 different amounts using the local ingredients and cooking procedures in 2016-2017. This study came up with a single recipe that was found to be most acceptable by the participants (BRAC Adolescent Clubs in nearby 'Gazipur' district), and we did not find drop-outs or reduced consumption due to palatability. Furthermore, it is important for the study to maintain its rigor and standardize the intervention recipe in order to capture the true effect of the Fe-fortified lentils compared to normal lentils and/or usual intake. So, the probability of any fluctuation of the feeding trial due to recipe would affect equally to each intervention arm provided that we use the same recipe for duration of the study among all arms. If we provided different recipes to increase the consumption, it is possible that either the study participants increase or decrease consumption compare to earlier recipe (even though probability of fluctuation remains the same) but that may influence the variation in study outcome.

Quality control will be ensured by following multiple steps. For instance, direct training of enumerators, data quality control supervisors, and data managers will be carried out prior to the commencement of data collection to adhere them to the consent, assent, and questionnaire equally with the same degree of questioning and understand the format accurately. Furthermore, in this face-to-face interview approach using a close-ended questionnaire, response options will be strictly-formatted. Field enumerators will be advised to cross-check the questionnaire within team members before sending it to the server using a cellular internet data connection. Additional training will be provided to the data quality control supervisors at the field level, and spot check the interview process in order to uncover any mistakes in the data collection procedures.

All interviews and arthrometric measurements will be taken by experienced female interviewers separately in a private setting with presence of a witness. Each arthrometric measurement will be taken 3 times to ensure consistency of the measurement. Survey and daily-wise data will be collected electronically by the Open Data Kit (ODK) app on an Android-based platform (Hartung et al., 2010; Raja, Tridane, Gaffar, Lindquist, & Pribadi, 2014). This customizable mobile or tablet-based app could work both online and offline allowing the use of GPS tracking, setting the condition of the responses and enable real-time data monitoring. An experienced data manager will be checking the received data thoroughly every day and will be providing feedback directly to the enumerators. Additionally, our data manager will be assessing the need for re-training the field staff.

Benefits of this study are three-fold. First, the results of the study will be used to garner support and substantiate large-scale market expansion of Fe-fortified lentils in Bangladesh and in other countries that consume lentils as a staple. Second, BRAC's extensive country-wide programmatic network has high potential for efficient marketing of high-Fe lentils throughout Bangladesh. Third, the results from this study will contribute to the knowledge on food-based approaches to enhance the Fe status of adolescents worldwide in resource-poor settings.

Compared to the effect of iron supplementation to improve Fe status, this study expects a smaller effect size. This study will require a higher number of samples to statistically detect the smaller difference between baseline, midpoint and endpoint, and between-groups status in mean serum ferritin. The small increase in mean ferritin status has the potential to reduce the prevalence of Fe deficiency by shifting a certain proportion of the deficient population above the cut off level (sFer>12 µg/L). Considering ~30% prevalence of Fe deficiency (sFer <12 µg/L) among rural female adolescents, shifting the population mean sFer from 22.5µg/L to 27.5µg/L would reduce the prevalence to about 20%.

Three different platforms will be used for dissemination of the study results. The study findings will be presented at academic seminars at the University of Saskatchewan. Furthermore, the same results will be presented in a seminar that will be organized by the BRAC Research and Evaluation Division. Furthermore, a layperson's summary of the results will be shared with BRAC for dissemination at the field level. The study findings will be presented at various conferences, such as the American Society for Nutrition (ASN), Canadian Society for Nutrition

(CSN), University of Saskatchewan annual Life and Health Science Expo, and other nutrition and public health conferences. Manuscripts will be written based on the study findings and submitted to high- impact factor, peer-reviewed scientific journals.

4.6 LIST OF ABBREVIATIONS

icddr,b: International Centre for Diarrhoeal Disease Research, Bangladesh; BRAC: It is not an abbreviation, it is known and identified as 'BRAC'; Usask: University of Saskatchewan; CDC: Crop Development Centre; , DPS: Department of Plant Sciences; VAS: Visual Analog Scales; AC: Adolescent Clubs; LSBE: Life Skill Based Education; NaFeEDTA: Sodium iron ethylenediaminetetraacetic acid; sTfR: Soluble transferrin receptor; TBI: Total body Fe; FFQs: Food Frequency Questionnaires; DDS: Dietary Diversity Score; sFer: Serum ferritin; DSMB: Data Safety Monitoring Board; GoB: Government of Bangladesh.

4.7 DECLARATIONS

4.7.1 Ethics approval and consent to participate

The study received ethical approval from the University of Saskatchewan, Canada (Ref: Bio#17-177), Marywood University, USA (Ref: IRB#1139116-2) and the Bangladesh Medical Research Council (BMRC; Ref: BMRC/NREC/2016-2019/455). Each ethical body will be informed separately for any changes in the trial protocol. Adolescents and their families will be informed separately about the purpose of the study, the procedures to be used, and the benefits to be derived from the study. Two separate written informed consent forms for parents/guardians and participants (adolescents) will be a prerequisite to recruitment of the subjects. The severely anaemic adolescents will be symptomatically treated for Fe deficiency. The study shall conform to all regulations of the Government of Bangladesh (GoB). A mid-term assessment of biochemical outcomes will be independently analyzed by the members of the Data Safety Monitoring Board (DSMB) to detect any adverse outcome. All participants will receive explanations about the objectives, importance, risks and benefits of the research before recruitment. Participation will be completely voluntary, and an appropriate written informed consent will be obtained from all participants.

4.7.2 Consent for publication

Not applicable

4.7.3 Availability of data and material

Data may be shared with interested researchers who may contact the corresponding author. After considering all aspects of ethical issues, and in consideration of the existing rules, anonymized data may be shared with qualified researchers.

4.7.4 Competing interests

The authors declare that they have no competing interests. CJ is an employee of Nutrition International (NI), which provided partial funding to the implementation of the study. However, the funding was provided after the study was conceptualized and the proposal drafted.

4.7.5 Funding

Global Institute for Food Security (GIFS) and Nutrition International (NI).

4.7.6 Authors' contributions

DMDV, CJ and AV conceptualized the study. DMDV, CJ and FMY designed the study. AV and RP carried out the lentil fortification, processing and shipping. FMY, CJ, KA, and DMDV drafted the manuscript. All authors approved the final version of the manuscript prior to submission.

4.7.7 Acknowledgements

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4.8 TRIAL STATUS: Not yet recruiting

CHAPTER 5: IRON PROFILE OF ADOLESCENT GIRLS IN RURAL NORTH-CENTRAL BANGLADESH–RESULTS FROM A CROSS-SECTIONAL SURVEY

This chapter reports major iron-related statistics related to adolescent girls in Bangladesh. A baseline survey carried out as part of a RCT was analyzed separately. This is a manuscript-formatted chapter intended for journal publication.

5.1 Abstract

Background:

Globally, iron deficiency (ID) is one of the most common micronutrient deficiencies. The most recent Bangladeshi iron status statistics were released seven years ago (in 2013). Because of the scarcity of recent iron status statistics in adolescent girls, we used valid iron biomarkers to assess the prevalence of ID in Bangladeshi adolescents with and without anemia. Data were collected as part of a community-based, double-blinded, cluster-randomized controlled trial (ClinicalTrials.gov NCT03516734).

Methods:

A cross-sectional survey was carried out among n=1195 Bangladeshi adolescent girls aged 10–17 years. Socio-demographic data, WASH information, and knowledge of ID and iron deficiency anemia (IDA) were collected electronically using seven-inch tablet computers programmed by the Android based ODK app. To assess the iron status of the adolescent girls, venous blood samples (6 ml) were collected and tested for the following: total blood count, hemoglobin (Hb), hematocrit/packed cell volume (PCV), mean corpuscular hemoglobin (MCH), mean corpuscular volume (MCV), red blood cells (RBC), white blood cells (WBC), total count, differential count, platelet count, sFer, soluble transferrin receptor (sTfR), and C-reactive protein (CRP).

Results:

We found prevalence of anemia (27%), anemia without ID (20.3%), IDA clinical (6.7%), IDA sub-clinical (10.7%), ID clinical (9.6%), and ID sub-clinical (26%). Furthermore, 3.7% of the adolescent girls had zero total body iron, and 1.8% were at risk for iron overload (sFer >150 μg/L). Inflammation was identified at CRP>5 mg/L 3.1% and/or WBC>11.5 (10^9/L) 20.6%. A multiple linear mixed model analysis (random effect at the level of the upazila [sub-district]) revealed that not having menarche was significantly different from having menarche. The estimates of the effects indicated that not having menarche was associated with higher serum ferritin, holding age and BMI constant.

Conclusion:

One in ten Bangladeshi adolescent girls in this study had iron deficiency, while one in four had IDA, numbers that were higher than anticipated, given recently reported national statistics.

Keywords: Micronutrient deficiencies, Iron, Women's health, Bangladesh, Anemia, Iron deficiency.

5.2 INTRODUCTION

Of all the global micronutrient deficiencies, iron deficiency (ID) is one of the most prevalent (Johnson-Wimbley & Graham, 2011), and iron deficiency anemia (IDA) is the most recognized (Schneider et al., 2005). IDA appears when the body's stored iron is too depleted to support erythrocyte production, and this shortfall depends on iron intake, existing stores, and excretion (Miller, 2013). An adequate amount of body iron is essential for respiration, energy production, DNA synthesis, and cell proliferation (Hentze et al., 2010b). Several studies have reported that ID and IDA are responsible for fatigue, tachycardia, muscle weakness, lack of endurance, cold intolerance, problems with physical performance, and cognitive, visual, and auditory impairment (Algarin C, Peirano P, Garrido M, Pizarro F, 2003; Brigham et al., 1996; Camaschella, 2017; DellaValle, 2013; DeLoughery, 2017; Haas, 2001; Halterman et al., 2001; Verdon F, Burnand B, Stubi CL, Bonard C, Graff M, Michaud A, 2003). Other studies have reported an increased risk of pre-term labour, low birth weight, and newborn and maternal mortality due to severe IDA during pregnancy (Anker et al., 2009; Camaschella, 2015). In addition to adverse health consequences, iron deficiency is responsible for US$ 4 per capita worth of productivity loss, and because South Asia has the world's highest prevalence of anemia, it is not surprising that it also has the highest productivity losses (J. Ross & Horton, 1998).

The World Health Organization's (WHO) Global Database on Anemia (1993-2005) has estimated that 1.6 billion of the global population (24.8%) suffer from anemia. School-age children contribute 25.4% of the global burden of anemia, with the majority being from south and southeast Asia (McLean et al., 2008). Recent WHO Global Health Estimates have shown that ~ 23,918 annual deaths are attributable to IDA, and also that the number of deaths has increased substantially since 2000 (World Health Organization, 2018a). The latest research on iron-related biomarkers in Bangladesh was carried out in 2011-12 using adopted WHO cut-off points and adjusted for inflammation [C-reactive protein (CRP >10mg/l) and Alpha-1-acid Glycoprotein (AGP >1 gm/l) (ICDDRB et al., 2013). Although it was assumed to be higher in Bangladesh, the prevalence of national ID (serum ferritin level <15.0 μg/L) was confirmed to be 7.1% among non-pregnant non-lactating (NPNL) women (aged 15–49 years) and 9.5% among children aged 12–14 years (ICDDRB et al., 2013). ID among NPNL and children aged 12–14 residing in rural Bangladesh was reported to be 6.7% and 10%, respectively (ICDDRB et al., 2013). However, reports of anemia (Hgb<12.0gm/dl) were much higher, with prevalence of 26% in NPNL women

and 17.1% in children aged 12–14 years. A similarly high prevalence was found among the rural population of NPNL women and children 12–14 years, at 27.4% and 18.1%, respectively. Among all risk groups, adolescent girls are at particularly high risk for developing ID with or without anemia because of extra iron demands due to their substantial growth and body development (McNulty et al., 1996; Zimmermann & Hurrell, 2007). However, to date, no data specific to the iron status of adolescent girls (10–17 years) in Bangladesh have been available. This study filled this gap in iron statistics by investigating the prevalence of ID and IDA among adolescent girls in Bangladesh, considering all major iron biomarkers.

5.3 METHODS AND MATERIALS

5.3.1 Study design and population

We conducted a cross-sectional study among adolescent girls aged 10–17 years residing in both urban and rural areas of the Mymensingh district from four upazilas (sub-districts) in Bangladesh. The study included n=1195 adolescent girls who were non-smoking, not pregnant, not breastfeeding, and generally healthy. Participating girls were enrolled from BRAC adolescent clubs. BRAC is earlier known as Bangladesh Rural Advancement Committee. These clubs provide platforms for locally resident adolescent boys and girls, regardless of whether they are enrolled in school, to gather and share cultural activities, mini-library facilities, indoor games, and national and international celebrations. These clubs open in the evening and use BRAC school infrastructures (BRAC, 2018). The study was carried out in the four upazilas in the Mymensingh district of Bangladesh in the month of November 2019. The study was part of a double-blind clustered community-based randomized controlled trial (ClinicalTrials.gov NCT03516734. Registered on 24 May 2018.) (Yunus, Jalal, et al., 2019). The sample size for the study was determined by the low expected serum ferritin (sFer) mean difference among the intervention groups (5±20 μg/L), inter-cluster correlation (ICC) 0.025, and 80% power. A total of 1195 adolescent girls were enrolled from 48 clubs, which were randomly selected from the sampling frame of 75 clubs (Yunus, Jalal, et al., 2019).

5.3.2 Data collection tools and techniques

Two types of data were collected. First, venous blood samples (6 ml) were collected after school hours from each participating adolescent girl, using two heparinized vacutainers (3 ml each) and butterfly needles. Two teams of three individuals, each consisting of one medical

technologist and two research assistants (1 female and 1 male), collected the samples. The teams first designed a blood sample collection schedule that was distributed to each club. On the scheduled date, adolescent girls were invited to gather at the club, and those who were interested in providing blood samples were assigned UID numbers. The UID was an eight-digit numeric identifier: the first two digits represented club ID, the next four represented the cluster number, and last two the participant number. All daily blood samples (maximum 50 samples per day) were then put into a cold box and delivered to a local field laboratory. Samples were separated (serum and cells) and stored overnight in a freezer at 2-8 ^{o}C. The next morning, a research assistant transferred the separated frozen serum and blood cell samples into insulated containers and delivered them to the ICDDRB headquarters in Dhaka for analysis. The aim was to test the participants' complete blood count (CBC), including the erythrocyte sedimentation rate, (ESR), hemoglobin (Hb), hematocrit/packed cell volume (PCV), mean corpuscular hemoglobin (MCH), mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), red blood cells (RBC), white blood cells (WBC), total count, differential count, platelet count, sFer, soluble transferrin receptor (sTfR), and C-reactive protein (CRP).

In the next stage, those who provided blood samples were invited to a face-to-face interview for survey data collection. Detailed socio-demographic WASH information, knowledge of ID and IDA, and deworming history were collected. The study also collected data using a food security questionnaire and a seven-day dietary recall (7DDR), which were adopted from the National Micronutrient Survey, 2011-2012 (ICDDRB et al., 2013). Survey data were collected electronically by seven-inch tablet computers with an Android operating system and internet connections linked to BRAC's secure server. All tablets were installed with Open Data Kit (ODK) applications, where the Bengali version of the questionnaire was uploaded, enabling us to set conditions for each question as appropriate to reduce data errors and follow real-life data monitoring. Furthermore, participants' height (cm), weight (kg), mid-upper arm circumference (MUAC in cm), hip circumference (cm), and waist circumference (cm) were measured by experienced research assistants using standard techniques and equipment. Details of the anthropometric measurement procedures were explained in our earlier paper (Yunus, Jalal, et al., 2019). Teen BMI was calculated in percentiles and z-scores (Centers for Disease Control and Prevention (CDC), 2017; Yunus, Jalal, et al., 2019).

5.3.3 Predictor variables

Socio-demographic data, such as age (World Health Organization, 2011b), education (Olesnevich et al., 2012), hand hygiene (Jayaweera et al., 2019), knowledge of anemia, and iron-containing foods (World Health Organization, 2001) were collected. Furthermore, food security status and dietary intake data were collected using a standardized food security and seven-day dietary recall (7DDR) questionnaire. Predictor variables were further categorized by age (10–13/14–17 years) based on Recommended Dietary Intake (RDA) (Institute of Medicine, 2003), education (primary/secondary), handwashing with soap (yes/no) as a proxy for hand hygiene, and knowledge of iron rich foods (yes/no). Additionally, detailed anthropometrics such as height (cm), weight (kg), mid-upper arm circumference (MUAC; cm), hip circumference (cm), and waist circumference (cm) were measured by experienced research assistants.

5.3.4 Outcome variables

sFer and anemia were the primary outcome variables. The study used WHO cut-off points for anemia (<12.0 g/dL), ID (sFer < 15µg/L), ID without anemia (sFer <15 µg/L + sTfR >5.0 µg/ml; sFer < 30 µg/L and sFer <30 µg/L + sTfR >5.0 µg/ml), and IDA (Hb <12.0 g/dL + sFer <15.0 µg/L) (World Health Organization, 2017). TBI was calculated using sTfR and sFer and coded as TBI <0 mg/kg. Combined inflammation indicators CRP (>5.0 mg/l) and WBC > 11.5 (10^9/L) were used to further adjust sFer to identify ID and IDA. To understand the Fe status of the adolescents, the study considered sub-clinical ID (<30 ug/L) and IDA (>8.0 ug/ml) (DeLoughery, 2017; Lopez et al., 2016).

5.3.5 Statistical analysis

Detailed demographics and iron status biomarkers were tabulated by number and percentage. We calculated the prevalence of key iron variables such as anemia, anemia without ID, IDA with and without anemia, and ID. Multiple definitions were used after the inflammation indicators were adjusted at CRP >5 and WBC> 11.5 (10^9/L). We defined anemia without ID (Hb <12.0 g/dL, sFer >15 µg/L, sub-clinical IDA (Hb <12.0 g/dL, sFer <15 µg/L), and clinical IDA (Hb <12.0 g/dL, sFer <30 ug/L). Both single and multiple criteria were used to define ID as <15 µg/L (clinical) and <30 µg/L (sub-clinical), sFer <15 µg/L, sTfR >5.0 µg/ml (clinical), and sFer <30 µg/L, sTfR >5.0 µg/ml (subclinical) (Camaschella, 2019). Multiple LMM models were run by entering different variables where the upazilla (sub-district) was set as the random effect

and other variables, such as age category, teen BMI, and experience of menarche, as the fixed effects. The outcome variables sFer and TBI were used to determine ID. Fer level was treated as a continuous variable after conversion to a natural logarithm (LnFer) (Govus et al., 2015).

Since sFer level is natural log transformed, results are presented as percent (%) change instead of beta coefficient estimates. To calculate the percent (%) change, given that the sFer level (dependent variable) and independent variables are in their original metrics, the coefficient values are exponentiated first and then one (1) is subtracted from the exponentiated number and multiplied by 100 (UCLA: Statistical Consulting Group, 2020). Multivariate analysis was carried out to investigate the independent association between the confounders and body iron storage. The explanatory variables significantly correlated to the outcome variable (sFer) were then entered into the LMM analysis. Four separate LMM models were run, where predictors were menarche (yes/no) and teen BMI (as continuous) with and without age, and the outcome variables were log ferritin (LnFer) and TBI (<0 mg/kg). Upazila was set to random effect, and the results were calculated through restricted maximum likelihood (REML) in all models. SPSS software for Windows PAWS version 25 was used to analyze the data, and all significance levels were set at <0.05

5.3.6 Ethical considerations

The study received ethical approvals from the University of Saskatchewan, Canada (Bio#17–177), from Marywood University, USA (IRB#1139116–2), and from the Bangladesh Medical Research Council (BMRC/NREC/2016–2019/455).

5.4 RESULTS

Table 5.1 presents the socio-demographics of the adolescent girls. The mean±SD age of the girls was 13.5±2.0 years, and 67.8% had experienced menstruation. More than half the girls were between the education classes of 5 and 10. None reported chronic disease. In the households of the adolescents, tube wells were the main source of drinking water (99.4%) and other household uses (97.2%), and pit latrines (62.3%) were commonly used. The majority of the participants used soap during handwashing (84.7%) and after defecation/or using the toilet (82.8%). Less than a third of the adolescent girls identified meat (25.6%) and eggs (32.3%) as sources of iron. Of the study participants, 40.1% had been dewormed at school. Adolescent BMI

was 18.0±3.1. The adolescents living in the household were Non-Food Insecure (NFI) (Food security score 0.9).

Table 5.1: Socio-demographic characteristics of the participants

Variables	**Yes (n= 1195)**
	N (%)
Age in years (mean±SD)	13.5±2.0
Menarche	810 (67.8)
Education (attending or completed)	
Primary (up to class 5)	360 (30.1)
Secondary (class 5–10)	738 (61.8)
College (class 11–12)	82 (6.8)
Kowmi (religion)	9 (0.8)
No response	6 (0.5)
Source of drinking water: Tube well	1188 (99.4)
Daily water HH use: Tube well	1162 (97.2)
Toilet facility HH use	44.6
Sanitary flush latrine	396 (33.1)
Pit latrine	745 (62.3)
Hand washing with soap	1012 (84.7)
Hand washing (after defecating/using toilet)	990 (82.8)
Ever heard of anemia or iron deficiency anemia (IDA)	657 (55)
Identify signs of anemia or iron deficiency anemia (IDA)	568 (47.5)
Knowledge of iron containing foods	
Meat (e.g., beef, goat)	306 (25.6)
Poultry	62 (5.2)
Dried or fresh fish	95 (7.9)
Organs (e.g., liver, kidney)	37 (3.1)
Green leafy vegetables	413 (34.6)
Other vegetables/fruits	327 (27.4)
Eggs	386 (32.3)
Milk	351 (29.4)

Pulse	181 (15.1)
Teen BMI (mean±SD)	18.0±3.1
Food security score	0.9
Fe intake (mg/day) from 7DDR (mean±SD)	10.5±2.4

Table 5.2 presents the mean SD with the reference values of the iron and inflammatory biomarkers.

Table 5.2: Iron and inflammatory biomarker status of the adolescent girls (10–17 years)

Biomarkers (Reference range)	**Mean±SD (n= 1195)**
Hemoglobin (12.0 – 16.0 (g/dl))	12.4±1.1
sFer (30-150 µg/L)	53.2±36.3
sFer (Ln)	3.8±0.7
TBI (mg/kg)	7.7±3.6
Hematocrit/PCV (36–46 %)	39.3±2.9
MCV (78 - 100 fl)	84.6±6.8
MCH (28.4-30.7 pg)	26.7±2.9
MCHC (31.0 – 36.0 %)	31.6±1.3
RDW-CV (11.5-14.5 %)	13.2±1.6
sTfR (1.9-5.0 µg/ml)	3.7±2.6
WBC [4.5 – 13.5 (10^9/L)]	9.9±2.3
CRP (<5.0mg/L)	1.2±3.6

List of abbreviations: hemoglobin (Hb); hematocrit/packed cell volume (PCV); mean corpuscular volume (MCV); mean corpuscular hemoglobin (MCH); mean corpuscular hemoglobin concentration (MCHC); red cell distribution width coefficient of variation (RDW- CV); serum ferritin (sFer); natural log of serum ferritin (sFerLn); soluble transferrin receptor (sTfR); total body iron (TBI); white blood cell (WBC), and C-reactive protein (CRP).

The prevalence of ID with or without anemia is presented in Table 5.3. ID without anemia was found to be much lower at the clinical level (3.0%) and higher at the sub-clinical level (15.3%).

Table 5.3: Prevalence of iron deficiency with or without anemia and iron biomarkers of the adolescent girls (10–17 years), adjusted for inflammation

Parameters	**Percent (%) (n= 915)**
Anemia (Hb <12.0 g/dL)*	27.0
Mild anemia (11.0-11.9 g/dL) (n=178)	19.5
Moderate anemia (8.1-10.9 g/dL) (n=61)	6.7
Severe anemia (<8.0 g/dL) (n=8)	0.9
Anemia without ID* (Hb <12.0 g/dL, sFer >15 µg/L) (n=186)	20.3
Iron deficiency anemia* (IDA)	
Hb <12.0 g/dL, sFer <15 µg/L (n=61)	6.7
Hb <12.0 g/dL, sFer <30 µg/L (n=98)	10.7
Iron deficiency without anemia* (ID w/o A)	
Hb >=12.0 g/dL, sFer <15 µg/L (n=27)	3.0
Hb >=12.0 g/dL, sFer <30 µg/L (n=140)	15.3
Iron deficiency*	
Single criterion	
sFer <15 µg/L (n=88)	9.6
sFer <30 µg/L (n=238)	26
Multiple criteria	
sFer <15 µg/L, sTfR >5.0 µg/ml (n=57)	6.2
sFer <30 µg/L, sTfR >5.0 µg/ml (n=73)	8.0
Risk of iron overload* (sFer >150 µg/L) (n= 15)	1.6
Iron deficiency biomarkers*	
TBI <0 mg/kg (n=37)	4.0
RDW- CV >16% (n=40)	4.4
MCV <75 fl (n=82)	9.0
Inflammatory biomarkers	
CRP >5 mg/L (n=37)	3.1
WBC > 11.5 (10^9/L) (n=245)	20.6

** After excluding inflammation, indicated at CRP >5 mg/L and WBC > 11.5 10^9/L*

Table 5.4 shows the differences of major iron and inflammatory biomarkers within each socio-demographics characteristic. Those who completed primary education had significantly higher sFer levels (7.42 μg/L) than those who completed secondary education. Similarly, those who had not experienced menstruation had significantly higher sFer levels (11.49 μg/L) than those who had not experienced menstruation. MCV and WBC levels were significantly different in the age and education categories. Teen BMI was significantly negatively correlated with sFer, and TBI was significantly positively correlated with MCV and WBC.

Table 5.4: Distribution of mean difference and correlation of iron biomarkers in different parameters of adolescent girls (10–17 years)

Variables	Mean difference (CI)								
	Hb (g/dL)	**Hematocrit (%)**	**sFer (µg/L)**	**TBI (mg/kg)**	**sTfR (µg/ml)**	**MCV (fl)**	**RDW-CV (%)**	**CRP (mg/L)**	**WBC (10^9/L)**
Age									
10-13 years 14-17 years	0.02 (-0.1, 0.1)	-0.05 (-0.4, 0.3)	2.42 (-2.4, 7.2)	0.32 (-0.2, 0.8)	-0.01 (-0.3, 0.3)	-2.44 (-3.3, -1.5) *	0.12 (-0.1, 0.3)	-0.12 (-0.2, 0.0)	0.25* (0.1, 0.4)
Education (completed)									
Primary Secondary	0.05 (-0.1, 0.2)	0.14 (-0.3, 0.6)	7.75 (2.4, 13.1) *	0.78 (0.3, 1.3) *	-0.21 (-0.6, 0.2)	-1.37 (-2.4, -0.4) *	-0.08 (-0.3, 0.1)	-0.04 (-0.1, 0.1)	0.40 (0.2, 0.6) *
Menarche									
No Yes	-0.12 (-0.3, 0.0)	-0.11 (-0.5 0.3)	-10.22 (-15.3, -5.2) *	-1.03 (-1.5, -0.5) *	0.19 (-0.2, 0.6)	2.50 (1.5, 3.5) *	0.09 (-0.1, 0.3)	0.11 (0.0, 0.2) *	-0.17 (-0.4, 0.0)
Handwashing with soap									
No Yes	-0.01(-0.2, 0.2)	-0.03 (-0.6, 0.5)	3.72 (-2.9, 10.4)	0.03 (-0.6, 0.7)	-0.16 (-0.6, 0.3)	-0.05 (-1.3, 1.2)	-0.11 (-0.4, 0.2)	0.08 (0.0, 0.2)	-0.05 (-0.3, 0.2)
Knowledge of iron-rich foods									
No Yes	-0.11 (-0.4, 0.2)	-0.29 (-1.1, 0.6)	5.69 (-5.1 16.5)	0.48 (-0.6, 1.6)	0.15 (-0.6, 0.9)	0.12 (-1.9, 2.2)	0.22 (-0.3, 0.7)	0.01 (-0.2, 0.2)	0.04 (-0.4, 0.5)
Teen BMI *(r)*	0.02	0.04	-0.07**	-0.08**	0.01	0.07**	-0.01	0.23**	0.08**
Fe intake (mg/day) from 7DDR *(r)*	-0.04	-0.06	0.00	-0.01	-0.01	0.07**	-0.04	0.04	-0.03

**Independent t test significant at <0.05*

*** Pearson correlation (r) significant at <0.05*

Linear Mixed Model (models 1–4) presented in Table 5.5 show that menarche had a strong effect on sFer (log) and TBI level. The sFer levels of those who had not experienced menstruation were 33.11% higher than those who had experienced menstruation after adjusting for age and BMI (model 1) and 31.24% higher after adjusting for BMI only (model 2). Similarly, a higher TBI of an average of 1.09 mg/kg and 0.96 mg/kg was noted among those who had not experienced menstruation compared to those who had experienced menstruation after adjusting for with or without age and BMI in models 3 and 4, respectively. However, age and BMI have no effect on sFer (log) and TBI at a significance level of $p<0.05$, holding menarche and BMI constant.

Table 5.5: Factors associated[Ψ] with serum iron and total body iron status after adjusting for inflammation.

Variable change	Serum Ferritin (sFer) µg/L										Total body iron (TBI) mg/kg									
	Model 1					Model 2					Model 3					Model 4				
	Δ (%)	Std error	p	95% CI		Δ (%)	Std error	p	95% CI		Est	Std error	p	95% CI		Est	Std error	p	95% CI	
				L	U				L	U				L	U				L	U
Intercept	3954.86	0.19	0.000[ϕ]	3.34	4.07	3858.46	0.18	0.000[ϕ]	3.32	4.03	7.97	0.91	0.000[ϕ]	6.18	9.76	7.75	0.88	0.000[ϕ]	6.02	9.49
Menarche (No)	33.11	0.07	0.000[ϕ]	0.16	0.41	31.24	0.06	0.000[ϕ]	0.16	0.39	1.09	0.32	0.001[ϕ]	0.47	1.71	0.96	0.29	0.001[ϕ]	0.4	1.53
Age (10-13 yrs)	-2.92	0.06	0.611	-0.14	0.08	-	-	-	-	-	-0.27	0.28	0.347	-0.83	0.29	-	-	-	-	-
BMI	-0.24	0.01	0.805	-0.02	0.02	-0.17	0.01	0.858	-0.02	0.02	-0.03	0.05	0.509	-0.12	0.06	-0.02	0.05	0.59	-0.11	0.07

Outcome variable sFer (Ln) and TBI

[Ψ] Linear mixed model; random effect: upazilla (sub-district); fixed effect: Menarche Y/N, Age Cat (10-13; 14-18 yrs), BMI Teen

[ϕ] Estimates of fixed effect significant at <0.05

5.5 DISCUSSION

Using major iron status biomarkers, this study reported the detailed iron status of Bangladeshi adolescent girls aged 10–17 years residing in both urban and rural areas. Overall, after adjustment for inflammation, 27.7% of adolescent girls were found to be anemic and 20.2% to be suffering from mild anemia. The study defined anemia without iron deficiency (ID) and ID with or without anemia, using a single criterion and multiple criteria for better understanding of the prevalence and the potential for types of anemia (Rangan, 1997). Clinical manifestation of iron deficiency prevalence was found to be approximately 10%, whereas sub-clinical ID was 26%. The higher prevalence of mild anemia and sub-clinical ID creates a window of opportunity for Bangladesh to substantially reduce iron deficiency anemia (IDA) since it is the most treatable of all types of anemias (DeLoughery, 2017).

The prevalence of ID with or without anemia in this research is similar to the prevalence of ID and IDA reported in the national micronutrient survey (NMS) in 2011-12 (ICDDRB et al., 2013); however, our data show a slight increase in anemia without ID (27.7% vs 26% in 2011-12) and ID (9.6% [clinical], 26% [sub-clinical] vs 7.1% in 2011-12). Caution is advised in interpreting the data when comparing them with NMS 2011-12. The study used only adolescent girls aged 10–17 years, whereas NMS 2011-12 considered two age categories: school-age children aged 6–14 years and non-pregnant non-lactating (NPNL) women of reproductive age 15–49 years, including both boys and girls. Another difference is that the current study used multiple criteria for defining anemia with ID, IDA, ID without anemia, and ID both at clinical and sub-clinical cut-offs. As well, this study reported iron overload among the adolescent girls, finding that although Bangladeshi adolescent girls had a high prevalence of deficiency indicators, 1.6% of the girls had been suffering from a higher risk of iron overload. Almost the entire study population drank tube well water (97.2%), and the prevalence of iron deficiency was still high, although an earlier study had reported that drinking tube well water lowers the chance of ID regardless of socio-economic status, geographical location, and food consumption status (ICDDRB et al., 2013), possibly because the presence of naturally containing iron in groundwater increases the bioavailability of iron (ICDDRB et al., 2013; Merrill et al., 2011). The NMS (2011-2012) report stated iron deficiency (sFer <15 μg/L) prevalence is low and not a problem of large magnitude in Bangladesh. This study opposes this statement because (1) ID remained the same in 2018 as it was in 2011-12 (9.6% vs 9.5%, respectively) and (2) the report did not include sub-

clinical ID (sFer <30 μg/L), which was found in 26% of adolescents in this study. Although the NMS survey does not explain generalizability, both the current study and the survey (NMS was a nationally representative survey) reported similar statistics on anemia, ID, and IDA. The data are insufficient to determine the cause; however other studies have reported that inadequate intake of dietary iron and low iron bioavailability of plant-based diets are the major reasons for ID (Zimmermann & Hurrell, 2007). To reduce the prevalence of ID in Bangladesh, several approaches have been considered. One is food-based fortification, an approach that does not single out groups at risk for ID. Another approach is iron supplementation, which requires active involvement in poorly resourced settings, particularly of low-income households (Davidsson & Nestel, 2004; D. Thavarajah et al., 2009; D. Thavarajah, Thavarajah, Wejesuriya, et al., 2011). Due to low compliance and implementation challenges, other studies have advised that iron fortification could be a long-term sustainable solution to mitigate ID (Huma et al., 2007; Hurrell, 1997b).

The current study's major limitation is that it does not explain the temporal relationship as data were collected at a single point in time from a specific population. Thus, the study's results cannot be generalized to other populations. Another limitation is that there were insufficient confounders to explain the reasons for the high prevalence of ID and IDA. However, the collection of direct blood samples to test all major iron biomarkers from a large sample of adolescent girls strengthens the study's findings. Additionally, the description of all iron biomarkers help to (1) assess the total amount of iron circulating in the blood, (2) total iron transport capacity, and (3) iron stored in the body of the sample of Bangladeshi adolescent girls. sFer and TBI as indicators for ID were included because these are the most reliable and cost-effective indicators for ID (Camaschella, 2017, 2019; Cook et al., 2003, 2005; Mei et al., 2005; Zimmermann & Hurrell, 2007). The study further included MCV, MCH sTfR, and RDW as these are reliable ID diagnostic parameters and enable the identification of specific types of anemia (Camaschella, 2017; DeLoughery, 2017; Harms & Kaiser, 2015). However, serum iron (SI) was not included in the study's diagnostic tools as it may falsely increase due to oral iron intake among the iron deficit population and can show low levels due to anemia with inflammation. Additionally, total iron-binding capacity (TIBC) was not included as it is sensitive to, but not specific to, ID testing (DeLoughery, 2017). Furthermore, the bone marrow iron stain is the most confirmatory and gold standard test to detect ID (DeLoughery, 2017).

5.6 CONCLUSION

One quarter of the adolescent girls in this study were found to be anemic and one tenth iron deficient; the majority of which were mild cases. Culturally-appropriate public health interventions may reduce these burdens in Bangladeshi teen girls.

CHAPTER 6 THE EFFECTS OF IRON-FORTIFIED LENTILS ON THE IRON (FE) STATUS OF ADOLESCENT GIRLS IN BANGLADESH: A DOUBLE-BLIND, COMMUNITY-BASED, CLUSTER-RANDOMIZED CONTROLLED TRIAL

This chapter presents an experimental study. It provides information on the study's rationale, materials and methods, as well as the efficacy of the consumption of iron-fortified lentils on the body iron stores of adolescent girls in Bangladesh. This is a manuscript-formatted chapter intended for journal publication.

6.1 ABSTRACT

Background:

Iron deficiency (ID) and iron deficiency anemia (IDA) are major public health concerns worldwide. The study attempted to reduce ID and IDA through iron-fortified lentils—a food-based sustainable micronutrient intervention.

Methods:

A double-blind, community-based, cluster-randomized controlled trial was designed and conducted with n=1195 Bangladeshi adolescent girls aged between 10–17 years. There were three intervention arms of the study: one arm received cooked iron-fortified lentils, one arm received cooked non-iron-fortified lentils, and one arm received no lentils (usual intake group). The two lentil groups were served either iron-fortified or non-iron-fortified lentils equal to the raw amount of 37.5 g (approximately 200 g of cooked lentils) five days per week for 85 feeding days (about four months). The lentils were incorporated into a standardized local dal recipe. The iron fortification of the lentils was carried out at the University of Saskatchewan, Department of Plant Sciences. Both socio-demographic information and venous blood (6 ml) were taken from the participants. Analysis was conducted at the baseline, midline, and end line of the study.

Results:

Adolescent girls who consumed ~ 200 g of cooked, iron-fortified lentils (equivalent to raw amounts of 37.5 g) over four months significantly increased ferritin levels by 21%, Hb by 0.15 g/dL, and total body iron (TBI) by 0.88 mg/kg compared to those who did not consume iron-fortified lentils, after adjusting for inflammation and holding upazilla as a random effect and age constant. Furthermore, the study found that those who consumed iron-fortified lentils, the odds of developing clinical ID (sFer <15 µg/L) rather than normal body iron (sFer 30-150 µg/L) were 57% less likely (OR 0.43; p=0.01) compared with the usual intake group after adjusting for age and baseline ID (<15 µg/L). The odds slightly increased [60% lower likelihood (OR 0.40; p=0.01)] of having sub-clinical ID (sFer 15 to <30 µg/L).

Conclusion:

The consumption of iron-fortified lentils is an effective intervention strategy for reducing iron deficiency among vulnerable adolescent girls.

Trial registration: ClinicalTrials.gov NCT03516734. Registered on 24 May 2018.

Keyword: Fortification, Micronutrient deficiency, Nutrition, Food-based approach, ID, IDA, etc

6.2 INTRODUCTION

Iron deficiency (ID) is one of the most persistent micronutrient deficiencies in the world. In a recent report, the national prevalence of anemia (< 12.0 g/dl) in Bangladesh was found to be 26% and 17.1% among non-pregnant non-lactating (NPNL) women aged 15–49 years and adolescent girls aged 12–14 years, respectively (ICDDRB et al., 2013). The report further stated that iron deficiency anemia (IDA) was much lower than expected, at 4.8% and 1.8% for the respective age groups. The definition of IDA was set to hemoglobin <12.0 g/dl plus serum ferritin (sFer) level <15.0 µg/L for NPNL and adolescents aged 12–14 years. Furthermore, the prevalence of ID defined by sFer level<15.0 µg/L was found to be 7.1% and 9.5% for the same respective age groups. This report covered only the clinical cut-offs of ID and reported that of all groups, adolescent girls are the most likely to suffer from ID (ICDDRB et al., 2013). Since it is the standard threshold for ID, holding the cut-off for ferritin at the sub-clinical level (sFer <30.0 µg/L) would likely increase both ID and IDA prevalence (Daru et al., 2017). However, no other study or report stating the situation with sub-clinical ID in Bangladesh was found. Additionally, the recommended dietary allowance (RDA) for iron increases almost two-fold during adolescence, due to the significant body growth that occurs during this period (Institute of Medicine, 2003; World Health Organization (WHO) & Food and Agricultural Organization of the United Nations (FAO), 2004). As well, the iron lost by adolescent girls during menstruation (roughly 0.5 mg Fe per day) plays a major role in their additional demands for iron and makes them more susceptible to ID (E. M. Miller, 2016). Although previous studies have reported that about 50% of anemia cases are linked to ID, recent studies have attributed only 25% and 37% of anemia cases among pre-school children and non-pregnant women, respectively, to ID (Chaparro & Suchdev, 2019; Petry et al., 2016).

The World Health Organization (WHO) has stated that food fortification technology has the potential to reduce global micronutrient deficiencies because fortified foods can reach a larger share of the population and be a more sustainable solution than other interventions (Allen et al., 2006; Tontisirin et al., 2002). Osendarp and colleagues supported this statement, pointing out that food fortification is one of the most cost-effective interventions to reduce micronutrient deficiencies (Osendarp et al., 2018). One potential candidate for fortification could be the lentils - a legume staple rich in protein and iron. Studies on Saskatchewan-grown lentils report varieties having high amounts of Fe (73–90 mg/kg), zinc (Zn, 44–54 mg/kg), and selenium (425–

673µg/kg) (D. Thavarajah, Thavarajah, Sarker, et al., 2011; D. Thavarajah, Thavarajah, Wejesuriya, et al., 2011). Lentils grown in Saskatchewan soil showed low anti-iron inhibitory compounds such as phytic acid (2.5–4.4 mg/g) compared to the mutant wheat (1.24-2.51 mg g^{-1} of total phytic acid P) and common bean (0.52-1.38 mg g^{-1} of total phytic acid P), making the Fe in the lentil relatively more bioavailable (DellaValle, Vandenberg, et al., 2013; D. Thavarajah, Thavarajah, Sarker, et al., 2011; D. Thavarajah, Thavarajah, Wejesuriya, et al., 2011). Other research has indicated that iron fortification of lentils has been successful at increasing the Fe concentration and relative bioavailability (Podder et al., 2017; Podder, Dellavalle, et al., 2018; Podder, Khan, et al., 2018). Therefore, the current study hypothesized that iron-fortified lentils could have substantial positive influence on adolescent girls' body iron status. If the data supported the hypothesis, iron-fortified lentils could be a sustainable food-based solution to minimize the global iron deficiency burden.

6.3 METHODS AND MATERIALS

6.3.1 Study setting and population

The study was conducted at the BRAC Adolescent Clubs (AC) in Bangladesh. The concept behind the clubs was to gather community adolescent boys and girls, regardless of their school, marital status, and socio-economic status, to socialize, receive life-skill-based education (LSBE) sessions, gain access to mini-library facilities, take part in cultural activities and indoor games, and celebrate national and international days. About 25–40 adolescent boys and girls belong to each club and use the physical structure of the BRAC School, which operates after school hours (Nawaz & Ahmed, 2009). A total of 48 clubs were randomly selected for this study from four sub-districts (Upazilas), i.e., Muktagacha, Mymensingh Sadar, Bhaluka and Gaffargaon in the Mymensingh district. Generally healthy adolescent girls aged between 10 and 17 years were enrolled from the randomly selected 48 BRAC AC. Participants had to be non-smokers and could neither be pregnant nor breastfeeding.

6.3.2 Study design

A double-blind, community-based, cluster-randomized controlled trial was designed to investigate the effect of iron-fortified lentils on adolescent girls' body Fe status. Both trial participants and outcome assessors were blinded on the intervention assignments. Details of the

design were explained in the earlier protocol paper (Yunus, Jalal, et al., 2019). The study was conducted from September 2018– April 2019.

6.3.3 Fe fortification of lentils

Saskatchewan-grown small red lentils (CDC Maxim) were fortified with iron at the Saskatchewan Food Industry Development Center Inc. (Food Centre) in collaboration with the Department of Plant Sciences of the Crop Development Centre (CDC) of the University of Saskatchewan, Canada. Lentils were fortified with NaFeEDTA, which contains approximately 13–14 mg of Fe per 100 kg of lentils (Podder et al., 2017). Details of the fortification process and methods were described in the earlier paper (Yunus, Jalal, et al., 2019). Both iron-fortified and non-iron-fortified lentils were packed in 20 kg bags and air-shipped to Bangladesh. Each bag of lentils had three layers of packing. First, the lentils were packed in polyvinyl bags and then packed in industrial food grade bags. Finally, each bag was placed in a stiff cardboard box before shipment. Two layers of colour coding were applied to each bag to ensure the accuracy of the intervention assignment. Inside, the boxes, the colour white represented iron-fortified lentils and yellow non-iron-fortified lentils. The outside of the box was marked with an adhesive coloured sticker (blue or green).

6.3.4 Intervention

There were three intervention groups in this study. One group consumed Fe-fortified lentils, the second group consumed non-Fe-fortified lentils, and the third group consumed no lentils, just their usual diet. The two intervention groups were served the same amount of cooked lentils (about 200 g), the equivalent of 37.5 g of raw lentils, five days a week for 85 feeding days (about four months) (Yunus, Jalal, et al., 2019). The study profile is presented in Figure 6.1. All lentils were cooked with standardized local daal recipes regardless of the intervention type. A similar recipe was used during the feasibility study (Yunus, 2018). Additionally, a standardized cup of cooked rice (locally known as ‘Baat’) was mixed with the lentils and given to both feeding groups.

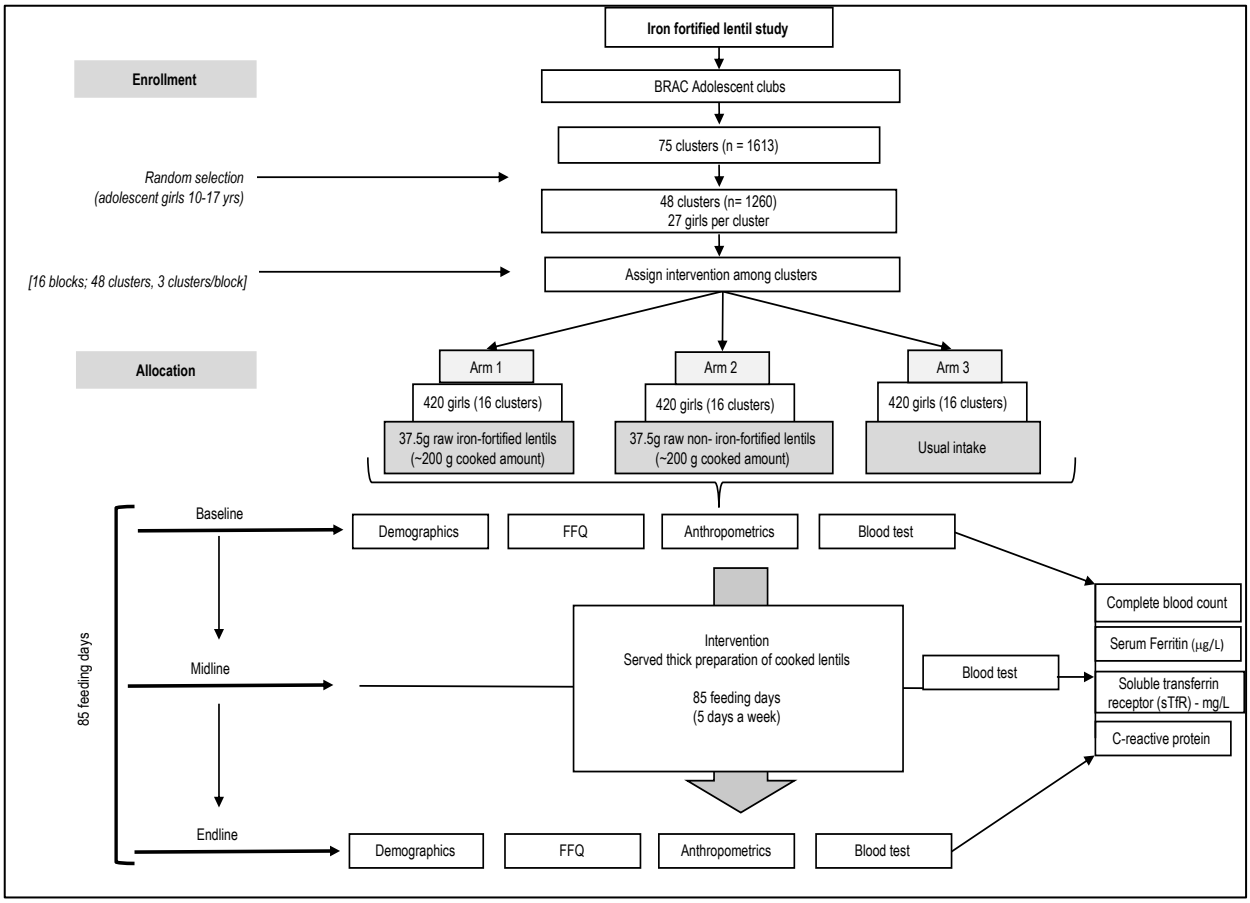

Figure 6.1: Efficacy trial profile [adopted from (Yunus, Jalal, et al., 2019)]

6.3.5 Sample size, randomization, and blinding

Considering the low expected mean serum ferritin (sFer) difference among groups (5 ±20 µg/L) with inter-cluster correlation (ICC) at 0.025 and 80% power at $p < 0.05$ significance level, a total of 48 clusters (clubs) were selected and further divided into 16 blocks (Yunus, Jalal, et al., 2019). Therefore, each block consisted of three adolescent clubs. Randomization was carried out in two phases using computer-generated random assignments. First, clusters (n = 48) were randomly assigned to blocks (n = 16), and, second, interventions (either Fe fortified, non-Fe fortified, or no lentils) were randomly assigned to each block (n = 3 clusters in each block). Clusters were the unit of randomization; however, data were collected at an individual level. This provided an equal number of clubs/clusters (n = 16) in each intervention group. Each group consisted of n=420 participants. The attrition rate was assumed to be 20%, leaving the total sample of n=1260 adolescent girls.

All the intervention assignments were carried out by a third party under the direct supervision of the principal investigator; however, blinding did not need to be broken down as there were no reported adverse effects relevant to lentil consumption during the study period. Details of the blinding break-down process were described in the earlier paper (Yunus, Jalal, et al., 2019). All participants in the study were assigned an eight-digit unique identity number (UID): the first two digits represented the club ID (01, 02, 03…….48), the middle four-digits the cluster (1001, 2002, 3003……1616), and the last two digits (01,02,03……) the participants.

6.3.6 Data collection tools and technique

Survey data, venous blood samples, and lentil consumption data were collected at baseline and end line; venous blood samples were also collected at midline. First, consent and assent were collected, and all potential participants were invited to come to the adolescent club for venous blood sample collection. Those who provided blood samples were considered as study participants. Venous blood samples of 6 ml were collected from each participant to test complete blood count (CBC), which included the erythrocyte sedimentation rate (ESR); hemoglobin (Hb); hematocrit; packed cell volume (PCV); mean corpuscular hemoglobin (MCH); mean corpuscular volume (MCV); mean corpuscular hemoglobin concentration (MCHC); red blood cell (RBC) and white blood cell (WBC) total count; differential count; platelet count; serum ferritin (sFer); soluble transferrin receptor (sTfR); C-reactive protein (CRP); and blood ABO typing. Blood samples were collected through aseptic procedures and a butterfly needle by heparinized vacutainers. All venous blood samples were collected in the afternoons or at noon as the adolescent girls were in school in the morning. Serum separation at 2-8 ^{o}C temperature was conducted at a field-based laboratory and stored locally. It was then transported to the central laboratory for analysis at the International Center for Diarrhoeal Disease Research, Bangladesh (ICDDRB) the next morning by maintaining a cold chain.

Survey data was collected electronically by 7” tablets built under an Android operating system using Open Data Kit (ODK) app-connected to the internet (Hartung et al., 2010; Raja, Tridane, Gaffar, Lindquist, & Prabadi, 2014). This electronic collection allowed for the monitoring of real-time data directly saved in BRAC’s secure server, once the research assistant had completed each interview. Those who did not have a history of taking anti-helminthic drugs in the four months prior to the lentil intervention were dewormed.

Those assigned to the two lentil groups were served about 200 g of cooked lentils (the equivalent of 37.5 g raw lentils). Research assistants measured the cooked amount served to each participant within +/- 1 g precision using a rechargeable digital scale. For instance, if a club had a total of 32 adolescent boys and girls, the total raw amount was determined as 37.5 g x 32 = 1200 g (1.2 kg) and cooked accordingly. Suppose the cooked amount totalled 5990 g (5 kg 990 g), it was then divided by total participants ($n = 32$) = 187 g, meaning that all club participants were served 187 g of cooked lentils. The number of participating adolescents in the club was fixed before the intervention, and it remained the same for the 85 days of the study. Participants were also given an option to have 'cooked lentils (dal) only' or 'dal and rice together.' Once participants finished eating the dal, the study's research assistant measured the residual amount to calculate the actual lentil consumption.

6.3.7 Variables assessed

Self-reported socio-demographic information, seven-day dietary recall (7DDR), and food security status data were collected from all participants. The study's primary objective was to understand if consuming iron-fortified lentils had an effect on body Fe status. To this end, lab blood tests were assessed for hemoglobin, hematocrit (volume of red blood cells), serum ferritin (sFer), serum transferrin receptor (sTfR), c-reactive protein (CRP), and calculated total body Fe (TBI, [log (TfR/ferritin ratio)) (Cook et al., 2003). Anemia, ID, and IDA were categorized according to the WHO cut-off points for anemia (<12.0 g/dL), ID (sFer < 15ug/L), and IDA (Hb <12.0 g/dL plus sFer <15.0 µg/L). Elevated CRP (>10.0 mg/l) and WBC (< 11.5 10^9L) were used to adjust sFer for inflammation (ICDDRB et al., 2013; World Health Organization, 2017). The sub-clinical cut-off points for sFer and sTfR were set at <30 ug/L and >8.0 ug/mL, respectively (Lopez et al., 2016). The study also looked at the changes to the adolescent girls' anthropometric data over the 4 months. The study measured height (cm), weight (kg), mid-upper arm circumference (MUAC; cm), hip circumference (cm), and waist circumference (cm). The participants' BMI (teen) was calculated in percentiles and z-scores (Centers for Disease Control and Prevention (CDC), 2017; Yunus, Jalal, et al., 2019).

6.3.8 Statistical analysis

First, group comparability was assessed by analyzing all baseline characteristics to determine if the random assignments were successful. Both descriptive and inferential statistics

were used. Serum ferritin data were log-transformed (natural) to avoid the violation of assumptions about the desired parametric analysis, and the study presented log-transformed data as percent change. This is a well-accepted and widely used data transformation procedure that allows the researcher to focus on the analysis of appropriate clinical measures of effect (Govus et al., 2015). Instead of using beta coefficient estimates, percent (%) change was used for this variable because it was natural log transformed. Regression model beta coefficient estimates were first exponentiated and then subtracted 1 from the exponentiated values and then multiplied by 100 (UCLA: Statistical Consulting Group, 2020). One-way ANOVA with post-hoc Tukey's test and chi-square test were used to determine the group mean differences among the continuous variables and association among categorical variables, respectively. Repeated measures ANOVA (RMANOVA) were used to test the group mean difference, time effect at baseline, midline, and end line, and group-by-time interactions were assessed to determine the changes in body iron status. Finally, multiple linear regression analysis [GLM, mixed models (with upazilla— sub-districts in Bangladesh—as the random effect)] were used to determine the effect of iron-fortified lentils on iron status after controlling for age only for the post-intervention data. Confounders such as menarche status, iron consumed from 7DDR (diff. baseline and end line), BMI teen, and drinking water (tube well) were not included since they were not eligible to be included in the final model. Since the study focused on body iron changes, ID was further categorized at clinical, sub-clinical, and iron overload cut-offs. Multinomial logistic regression was carried out to calculate the likelihood of iron-fortified lentils to increase sFer on the various ID cut-offs after adjusting for age and baseline iron biomarkers. Combined inflammation indicated at CRP >5 mg/L and WBC >11.5 (10^9/L) was adjusted for in all analyses. SPSS for Windows PAWS version 25 was used to analyze data, and $p < 0.05$ was set as the significance level.

6.3.9 Ethical approval

Ethical approvals were received from the University of Saskatchewan, Canada (Bio#17–177), Marywood University, USA (IRB#1139116–2), and the Bangladesh Medical Research Council (BMRC/NREC/2016–2019/455) as per their respective protocols. Informed written consent and assent were taken from each participant and their respective parents, and a copy of the signed assent and consent form was given to the participants and parents.

6.4 RESULTS

Table 6.1 presents the mean±SEM of the vital iron biomarkers at three-time points after adjusting for inflammation (CRP > 5 mg/L and WBC >11.5 10^9/L) at each of the three time points. A declining trend of all biomarkers was observed among all study groups over time; however, the biomarkers of the usual intake group significantly declined compared to those of the iron-fortified lentil consumption group. Confidence interval plot for sFer is presented in figure 6.2.

Table 6.1: Comparison (Mean±SEM) of iron biomarkers in Bangladesh adolescent girls during a ~4-month iron-fortified lentil intervention in the form of NaFeEDTA.

Variables	**Total (n)**	**Iron-fortified lentils**	**Non-iron-fortified lentils**	**Usual intake**	**P value**
Hb (g/dL)					
Baseline	1177	12.4 (±0.1)	12.4 (±0.1)	12.4 (±0.1)	0.593
Midline	871	12.3 (±0.1)	12.2 (±0.1)	12.1 (±0.1)	0.274
End line	936	12.2 (±0.1) $^{\Psi}$	12.1 (±0.1)	12.0 (±0.1) $^{\Psi}$	0.053
Ferritin (µg/L)					
Baseline	1178	55.6 (±1.8)	52.9 (±1.8)	53.6 (±1.9)	0.415
Midline	871	51.7 (±1.9) $^{\Psi}$	43.0 (±1.5)	45.6 (±2.0) $^{\Psi}$	0.002*
End line	936	49.5(±1.7) $^{\Psi}$	38.7 (±1.3) $^{\Psi}$	39.6 (±1.7) $^{\Psi}$	<0.001*
sTfR (µg/ml)					
Baseline	1178	3.6 (±0.1)	3.7 (±0.1)	3.7 (±0.1)	0.956
Midline	871	3.6 (±0.1)	3.9 (±0.2)	3.7 (±0.2)	0.202
End line	936	3.8 (±0.1)	4.2 (±0.2)	4.0 (±0.2)	0.192
TBI (mg/Kg)					
Baseline	1178	7.8 (±0.2)	7.6 (±0.2)	7.6 (±0.2)	0.628
Midline	871	7.6 (±0.2) $^{\Psi}$	6.7 (±0.2) $^{\Psi}$	7.0 (±0.2)	0.007*
End line	936	7.3 (±0.2) $^{\Psi}$	6.1 (±0.2) $^{\Psi}$	6.2 (±0.2) $^{\Psi}$	<0.001*

All numbers represent Mean±SEM
Combined (baseline-midline-end line) inflammation (CRP > 5 mg/L and WBC >11.5 (10^9/L) adjusted
** One-way ANOVA test significant at p <0.05*
$^{\Psi}$Post-hoc Tukey's test significant at p <0.05

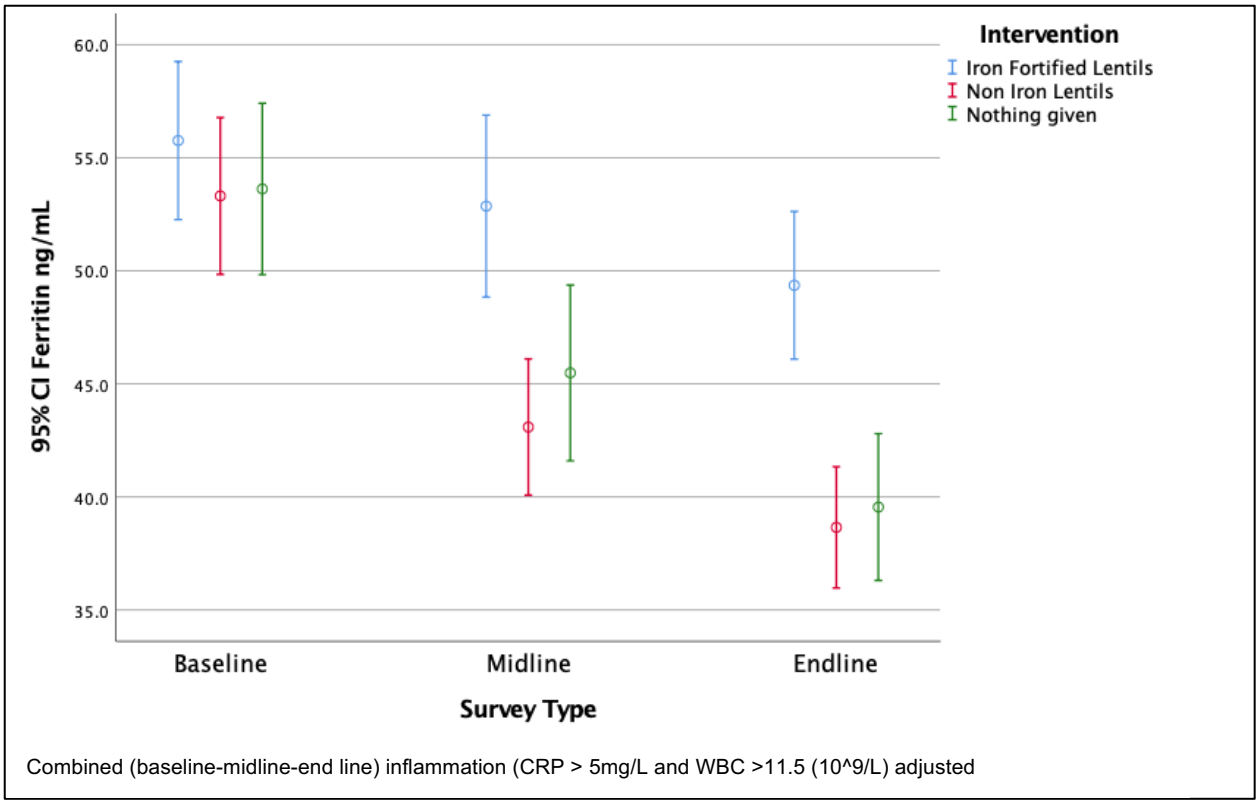

Figure 6.2: Confidence interval plot for sFer for three intervention groups. Based on a 95% confidence interval plot after adjusting for inflammation, the upper and lower border of the sample means (serum ferritin) of the iron-fortified lentil group, non-iron-fortified lentil group, and the usual intake group fall within each other at the baseline data point. Therefore, it is plausible that the true means of all intervention groups are equal at baseline. At the midline data, similarly, the upper and lower borders of the sample mean of the iron-fortified lentil group fall within the area of the upper and lower borders of the sample mean of the usual intake group. Therefore, they are equal. However, the upper and lower borders of the sample mean of the iron-fortified lentil group do not overlap with the sample mean of the non-fortified lentil group; hence, it is plausible that two populations have different means. At the end line, the upper and lower borders of the sample mean of the iron-fortified lentil group do not overlap with the upper and lower borders of the sample mean of the non-fortified lentil and usual intake groups; hence, it is plausible that the true mean of the iron-fortified lentil group is different from that of the other two populations. The sample mean of the non-iron-fortified lentil and usual groups fall within each other's upper and lower boundaries, both at midline and end line. Thus, the true mean of both groups is equal.

The prevalence of anemia, ID, and IDA was calculated after adjusting for inflammation (CRP > 5 mg/L and WBC >11.5 (10^9/L) at respective time points (Table 6.2). Prevalence was observed to increase over time points, but at a less significant level in the iron-fortified lentil group compared to the non-iron-fortified and usual intake groups. Less anemia (32.2%) was found in the iron-fortified lentil consumption group than what was expected, and more anemia

(47.5%) was found in the usual intake group at end line than what would have been expected if there were no relationship. A similar trend was observed in anemia without ID, ID without anemia, and sub-clinical ID. Anemia with ID prevalence was much higher (20.4% at baseline) than IDA (6.1% at baseline). Although ID at the clinical level was 9.4%, sub-clinical ID was more than two-fold (25.9%), suggesting that about one-third of the population had sub-clinical ID. The study observed consistent low prevalence (~1%) of active body infection among the adolescent girls over time.

Table 6.2: Prevalence of anemia, ID, and IDA in Bangladeshi adolescent girls during a ~4-month iron-fortified lentil intervention in the form of NaFeEDTA

Variables	**Total %**	**Iron-fortified lentils %**	**Non-iron-fortified lentils %**	**Usual intake %**	**P**
Anemia (<12 g/dL)					
Baseline	26.5	25.8	24.4	29.9	0.205
Midline	35.9	31.9	35.3	41.1	0.058
End line	38.6	32.2	37.6	47.5	0.001*
Anemia without ID (Hb <12.0 g/dL, sFer >15 µg/L)					
Baseline	20.4	20.3	19.5	21.7	0.754
Midline	27.9	25.8	27.4	31	0.346
End line	30.8	26.6	29.7	37.1	0.017*
Iron deficiency anemia					
Clinical (Hb <12.0 g/dL, sFer <15 µg/L)					
Baseline	6.1	5.5	4.9	8.2	0.13
Midline	7	5.4	7.3	8.5	0.279
End line	7.4	5.3	7.6	9.7	0.11
Sub-clinical (Hb <12.0 g/dL, sFer <30 µg/L)					
Baseline	10.5	10.5	9.2	12.2	0.383
Midline	13.1	12.4	13.5	13.5	0.883
End line	13.8	11.5	14	16.3	0.225
Iron deficiency without anemia					
Clinical (Hb <12.0 g/dL, sFer <15 µg/L)					

Baseline	3.3	3	4.7	2	0.101
Midline	2.1	2.8	2.1	1.3	0.362
End line	2.2	3.2	2.2	1	0.174
Sub-clinical (Hb <12.0 g/dL, sFer <30 µg/L)					
Baseline	15.4	14.8	18.5	12.2	0.047*
Midline	11.2	11.8	12.7	8.7	0.236
End line	11.3	13	13.2	6.9	0.021*
Iron deficiency					
Single criterion: Clinical (sFer <15 µg/L)					
Baseline	9.4	8.5	9.6	10.2	0.71
Midline	13.2	12	13.4	14.2	0.707
End line	12.2	9	13.9	13.7	0.097
Single criterion: Sub-clinical (sFer <30 µg/L)					
Baseline	25.9	25.3	27.7	24.4	0.548
Midline	34.1	27.9	36.1	38.7	0.012*
End line	39.2	28.6	44.8	44.6	<0.001*
Multiple criteria: Clinical (sFer <15 µg/L, sTfR >5.0 µg/ml)					
Baseline	5.9	5.5	6.6	5.7	0.784
Midline	5.9	5.7	7	4.7	0.425
End line	5.6	4.7	6.3	5.9	0.645
Multiple criteria: Sub-clinical (sFer <30 µg/L, sTfR >5.0 µg/ml)					
Baseline	7.6	8.3	8	6.3	0.536
Midline	7.7	8.6	8.4	6	0.387
End line	8.3	8.8	9	6.9	0.58
Inflammation [(CRP > 5mg/L and WBC >11.5 (10^9/L)]					
Baseline	1.3	0.7	1.4	1.9	0.35
Midline	0.7	1.4	0.3	0.6	0.191
End line	0.4	0	0.05	0.7	0.341

**Chi-square test significant at p <0.05*
All percentages stated after adjusting for inflammation (CRP > 5 mg/L and WBC >11.5 (10^9/L) except the inflammation variable itself.

Table 6.3 presents the percent difference in sFer levels and parameter estimations of Hb and TBI levels. Of those who consumed about 200 g of cooked iron-fortified lentils (equivalent

to the raw amount of 37.5 g) five days a week over 85 days (about four months), the average sFer level increased by 22.4 % ($p<0.001$; CI 0.14, 0.26) compared to those who consumed a similar amount of non-iron-fortified lentils for a similar duration after adjusting for inflammation, holding age constant, and treating upazilla as the random effect. An increment of 21.2% ($p<0.001$; CI 0.13, 0.26) of sFer was observed in the iron-fortified lentil consumption group compared to those who did not received lentils (either iron-fortified or non-iron-fortified) as a part of the intervention (usual intake group).

On average, Hb levels increased by 0.15 g/dL in the iron-fortified lentil group (p 0.004; CI 0.05, 0.25) compared to those who consumed non-iron-fortified lentils over the study duration of four months. A slightly higher increment of Hb level (0.17 g/dL) was observed (p 0.001; CI 0.07, 0.27) in the iron-fortified lentil consumption group than in the usual intake group.

TBI increased an average of 0.92 mg/kg ($p <0.001$; CI 0.66, 1.18) among those who consumed iron-fortified lentils of similar doses and duration compared to the non-iron-fortified lentil consumption group. In comparison with the usual intake group, TB increased an average of 0.82 mg/kg ($p <0.001$; CI 0.54, 1.10) in the iron-fortified lentil group. The majority of the changes to the sFer, Hb, and TBI levels in the iron-fortified lentil group occurred during the first two months of consumption. There were no significant changes in sFer, Hb, and TBI levels observed in the either the non-iron-fortified lentil or usual intake groups.

Table 6.3: Percent (Δ %) and estimates (est) change at 95% confidence intervals of ferritin, hemoglobin, and total body iron at three timepoints in the form of NaFeEDTA

Biomarkers	**Iron-fortified lentils vs Non-iron-fortified lentils**			**Iron-fortified lentils vs Usual intake**			**Non-iron-fortified lentils vs Usual intake**		
Ferritin (Ln) µg/L	**Δ (%)**	**p**	**CI 95%**	**Δ (%)**	**p**	**CI 95%**	**Δ (%)**	**p**	**CI 95%**
0-4 months	22.4	<0.001*	[0.14, 0.26]	21.2	<0.001*	[0.13, 0.26]	-1.4	0.657	[-0.08, 0.05]
0-2 months	13.3	<0.001*	[0.06, 0.19]	10.8	0.003*	[0.04, 0.17]	-0.2	0.956	[-0.07, 0.07]
2-4 months	8.5	0.010*	[0.02, 0.14]	10.0	0.005*	[0.03, 0.16]	-0.4	0.907	[-0.07, 0.06]
Hemoglobin g/dL	**Est**	**p**	**CI 95%**	**Est**	**p**	**CI 95%**	**Est**	**p**	**CI 95%**
0-4 months	0.15	≤0.004*	[0.05, 0.25]	0.17	0.001*	[0.07, 0.27]	0.01	0.785	[-0.09, 0.11]
0-2 months	0.15	≤0.004*	[0.05, 0.25]	0.13	0.007*	[0.03, 0.23]	0.00	0.960	[-0.10, 0.11]
2-4 months	0.01	≤0.871	[-0.08, 0.10]	0.05	0.313	[-0.05, 0.15]	0.04	0.423	[-0.06, 0.14]
TBI (mg/kg)	**Est**	**p**	**CI 95%**	**Est**	**p**	**CI 95%**	**Est**	**p**	**CI 95%**
0-4 months	0.92	<0.001*	[0.66, 1.18]	0.82	<0.001*	[0.54, 1.10]	-0.13	0.354	[-0.40, 0.14]
0-2 months	0.62	<0.001*	[0.34, 0.89]	0.33	0.021*	[0.05, 0.60]	-0.19	0.187	[-0.47, 0.09]
2-4 months	0.34	0.008*	[0.09, 0.59]	0.52	<0.001*	[0.25, 0.79]	0.09	0.489	[-0.17, 0.36]

** Mixed model significant at p <0.05 with 'upazilla' (sub-districts in Bangladesh) as the random factor effect and 'age' as the fixed factor*

Combined (baseline-midline-end line) inflammation (CRP > 5 mg/L and WBC >11.5 (10^9/L) adjusted

Table 6.4 presents the likelihood of the iron-fortified lentil group having clinical ID, sub-clinical ID, or iron overload at end line. Holding age and baseline ID (sFer <15 µg/L) constant, the odds of the adolescent girls who only received iron-fortified lentils developing clinical ID (sFer <15 µg/L) rather than normal body iron (sFer 30-150 µg/L) were 57% less likely (OR 0.43; p=0.01) than the odds for adolescent girls who consumed only their usual intake. The odds of developing clinical ID (sFer <15 µg/L) compared to normal iron status (sFer 30-150 µg/L) for the girls who received iron-fortified lentils were about 60% lower (OR 0.40; p=0.01) than the odds for those in the usual intake group. The relationship between consuming iron-fortified lentils and iron overload was not significant.

Table 6.4: Likelihood of those in the iron-fortified lentil group falling into three ID categories, after adjusting for age and baseline iron biomarkers

ID category[Ψ]	**Variables**	**Est**	**SE**	**OR**	**p**	**CI 95%**	
						Lower	**Upper**
Clinical ID (<15 µg/L) End line	Age (10–13 years)	-0.17	0.28	0.85	0.546	0.49	1.45
	Age (14–17 years)	--------- Ref ---------					
	ID (<15 µg/L) – Baseline	6.94	1.02	1036.91	<0.001*	139.47	7709.32
	ID (else) – Baseline	--------- Ref ---------					
	Iron-fortified lentils	-0.85	0.36	0.43	0.019*	0.21	0.87
	Non-iron-fortified lentils	0.20	0.32	1.23	0.523	0.66	2.29
	Usual intake	--------- Ref ---------					
Sub-Clinical ID (15 - 30 µg/L) End line	Age (10–13 years)	-0.14	0.17	0.87	0.418	0.63	1.21
	Age (14–17 years)	--------- Ref ---------					
	ID (15–30 µg/L) – Baseline	2.43	0.24	11.40	<0.001*	7.12	18.26
	ID (else) – Baseline	--------- Ref ---------					
	Iron-fortified lentils	-0.91	0.21	0.40	<0.001*	0.26	0.61
	Non-iron-fortified lentils	-0.11	0.19	0.90	0.574	0.61	1.31
	Usual intake	--------- Ref ---------					
Iron overload (>150 µg/L) End line	Age (10–13 years)	-1.10	0.48	0.33	0.020*	0.13	0.85
	Age (14–17 years)	--------- Ref ---------					
	ID (>150 µg/L) – Baseline	3.75	0.64	42.42	0.000*	12.10	148.70
	ID (else) – Baseline	--------- Ref ---------					
	Iron-fortified lentils	-0.96	0.55	0.38	0.077	0.13	1.11

	Non-iron-fortified lentils	-1.09	0.61	0.34	0.073	0.10	1.11
	Usual intake	--------- Ref ---------					

[Ψ]*Reference category: Normal body iron stores at serum ferritin 30-150 ng/ml*
Combined (baseline-midline-end line) inflammation (CRP > 5mg/L and WBC >11.5 (10^9/L) adjusted
**Significant at p<0.05*

As shown in the sub-category of anemia in Table 6.5, the adolescent girls who consumed iron-fortified lentils were 42% (OR 0.58; p = 0.006) less likely to develop mild anemia (Hb 11.0-11.99 g/dL) rather than having normal Hb levels (Hb >12 g/dL). The likelihood of suffering from moderate anemia (Hb 8.01- 10.99 g/dL) compared to having normal Hb levels is even lower in the iron-fortified lentil group (77%; OR 0.23; p <0.001), relative to the usual intake group. Exposure to iron-fortified lentils had no effect on severe anemia.

Table 6.5: Relationship of iron-fortified lentils to anemia categories after adjusting for age and baseline anemia

ID category[Ψ]	**Variables**	**Est**	**SE**	**OR**	**P**	**CI 95%**	
						Lower	**Upper**
Anemia – Mild (Hb 11.0-11.99 g/dL)	Age (10–13 years)	0.23	0.16	0.16	1.253	0.92	1.72
	Age (14–17 years)	--------- Ref ---------					
	Anemia – Mild – Baseline	-2.12	0.20	0.12	0.000*	0.08	0.18
	Anemia (else) – Baseline	--------- Ref ---------					
	Iron-fortified lentils	-0.54	0.20	0.58	0.006*	0.40	0.86
	Non-iron-fortified lentils	-0.29	0.19	0.75	0.130	0.52	1.09
	Usual intake	--------- Ref ---------					
Anemia – Moderate (Hb 8.01-10.99 g/dL)	Age (10–13 years)	-0.02	0.31	0.99	0.960	0.54	1.79
	Age (14–17 years)	--------- Ref ---------					
	Anemia -–Moderate – Baseline	-5.34	0.64	0.01	0.000*	0.00	0.02
	Anemia (else) – Baseline	--------- Ref ---------					
	Iron-fortified lentils	-1.46	0.39	0.23	0.000*	0.11	0.50
	Non-iron-fortified lentils	-0.86	0.35	0.42	0.014*	0.21	0.84
	Usual intake	--------- Ref ---------					
Anemia – Severe (<8.0 g/dL)	Age (10–13 years)	0.23	0.78	1.26	0.769	0.27	5.76
	Age (14–17 years)	--------- Ref ---------					
	Anemia – Severe – Baseline	-20.79	916.92	0.00	0.982	0.00	.[b]
	Anemia (else) – Baseline	--------- Ref ---------					

	Iron-fortified lentils	0.01	0.97	1.01	0.996	0.15	6.67
	Non-iron-fortified lentils	0.12	0.93	1.13	0.896	0.18	7.00
	Usual intake	--------- Ref ---------					

[Ψ]Reference category: No anemia (Hb >12 g/dL)
Combined (Baseline-midline-end line) inflammation (CRP > 5mg/L and WBC >11.5 (10^9/L) adjusted
**Significant at p<0.05*

As shown in Table 6.6, the adolescent girls who received iron-fortified lentils were 70% (OR 0.30; p=0.01) less likely to develop sub-clinical IDA (Hb < 12 and sFer 15-30 µg/L) at end line compared to the adolescent girls who received no lentils (usual intake). Clinical IDA (Hb < 12 g/dL and sFer < 15 µg/L) and iron overload with anemia (Hb < 12 and sFer >150 µg/L) had no effect on any of the study groups.

Table 6.6: Relationship of iron-fortified lentils to IDA categories, after adjusting for age and baseline anemia

ID category[Ψ]	**Variables**	**Est**	**SE**	**OR**	**P**	**CI 95%**	
						Lower	**Upper**
IDA–Clinical (Hb < 12 g/dL & sFer < 15 µg/L)	Age (10–13 years)	-0.452	0.334	0.64	0.176	0.331	1.225
	Age (14–17 years)	--------- Ref ---------					
	IDA–Clinical–Baseline	-5.373	1.032	0.00	0.000*	0.001	0.035
	IDA–Else–Baseline	--------- Ref ---------					
	Iron-fortified lentils	-0.715	0.447	0.49	0.109	0.204	1.174
	Non-iron-fortified lentils	0.337	0.377	1.40	0.371	0.670	2.932
	Usual intake	--------- Ref ---------					
IDA-–Sub-Clinical (Hb < 12 & sFer 15-30 µg/L)	Age (10–13 years)	-0.340	0.365	0.71	0.351	0.348	1.456
	Age (14–17 years)	--------- Ref ---------					
	IDA–Sub-clinical–Baseline	-2.040	0.567	0.13	0.000*	0.043	0.395
	IDA–Else–Baseline	--------- Ref ---------					
	Iron-fortified lentils	-1.218	0.443	0.30	0.006*	0.124	0.705
	Non-iron-fortified lentils	-1.167	0.443	0.31	0.008*	0.131	0.741
	Usual intake	--------- Ref ---------					
Iron overload with anemia (Hb < 12 &	Age (10–13 years)	-0.317	1.574	0.73	0.840	0.033	15.925
	Age (14–17 years)	--------- Ref ---------					
	IDA–Overload–Baseline	-15.593	0.000	0.00	.	0.000	0.000
	IDA–Else–Baseline	--------- Ref ---------					

sFer >150 µg/L)	Iron-fortified lentils	-0.249	1.996	0.78	0.901	0.016	38.974
	Non-iron-fortified lentils	-0.089	1.815	0.915	0.961	0.026	32.049
	Usual intake	--------- Ref ---------					

ᵠReference category: No IDA (Hb >12 & sFer 30-150 µg/L)
Combined (baseline-midline-end line) inflammation (CRP > 5mg/L and WBC >11.5 (10^9/L) adjusted
**Significant at p<0.05*

Table 6.7 presents the effect size of the iron-fortified lentils on sFer, Hb, and TBI levels. On average, 5.7 µg/L ferritin was a significant increase among the adolescents who consumed iron-fortified lentils for 85 days compared to the usual intake group. Although the ferritin levels of those in the non-iron-fortified group decreased more than the levels of those in the usual intake group, this was not significant. Similarly, TBI increased by 0.84 mg/kg in the iron-fortified lentil group compared to the usual intake group. Decreased TBI levels between the non-iron fortified and usual intake groups were not significant.

Table 6.7: Effect size of the key outcome variables

Outcome variables	Study groups	N = 871	Baseline Mean±SEM	Midline Mean±SEM	End line Mean±SEM	Mean±SEM diffirence (2 months)	Mean±SEM difference (4 months)	Effect size at 4 months ¥	CI 95%	
									Lower	Upper
Ferritin (µg/L)	Iron-fortified lentils	301	56.0 (±2.1)	51.7 (±1.8)	48.8 (±1.6)	- 4.3 (±1.4) *	- 7.2 (±1.3) *	5.7**	1.7	9.5
	Non-iron-fortified lentils	327	52.5 (±2.0)	42.6 (±1.8)	38.2 (±1.5)	- 9.9 (±1.3) *	- 14.3 (±1.3) *	-1.5	-5.4	2.4
	Usual intake	243	52.8 (±2.4)	45.0 (±2.1)	40.0 (±1.8)	- 7.7 (±1.5) *	-12.8 (±1.5) *	-	-	-
Total body iron (TBI) mg/kg	Iron-fortified lentils	301	7.8 (±0.2)	7.5 (±0.2)	7.3 (±0.2)	- 0.21 (±0.1) *	- 0.48 (±0.1) *	0.84**	0.56	1.12
	Non-iron-fortified lentils	327	7.5 (±0.2)	6.6 (±0.2)	6.1 (±0.2)	- 0.83 (±0.1) *	- 1.36 (±0.1) *	-0.04	0.02	0.02
	Usual intake	243	7.6 (±0.2)	7.0 (±0.2)	6.3 (±0.2)	- 0.61 (±0.1) *	- 1.32 (±0.1) *	-	-	-

**Pairwise comparison significant at p <0.05*
*** Two-Sample Independent t Test significant at p <0.001*
¥ Compared to usual intake group
Combined (baseline-midline-end line) inflammation (CRP > 5mg/L and WBC >11.5 (10^9/L) adjusted

6.5 DISCUSSION

This study consisted of an experiment with three groups of rural Bangladeshi adolescent girls aged 10-–7 years for about a four-month period. Those in the first group consumed a diet with iron-fortified lentils, those in the second a diet with non-iron-fortified lentils, and those in the third their usual diet. At end line, differences in the body iron levels of those in the three groups were calculated. To the best of my knowledge, no other research has been conducted on the efficacy of iron-fortified lentils.

The study found that the iron status of the adolescent girls in the iron-fortified lentil group was protected from decreasing compared to the other groups. These results suggest that chronically iron-depleted populations and/or high-risk ID groups would significantly benefit from consuming iron-fortified lentils. Although the the iron biomarkers decreased over time across all study groups, less decline occurred in the iron-fortified lentil group. Additionally, serum ferritin levels were found to increase by 21.6% in the iron-fortified lentil group over the four-month period, but a decreasing trend of ferritin was seen in the non-iron-fortified lentil and usual intake groups. This declining trend is a major concern since the results are contrary to those the study expected, i.e., a declining trend of the key iron variables (sFer) from baseline to end line among all study groups. Given the pattern of decline, this unexpected result can be explained by a regression toward the mean (RTM). This widespread statistical phenomenon occurs with repeated measurements on the same subjects (Barnett et al., 2005). Several research articles have reported such a phenomenon (Ahmed et al., 2001; Cameron et al., 2005; Cummings et al., 2000; Finney, 2008; Martínez-Yélamos et al., 2006). The study was careful to reduce the RTM effect by comparing the data with the appropriate control group since the previous study reported that the RTM phenomenon may misguide the true intervention effect in the absence of a control group (Linden, 2013).

The adolescent girls who were clinically and sub-clinically iron deficient at the beginning of the study were most likely to benefit from consuming iron-fortified lentils. We found substantially-reduced risk of developing clinical ID (57%), sub-clinical ID (60%), sub-clinical IDA (70%), mild anemia (42%) and moderate anemia (77%). This may have been because the rate of iron absorption increases among the most iron-depleted individuals as part of the body's natural iron hemostasis (Abbaspour et al., 2014; Byrnes et al., 2002; Zijp et al., 2000). The

findings of the study are consistent with previous studies, which have confirmed that iron fortification using various food vehicles could increase iron stores in the body (Andang'o et al., 2007; Biebinger et al., 2009; Van Thuy et al., 2003; Ziegler et al., 2009; Zimmermann et al., 2003). Additionally, much difference in Hb levels was found between and within groups as opposed to the ferritin findings. These differences were likely seen because decreased iron stores in non-depleted individuals would not change their Hb levels (causing anemia) as much as they would in depleted individuals, who become non-depleted since erythrocyte production weakens when iron stores fall to support erythropoiesis (Abbaspour et al., 2014; Byrnes et al., 2002; Chaparro & Suchdev, 2019; Johnson-Wimbley & Graham, 2011; Zijp et al., 2000). Furthermore, at baseline, the study found 26.5% of anemia prevalence among adolescent girls in Bangladesh. aged 10–17 years.

The most recent, reliable, nationally representative report, published in 2013, indicated 27.4% anemia prevalence among women 15–49 years (non-pregnant and non-lactating) and 18.1% among children 12–14 years (ICDDRB et al., 2013). Using the WHO cut-offs, the current study noted that anemia prevalence had remained almost static for over seven years (since the publication of the national study in 2013). However, anemia with ID prevalence was found to be much higher (20.4% at baseline) than IDA (6.1% at baseline). These findings suggest that a high prevalence of anemia may not be due to iron deficiency.

Furthermore, the 2013 national survey reported an IDA of 4.8% among women of 15–49 years (non-pregnant and non-lactating) and of 1.8% among children of 12-–14 years). The report also found that among girls aged 10–17 years, 6.1% had clinical IDA (Hb <12.0 g/dL, Ferritin <15 µg/L) and 10.5% sub-clinical IDA (Hb <12.0 g/dL, Ferritin 15 to <30 µg/L). The range of age groups in this report and the current study overlapped and additional sub-clinical cut-offs were used in our study. Our findings indicate that IDA has increased over the past seven years since the publication of the NMS 2013 survey, even though the rate of IDA in Bangladesh has been assumed to be much lower. Besides, there has been growing concern about high prevalence of IDA in Bangladesh since the level of iron in groundwater has been found to be directly linked to a lower prevalence of IDA than what has been observed in other developing countries (S. Rahman & Ireen, 2019). Bangladeshi drinking water may be high in iron because of

contaminated tube wells; about 44.6% tube wells in Bangladesh exceed the Bangladesh standards for iron in drinking water (>1. mg/L Fe) (M. A. Rahman & Hashem, 2019).

The current study investigated whether randomization worked among the study population. No significant mean difference of baseline ferritin among the study population was found, meaning that the randomly assigned groups did not differ from one another. Furthermore, outcome variables were naturally log-transformed to reduce the variability of the data. The body's active inflammation was controlled for during the analysis since inflammation alters the level of iron biomarkers, making it difficult to confirm identification of iron deficiency (Archer & Brugnara, 2015).

Although the results of this study, which took place in a controlled environment, suggest that iron-depleted populations would benefit from consuming iron-fortified lentils, implementing a program in the natural environment would face major challenges. For example, the study provided the participants with a specific amount of lentils that had already been prepared and cooked. In a natural setting, for this intervention to work, the population would have to be amenable to incorporating iron-fortified lentils into their diets at home. Although people in Bangladesh are accustomed to preparing and cooking lentils, they would have to change their routines to deal with iron-fortified lentils. As in many cultures, it is common practice in Bangladesh to wash and rinse lentils prior to preparing and cooking them. However, washing and rinsing lentils after they have been fortified with iron reduces the iron content since the outer layer of the lentils are sprayed with iron fortificants (NaFeEDTA), and this fortificant would wash off during this common practice. Although this study did not calculate how much iron would be lost after each washing, an informal discussion with the researcher who carried out the iron-fortification revealed that about 25% of iron could be eliminated each time the lentils are washed or rinsed. Thus, if the evidence is transferred to population-level action, This factor needs to be considered. To minimize the challenges with washing, The University of Saskatchewan researchers have launched a new study to understand the factors associated with large-scale marketing and acceptability for consumption of iron-fortified lentils at the household level.

6.6 CONCLUSION

As this study has demonstrated, iron-fortified lentils have a high impact on the iron status of adolescent girls in Bangladesh. An effective intervention strategy involving the consumption

of iron-fortified lentils reached this vulnerable adolescent population. This study has generated new evidence about the efficacy of iron-fortified lentils which could be useful for future lentil-based public health initiatives to reduce iron deficiency on a global scale. National and international non-government organizations (NGO), as well as humanitarian organizations, could use iron-fortified lentils as a public health commodity to benefit iron-depleted populations and those who are at-risk in poorly-resourced settings. The findings of this study could inform new policies in lentil fortification, complement scientific evidence on iron-fortified lentils, and create a window of opportunity for a future market for iron-fortified lentils. However, before commercial enterprises can be launched, such matters as cost-effectiveness, the consumer's perspective, finding an ideal stakeholder, and government policy need to be considered.

CHAPTER 7: GENERAL DISCUSSION AND CONCLUSION

This chapter summarizes the findings of the two studies, as well as the significance of the results, considering knowledge from previous studies. The chapter discusses the studies' validities, biases, strengths, and limitations and ends with the practical implications of the results, recommendations, future research opportunities, and conclusion.

7.1 GENERAL DISCUSSION OVERVIEW

The current study used a cluster feeding RCT design to report the current and updated iron profile of adolescent girls aged 10–17 years in Bangladesh, as part of an iron-fortified lentil intervention. There have been no recent reliable iron data on adolescent girls in Bangladesh, with the last report published seven years ago (ICDDRB et al., 2013). A double-blind, community-based, randomized controlled trial was conducted to investigate the efficacy of the consumption of iron-fortified lentils in improving body iron stores of non-pregnant non-lactating adolescent girls in Bangladesh. There were three intervention groups: one group was served 200 g of iron-fortified cooked lentils (from 37.5 g of raw iron-fortified lentils), one group was served 200 g of non-iron-fortified cooked lentils (from 37.5 g of raw non-iron-fortified lentils), and one group received no intervention (usual intake). The participants in the first two groups consumed a thick preparation of cooked lentils five days a week for 85 feeding days (around four months). Socio-demographics, anthropometrics, and 7DDR were collected before and after the intervention, and venous blood samples (6 ml) were collected at baseline, at midpoint, and at end line. The study established a causal relationship between the consumption of iron-fortified lentils and the body iron status of the adolescent girls.

7.2 SYNTHESIS OF KEY FINDINGS

7.2.1. Cross-sectional study

Cross-sectional data were used to determine the iron profile of a sample of Bangladeshi adolescent girls since there are no recent reliable data on iron profiles in Bangladesh. The study found that 27% of adolescent girls had anemia (Hb <12.0 g/dL) and 20.3% had anemia without

iron deficiency (ID), indicated as Hb <12.0 g/dL and sFer >15 μg/L. The study reported both clinical (9.6% at sFer <15 μg/L) and sub-clinical (26% at sFer <30 μg/L) ID prevalence. Clinical iron deficiency anemia (IDA) (Hb <12.0 g/dL, sFer <15 μg/L) was found in 6.7% of the participants and sub-clinical IDA (Hb <12.0 g/dL, sFer <30 μg/L) in 10.7%. Multiple cut-offs were used for clinical (6.2% at sFer <15 μg/L, sTfR >5.0 μg/ml) and sub-clinical (8.0% at sFer <15 μg/L, sTfR >5.0 μg/ml) iron deficiency in a given population. The study controlled for active inflammation [CRP >5 mg/L 3.1% and WBC > 11.5 (10^9/L)] in all analyses. It was found that menarche had a strong effect on sFer and total body iron (TBI) levels, after holding age and BMI constant. The prevalence data of the study were based on primary blood sample analyses.

Results of the cross-sectional analyses were consistent with previous studies that used similar study designs and settings, biomarkers, and WHO cut-offs (Akramipour et al., 2008; Andriastuti et al., 2019; Ramzi et al., 2011; Seyoum et al., 2019). In Bangladesh, the most recent and reliable nationally representative 'National Micronutrient Survey' (NMS) 2011-12 reported iron prevalence of 26% and anemia prevalence of 17.1% in Bangladesh among NPNL women (15–49 years) and children aged 12–14 years, respectively (ICDDRB et al., 2013). This survey further reported 7.1% and 9.5% ID and 4.8% and 1.8% IDA among NPNL women (15–49 years) and children aged 12–14 years, respectively. The current study found a similar prevalence as that reported in the 2013 NMS. Although the NMS survey was conducted on a nationally-representative population, the likely reason for the similar results is that both the NMS and this study used the same iron biomarkers and testing methods. However, the major difference between the NMS and this study was in the age groups of participants. The NMS used school-aged children of 6–14 years and NPNL women of 15–49 years, and this study used adolescent girls of 10–17 years. The major implication of the use of different age groups is for iron RDA. The iron RDA is 8 mg/d and 15 mg/d for adolescents 9–13 years and 14–18 years, respectively (Institute of Medicine, 2003). To comply with age-specific iron RDA, the girls in this study were categorized according to age: 10–13 years and 14–17 years. In the study, iron intake was collected from 7DDR and adjusted during the analysis, but data was lacking on groundwater iron consumption.

The presence of a naturally high concentration of iron in the groundwater may be another factor that influences low iron deficiency in Bangladesh. Studies have reported that the natural

presence of iron in drinking water is linked to a lower prevalence of IDA in Bangladesh (S. Rahman & Ireen, 2019). Tube wells are a major source of drinking water in rural Bangladesh, and it is reported that almost half of the country's tube wells (44%) exceed the Bangladesh standard of iron content in drinking water (>1. mg/L Fe) (M. A. Rahman & Hashem, 2019). Additionally, the iron in groundwater is in the absorbable form of iron (Fe^{2+}), therefore having high bioavailability (ICDDRB et al., 2013; Merrill et al., 2011).

The baseline results from the current study indicate that iron contributed to less than 50% of the high anemia in Bangladesh. This means that other causes of anemia such as socio-demographics factors, other nutritional anemia (vit A, vit B_2, B_6, B_9, B_{12}, vit C, vit D, vit E, copper), and anemia associated with chronic disease may contribute to this high prevalence (Pasricha et al., 2013).

7.2.2. Cluster-randomized trial

This efficacy feeding trial shows that 22.9% and 21.2% sFer increased among the adolescent girls who consumed iron-fortified lentils compared to those who consumed non-iron-fortified lentils and usual intake group, respectively after adjusting for the upazilla (sub-districts in Bangladesh) as the random effect and age as the fixed effect constant. Cases of active inflammation [3.1% CRP >5 mg/L and 20.6% WBC > 11.5 (10^9/L)] were excluded during the analysis. The study expected to have a 5 μg/L difference in mean sFer over 85 days among the iron-fortified lentil group; however, the result indicated a slightly higher effect size than was expected. This would mean that consumption of iron fortified lentils has stronger positive effect on iron stores. sFer increased in this group compared to the usual intake group, in which a decrease of sFer was observed. It was further noted that the iron-depleted (<15 μg/L) girls benefited the most from consuming iron-fortified lentils, and, at end line, they were 57% less likely to be iron deficit than they were at baseline compared to the usual intake group. Furthermore, consuming iron-fortified lentils made girls 42% less likely to suffer from anemia compared to the usual intake group than had they not consumed the fortified lentils. They were even less likely (70%) to suffer from IDA. These efficacy trial results are consistent with earlier global trials on iron fortification; however, this is the only study that used lentils as a food vehicle for iron fortification.

Several studies conducted on the iron fortification of various food vehicles have found positive effects of iron fortification on the body's iron status. For instance, randomized controlled trials carried out in Vietnam, Morocco, China, Thailand, Kuwait, and Cote d'Ivoire used iron-fortified fish sauce, bread, fava beans, soy sauce, white flour, and biscuits and reported increased Hb and sFer (Biebinger et al., 2009; Chen et al., 2005; Van Thuy et al., 2003; Zimmermann et al., 2003, 2005, 2010). Iron status was positively affected in these studies because they used staple foods and bioavailable iron fortificants such as NaFeEDTA, Ferrous sulfate hydrate encapsulated, Ferrous sulfate, electrolytic Fe, and hydrogen-reduced Fe.

Similarly, the current study used the NaFeEDTA iron fortificant, in which EDTA inhibits anti-iron absorbent factors (phytate and polyphenols) and results in increased iron absorption. However, it is important to note that the body has a well-regulated iron hemostasis mechanism, which maintains a standard balance among iron uptake, transport, storage, and utilization (Lieu et al., 2001). Regardless of the dose of the fortificant and its level of bioavailability, ethnicity, age group or gender, the body only absorbs iron that is required for its daily biological activities, meaning that the iron-depleted body absorbs more iron than does the body that is iron replete (Abbaspour et al., 2014; Byrnes et al., 2002; Zijp et al., 2000). Thus, the efficacy trial results indicates that those with depleted iron status are likely to benefit most from consuming iron-fortified lentils.

Additionally, the consumption of iron-fortified lentils is likely to cover a major portion of the iron RDA for adolescent girls and women of reproductive age (15–18 mg Fe/day). However, pregnant women may not receive full benefits from consuming iron-fortified lentils since the RDA during pregnancy is 27 mg Fe/day; for them, supplemental iron may be necessary to maintain iron homeostasis. Furthermore, men may not benefit from iron-fortified lentil consumption because iron from regular dietary intake may be enough to meet their body demands. On the other hand, the consumption of iron-fortified lentils is unlikely to be harmful to men because iron has upper intake levels (UL 45 mg/day) four-fold the daily RDA (8 to 11 mg Fe/day for men of aged 9 to 50 years).

Participants were served ~ 234.0 g of cooked lentils per meal, and consumed ~222.3 g of cooked lentils per meal, after deducting an average residual amount of ~11.7 g/meal. Considering

the iron content of 7.5 mg in the 37.5 g raw portion, reported by Podder and colleagues (Podder et al., 2017), participants consumed ~ 7.1 mg of iron from the study intervention.

Furthermore, the study collected food consumption history of the adolescent girls at baseline and end line through standardized 7DDR questionnaire and iron intake was estimated over 4 months, as shown in Table 7.1. Significant differences in Fe intake (mg/d) were noted between groups at end line, as well as between baseline and end line among study groups. This would mean that iron-fortified lentils groups consumed significantly higher amount of iron (mg) per day compare to non-iron-fortified lentils and usual intake group. This Fe intake data from the 7DDR was used in regression models as a confounding factor.

Table 7.1: Average iron intake mg/day of the adolescent girls

Variables	**Total (N)**	**Average iron intake mg/day [Mean (±SEM)]**	**CI 95%**		**P value**
			Lower	**Upper**	
Baseline					
Iron-fortified lentils	403	10.3 (±0.1)	10.1	10.6	0.169
Non-iron-fortified lentils	432	10.6 (±0.1)	10.4	10.9	
Usual intake	359	10.6 (±0.1)	10.3	10.9	
End line					
Iron-fortified lentils	377	12.9 (±0.2) [#]	12.6	13.3	0.000*
Non-iron-fortified lentils	409	11.5 (±0.1) [#†]	11.3	11.8	
Usual intake	320	12.3 (±0.2) [†]	12.0	12.7	
Difference between baseline and end line					
Iron-fortified lentils	377	2.6 (±0.2) [#]	2.2	3.0	0.000*
Non-iron-fortified lentils	409	0.9 (±0.2) [#]	0.6	1.3	
Usual intake	320	1.8 (±0.2) [#]	1.3	2.2	

** One-way ANOVA test significant at p <0.05*
[#†] Post-hoc Tukey's test significant at p <0.05

Although the study found a positive causal effect of iron-fortified lentils on sFer, Hb and TBI, a declining trend for these outcome variables was observed from baseline to end line among all study groups (Figure 7.1). The reason for declining sFer, Hb, and TBI mean (avg.) from baseline to endpoint in all three intervention groups may be explained by 'regression toward the mean' (RTM). In other words, following an extreme random event, the next random event is likely to be less extreme., and unusually large or small measurements of a dataset tend to be

closer to the mean values. This statistical phenomenon occurs with repeated measurements taken from the same participants or having the same unit of observations. It develops because the values are observed with random errors. To confirm if this is the case in this study, sFer levels were categorized at <15 µg/L, between 15-30 µg/L, and >150 µg/L. To solve the problem of regression towards the mean, a sub-group analysis was undertaken without the outlier group to determine the group most likely to benefit; this entailed dropping the high blood sFer group (>150 µg/L). It was found that in the iron-fortified lentil group, those who began the study with sFer levels between 0 to <15 µg/L and 15 to <30 µg/L significantly increased their sFer level from baseline to end line. However, in the non-fortified lentil and no lentil group, those who started the study with sFer levels of 0 to <15 µg/L had slightly increased levels at the endpoint but not at the midpoint. Nevertheless, in the groups with sFer 15- <30 µg/L and sFer 30 to 150 µg/L, sFer decreased from baseline to end line, suggesting that those with the most potential to benefit from intervention had clinical and sub-clinical iron deficits at baseline (Figure 7.2).

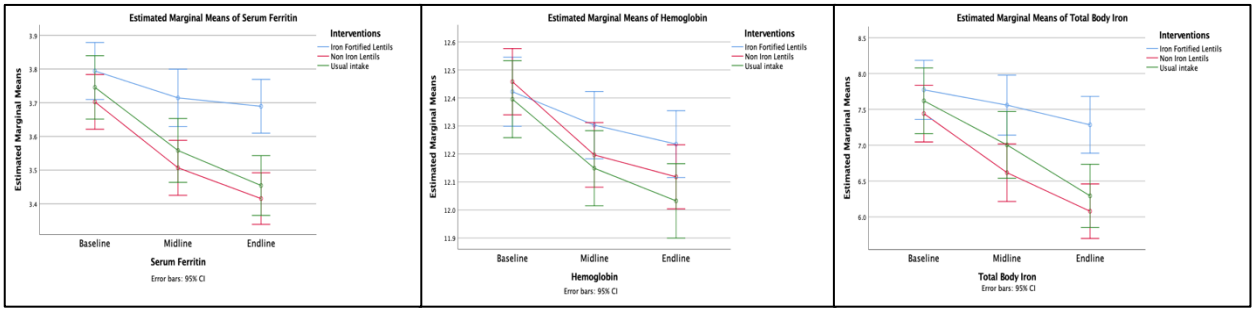

Figure 7.1: Changes of sFer over time in three intervention groups after adjusting for combined inflammation indicators (>5.0 mg/l) and WBC > 11.5 10^9 L.

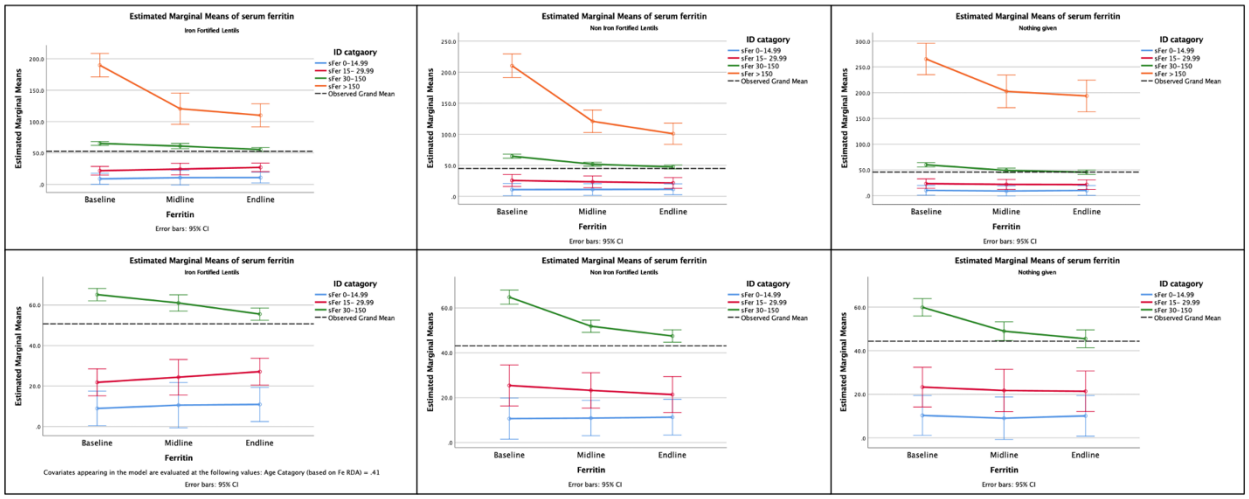

Figure 7.2: The graph shows the regression towards the mean trend among the groups. The first row of the confidence interval plot shows the unusually high mean among those who had sFer levels greater than >150 µg/L after adjusting for combined inflammation indicators (>5.0 mg/l) and WBC > 11.5 10^9 L. The second row shows the adjusted value among those who had sFer levels greater than >150 µg/L and combined inflammation indicators (>5.0 mg/L) and WBC > 11.5 10^9 L to confirm if the regression towards the mean occurs in the dataset.

Additionally, anthropometric measurements (presented as measurement-for-age *z*-scores) did not significantly change over the course of the study, and were not significantly different between groups, as shown in Table 7.2.

Table 7.2: Mean and standard deviation of anthropometric *Z*-scores

***Z*-score**	**Iron-fortified lentils**		**Non-iron-fortified lentils**		**Usual intake**		**p**
	Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**	
Height-for-age *Z*-score							
Baseline	-1.26	1.15	-1.18	1.12	-1.22	1.13	0.566
End line	-0.97	1.16	-0.89	1.14	-0.87	1.08	0.428
BMI-for-age *Z*-score							
Baseline	-0.55	1.13	-0.45	1.08	-0.56	1.15	0.270
End line	-0.41	1.06	-0.34	1.05	-0.45	1.10	0.409

One-way ANOVA test significant at p <0.05

Regarding the iron fortification, the dehulled red football lentil was chosen to be fortified with 160 ppm Fe from NaFeEDTA per 1000 mg (1 kg) of the lentils (160 mg Fe added to fortify

1 kg of lentils). This method provided approximately 210–220 mg of Fe/kg lentils, including the natural presence of the lentils' iron content. The study estimated that 50 g of raw iron-fortified lentils could provide approximately 11.0–11.5 mg of Fe, of which 8 mg of Fe would come from the NaFeEDTA fortificants and 3.5 mg of Fe from the natural iron content of the lentils (Podder et al., 2017). Since iron has a high tolerable upper intake level of Fe (45 mg/day), iron-fortified lentils with a given amount of fortificants, would be unlikely to adversely affect human health even with high iron bioavailability (Institute of Medicine, 2003; Podder, Dellavalle, et al., 2018).

The iron fortification of the lentils was carried out by following the food fortification policy principles of The United States Food and Drug Administration (FDA) (Dwyer et al., 2015). Based on the personal communication of the research technician, it was suggested that Fe fortified lentils should be stored in air sealed bags and have storage ability for at least 18 months after fortification (R. Podder, personal communication, June 18, 2019). It is estimated that a bulk of iron-fortified lentils have 13–14% moisture, 5% (max) discoloured lentils, 0.2% (max) inorganic matter, 2.5 g/100 g fat, 28.3 g/100 g protein, 67.1 g/ 100 g carbohydrate, 12.2 g/100 g total fibre, 83 mg/ 100g EDTA and 67.1 g/ 100 g selenium (R. Podder, personal communication, June 18, 2019). The study used the colorimetric method for fortifying lentils with iron, which is commonly known as spray technology (Podder et al., 2017).

As for food fortification technology, multiple experiments have been undertaken, among which hot extrusion, cold extrusion, and coating for iron fortification have shown promising results on rice. Additionally, a new chelation-redox modulation technology known as 'GrowthPlus' reduces adverse taste and appearance, improves product stability, and does not interfere with bioavailability (Mehansho, 2006). This new approach resolves alteration of colour due to iron oxidation and the metallic after taste without compromising iron bioavailability. Since lentil fortification with iron is a new and innovative approach, as is the use of spray technology to fortify lentils, no other information was found on technologies for the successful iron fortification of lentils. The study found that iron-fortified lentils could increase body iron status; however, other potential technologies may alter the bioavailability.

Irrespective of fortification technology, universal fortification challenges and benefits remain applicable to the iron fortification of lentils. For instance, challenges include the following: differences in consumption amounts by age may not fulfil the body's demand; nutrient

fortifications to date have a limited scope; price increases impact use; and it is difficult to find appropriate food vehicles for fortification. Benefits include a population's regular consumption of the fortified staple food and the fact that the food itself is not altered (Allen et al., 2006).

7.3 STUDY VALIDITIES: CROSS-SECTIONAL SURVEY AND EFFICACY TRIAL STUDY

Since data were cross-sectional, meaning that data were collected at specific points on a specific population, causality cannot be explained through this study. Put another way, the nature of the data does not meet the causality conditions. The condition of causality are as follows (Chambliss & Schutt, 2013; Oppewal, 2010):

(1) Covariation / empirical association: This association means that if one variable varies, then the other variable varies too. Both studies complied with this condition of association for causality. In the cross-sectional paper, the study measured the association between body iron stores (ferritin level) and menarche of the adolescent girls and found these two variables to be negatively associated. In the efficacy trial study, the association was measured between consumption of iron-fortified lentils over time (four months) and an adolescent's body iron status (ferritin level), and positive association was found between them.

(2) Temporal precedence / temporal priority of the independent variable / Time order: Temporal precedence simply means that the cause must have occurred before the effect; therefore, time order should exist. In the cross-sectional study, temporal precedence was violated since data were collected at a single point of time, and the relationship was measured at that single data point. For this reason, what happened before and after the data point is uncertain. However, the feeding trial fulfilled the time order condition. Based on this condition, in the efficacy trial-feeding trial, the cause is the consumption of the iron-fortified lentils and the effect is the changes in the body iron stores, and the cause occurred before the changes in the body iron stores. To comply with the time order condition, the participants' body iron status was first measured, and then the iron-fortified lentils were served to them for a certain period of time (85 days), and after the feeding trial was completed, their body iron status was measured again. In this way, the changes to body iron status over the time period before and after the intervention could be observed.

(3) Control for 'third variables' / non-spuriousness: This control demarcates whether the given intervention caused the outcome (and not the third variable). In the cross-sectional survey, the third variables such as status of menarche, age category, and BMI were adjusted to determine the true association between iron biomarkers and other independent variables. This method provided us with one-to-one association between dependent and independent variables, holding other factors constant. In addition, for the efficacy trial study, third variables (e.g., age) were controlled for to determine the direct association between the body iron status (sFer, Hb, and TBI levels) and the consumption of iron-fortified lentils.

To comply with requirements for rigorous research, it is critical to determine if the two studies in this research (*1)* identified a causal mechanism, and *(2)* identified the context in which the effect occurs, thus strengthening the causal clarification. Because the data was collected at a specific point of time from a specific population, it was not possible to identify the causal mechanism of the cross-sectional study (baseline study). For a similar reason, the baseline study did not identify the context, in effect because this study did not follow the time order condition. However, a causal mechanism was identified for the efficacy trial study: Adolescent girls consumed iron-fortified lentils, plant-based iron, and, given iron's known bioavailability and absorption mechanism, this consumption increased their body iron stores. In addition, identification of the context was irrelevant in the efficacy trial since it explained the biological causal pathway.

7.3.1 Internal validity of the cross-sectional study and efficacy study

Since this study was a cluster-randomized trial, it was important to maintain internal validity. The Cochran handbook identifies four major distortions of cluster RCT: *(1)* recruitment/selection bias, *(2)* baseline imbalance among groups *(3)* loss of clusters, and *(4)* incorrect analysis (Higgins & Green, 2011). The *selection* and *information / measurement / observation biases* were carefully minimized. To randomize the efficacy trial study, two steps were followed: first, adolescent girls were randomly selected from a poll of adolescent girls, who fell under same inclusion criteria, and then the interventions were randomly assigned (either iron-fortified or non-iron-fortified or no lentils) to the participants by BRAC AC. This method allowed both studies to be free from *selection bias*. The only difference between the two studies was the sampling techniques: clustered random sampling in cross-sectional study and a

randomization technique in the efficacy trial. The rest of the study methods and the materials were the same. As for baseline imbalance and cluster loss, there were no significant differences in sFer among the adolescent girls randomly assigned to three intervention groups, and clusters were not lost. For the analysis, appropriate statistical tests were used similar to those used in earlier studies (Van Thuy et al., 2005; Zimmermann et al., 2005). For instance, association was first measured between the outcome variables (sFer, Hb, and TBI) and all possible confounders. Those confounders that were found to be associated with the outcome variables were included in the final regression model. The adjusted regression model was run because association does not point out the magnitude of the association and by chance probability. The regression model explained the magnitude of association by controlling for the effects of confounders on the outcome variables. The study clearly defined the 'standards' for the reference values of the iron biomarkers as per WHO guidelines and followed 'standard' data collection procedures. Each participant followed the same steps at each of the data points, and there was 'standard reporting' of the results of the study.

A major concern of any efficacy trial is the retention of the participants. In this study, total sample size was n=1256 adolescent girls including an assumptive 20% attrition rate, leaving the actual sample size of n=1005 (without the attrition rate). The study enrolled a total of n=1195 adolescent girls at baseline and followed-up n= 986 adolescent girls at end line. A total of n= 270 (21.5 % attrition rate) adolescent girls were lost to follow up. Regardless, the sample holds enough power to make causal inferences, as calculated in the original sample size since the study lost very small fraction (1.5%) beyond the 20% attrition rate (Table 7.3).

Table 7.3: Attrition rate of the efficacy study

Data points	**Iron-fortified lentil**	**Non-iron-fortified lentil**	**Usual intake**	**Total sample size**	**Dropouts (N)**	**Attrition rate (%)**
Total sample size (with 80% power)	335	335	335	1256 [335(100/80)3]	-	-
Baseline	404	432	359	1195	-	-
Midline	332	358	284	974	282	22.4
End line	335	362	289	986	270	21.5

Dropouts and attrition rate are based on total sample size (N 1256)

The accuracy of the measurements of sFer, Hb, and other iron biomarkers were ensured to reduce the *information / measurement / observation biases.* For instance, the current study relied entirely on laboratory-based biological specimen test results. It is possible to misclassify results, but even if errors occurred in the results of the biological specimens (venous blood), the likelihood of there being the same error in all samples was extremely low because the same test methods, reagents, kits, and cut-off standards were used to test the parameters. Furthermore, differential misclassification was unlikely to occur because the outcome variables (sFer, Hb, and TBI) were not self-reported. However, socio-demographic information and potential confounders were collected as self-reported data. These data were collected electronically using an ODK (open data kit) application installed in a 7-inch tablet built under the Android operating system. Each question was set to conditions, meaning that research assistants could not put in unrealistic numbers as a response, thus minimizing the chance of error. Furthermore, the study adopted a standardized 7DDR questionnaire for the adolescents in Bangladesh from the NMS survey (2013) (ICDDRB et al., 2013). Additionally, the study collected anthropometrics using standard tools. Age, education, menarche, handwashing with soap as a proxy for hygiene, knowledge of iron-rich food, BMI, and daily average iron intake were considered as possible confounders and controlled using appropriate statistical tests.

Because acute infection in the body naturally increases hepcidin secretion and reduces iron absorption, any acute inflammation of the study participants was adjusted during data analysis (De Domenico et al., 2007; Nemeth & Ganz, 2006). WHO has advised that C-reactive protein (CRP) and α1-acid-glycoprotein (AGP), two acute phase proteins, should be considered when sFer values are measured (World Health Organization, 2011a). The current study used one of the methods of dealing with inflammation suggested by WHO, which excludes those participants with increased CRP or AGP from ID (World Health Organization, 2011a). Although these two inflammation indicators are commonly used during sFer calculation, only CRP was used in this study because it measures acute body infection, which is not uncommon in adolescent girls. A clinical cut-off for CRP of > 5 mg/L was used (Iqbal et al., 2015; Turgeon O'Brien et al., 2016). AGP, which measures chronic inflammation, was not controlled for because the majority of chronic disease occurs in adults between the ages of 30 and 69 years (Northrop-Clewes, 2008; Suchdev et al., 2017; World Health Organization, 2018b) Therefore, anemia, ID, and IDA prevalence presented in this study is a good representation of the iron

biomarker statistics in adolescent girls in Bangladesh. However, it would have been ideal to have considered both acute and chronic inflammation since the Bangladesh National Nutrition Survey (NMS 2013) used both, perhaps because it covered all ages (ICDDRB et al., 2013). Nevertheless, the current study controlled for the WBC count [>11.5 (10^9/L)] because it is an indicator of chronic inflammatory conditions (Riley & Rupert, 2015; Rodak et al., 2017).

7.3.1.1 Interval validities of the iron biomarkers

Complete blood count (CBC): A white blood cell (WBC) count and differential counts were performed with an optical detector block based on the flow cytometry method, using a semiconductor laser. The red blood cell (RBC) detector using the Hydro Dynamic Focusing method analyzed RBCs and platelets. The HGB detector based on the sulfolyser hemoglobin detection method analyzed hemoglobin (HGB). When samples were received in the laboratory, the blood specimens were placed on an orbital shaker for a minimum of 30 minutes each. The blood specimens were then run for CBC measurements on an Automated Hematology Analyzer equipped with five-part differential functionality, XS-800i, according to the manufacturer's instructions. Commercial internal quality control material, E-check, was used to monitor and verify the performance of the Sysmex Hematology analyzer. It provided assay data for the CBC parameters and the WBC differential count. This controlled material was run at the beginning of the analysis, and results were reviewed. If all the parameters fell within the manufacturer's recommended ranges, then study samples were run. Otherwise, necessary corrective action was executed for out-of-range parameters until all the parameters fell within the acceptable range.

Serum ferritin (sFer), C-reactive protein (CRP), and serum transferrin receptor (sTfR): Ferritin was measured by an electrochemiluminescence immunoassay (ECLIA) with a Roche automated immunoassay analyzer, Cobas e601, using a commercial kit (Roche Diagnostics, GmbH, 68305 Mannheim, Germany), according to the manufacturer's instructions. CRP and sTfR were measured by the Particle-enhanced immunoturbidimetric method using a commercial kit from Roche diagnostics in a fully automated Clinical Chemistry analyzer Cobas c311 (Roche Diagnostics GmbH D-68298 Mannheim, Germany), according to the manufacturer's instructions. The frozen samples (stored at -20º C) were brought to room temperature and then homogenized through vortex mixing. A small portion of each sample was transferred to a micro-centrifuge tube and used for the above test in the analyzer, according to the manufacturer's instructions and

by following the above-mentioned principles. The analyzers automatically calculated the results via instruments specifically generated for respective calibration curves. Two levels of commercially available quality control serums (Roche Diagnostics, GmbH, and D-68298 Mannheim, Germany) were used to check the accuracy and precision of the assays as an internal quality control. These QC samples were run at the beginning of the analysis and their results reviewed. If these values were within an acceptable limit, then study samples were run. Corrective actions were taken in those cases where unacceptable QC values were obtained.

7.3.2 External validity of the cross-sectional (baseline) and efficacy trial study

For the baseline cross-sectional study, generalizability cannot be drawn from the study results since the study sample was not nationally representative. As a result, the prevalence of anemia, ID, and IDA found in the study cannot be generalized for all Bangladeshi adolescent girls. Caution must therefore be advised when interpreting the results. Nonetheless, the results could hint at the scale of the magnitude of iron-induced micronutrient deficiencies in Bangladesh. Furthermore, neither biological nor behavior pathways can be explained by the association between iron biomarkers and socio-demographics because the cross-section study design does not allow for the investigation of the before and after status of the variables.

For the efficacy trial study, even though the samples were the same as those used for the baseline study, the universal biological response between the consumption of iron-fortified lentils and body iron stores can be generalized, a conclusion based on the natural consequence assumption from the earlier study. This assumption is that causal inference in a study ensures the external validity if it has a short causal pathway and simple impact model (Victora et al., 2004). Through the efficacy trial design, a simple and short causal pathway between the consumption of iron-fortified lentils and body iron stores was established by comparing the results of the iron-fortified lentil group with those of the non-iron-fortified lentil and usual intake groups after controlling for confounders.

However, the generalizability of the study is limited to other populations due to race-ethnicity, socio-demographics, and variations in lifestyle characteristics of major iron biomarkers such as sTfR and sFer (C. M. Pfeiffer et al., 2013). As well, because of the nature of natural iron hemostasis, the causal inference applies only to those adolescent girls whose body iron status falls within similar ranges as the study participants. The causal inference is restricted because

those who are iron depleted absorb more iron than those who are iron replete (Anderson & Frazer, 2017). Therefore, even though a causal relationship has been established, the generalizability of the efficacy trial study is limited. Another aspect of generalizability was the selection of participants. Generally healthy adolescent girls whose body iron status was unknown were selected, suggesting that the biological pathway could have developed in the way it did because only healthy adolescents participated. Had the participants been selected based on their low iron status, the results could have been higher efficacy because of natural iron homeostasis, but study generalizability would have been further limited to only iron- depleted adolescent girls.

7.4 POSSIBLE BIASES, STUDY LIMITATIONS, AND STRENGTHS

The studies were prone to certain biases. Although *selection bias* can occur in all studies, it likely did not occur in this study because participants were selected randomly from a poll of adolescent girls belonging to the BRAC school. As for the RCT, there was the possibility of *performance bias* because the iron-fortified lentil intervention group may have been motivated to perform better than the other two groups. To address the *performance bias* among the participants, the researcher ensured that both the iron-fortified lentils and non-iron-fortified lentils were identical in visual appearance, so the groups could not tell them apart. Additionally, the intervention information was double-blinded at both the researcher and research assistant level, meaning that both participants and outcome assessors were not aware of who was receiving what. This double-blind strategy also minimized the *ascertainment bias*— that the study could be systematically distorted by knowledge, by either the study investigators or the study participants. Moreover, the double-blind strategy prevented allocation concealment in the RCT study because the randomization codes were hidden (blinded) at the investigator level. Furthermore, *self-reported bias* was minimized by relying on biological outcome measurements. *Attrition bias* was another concern for the RCT. The researcher was aware of the *attrition bias* and thus allowed for a 20% attrition rate when calculating the sample size. The actual attrition rate was 18.6% from baseline to midline and 17.6% from baseline to end line. Additionally, a mixed model linear regression model was used that only deleted the missing point data. The rest of the information belonging to each participant remained in the dataset and was automatically counted during the analysis. The study protocol was published in *Trials* journal (published by BioMed Central), and the trial was registered in ClinicalTrials.gov (NCT03516734. Registered on 24 May, 2018)—a

USA-based, well-accepted trial registry website. As a result, *outcome reporting bias* was unlikely. *Publication bias* was and is also unlikely to be a problem because the results of the study will be publicly shared in the trial registry website since it is registered in the clinicaltrial.gov database. Another common concern was the spill-over effect of the RCT. In this RCT, it was possible that adolescent girls who received either iron-fortified lentils or non-iron-fortified lentils from different adolescent clubs had interacted with each other (*'contagion effects'* or *'synergistic effects'*—within intervention interaction). They may have interacted socially or at community events or may have attended the same school. If this were the case, their knowledge and/or discussion (either positive or negative) about the consumption of iron-fortified lentils may have affected the amounts consumed by the intervention groups. Additionally, the intervention groups could have interacted with the non-intervention group ('contamination'—intervention affects control group), resulting in those in the non-intervention group consuming more lentils at home since they were not getting lentils in the study. If they took place, these interactions could have resulted in a deviation from the natural differences between daily consumption and usual consumption. All these interactions between those from the three groups were possible by random chance because the study randomly assigned the interventions among 48 clubs regardless of their administrative boundaries in four upazilas (administrative regions in Bangladesh). However, since the interventions were randomly assigned among the clubs, any behavioural deviation would have affected each group equally (either within intervention groups or among groups); therefore, they were unlikely to bias the result towards certain directions.

Another major issue of the study was that it was an efficacy trial. Because it was carried out under an ideal and controlled research setting, it remained unknown how participants' consumption patterns/frequency and consumption amounts would have varied in the natural 'real world' setting. Furthermore, the participants' attitudes towards the purchase and consumption of lentils was uncertain because of the controlled research environment. The lentils were free, cooked, and served as part of the research protocol, but it is not known what preparation techniques would have been used in natural settings. This is crucially important because of the level of lentil concentration in cooking preparation: a thin concentration is a watery soup, and a thick concentration more of a semi-solid soup. In thin-cooked lentils, the concentration of lentils in the lentil soup is noticeably less than that in thick preparation.

Another possible limitation concerns the group included in the study. Since the adolescent girls were the members of a well-established NGO in Bangladesh, it is possible that they were exposed to public health awareness information delivered by BRAC as a part of its regular public health activities. Adolescent girls at BRAC participate in life-skill-based education (LSBE) sessions, and they have access to mini-library facilities, perform cultural activities, celebrate national days, and play sports in the adolescent clubs. These activities may have prompted the girls in the study to participate in the research project and comply with the protocol; however, those not exposed to such NGO activities may have behaved differently. Although these adolescent girls may not represent rural, underprivileged adolescent girls in Bangladesh, this fact likely did not affect their iron absorption because, as we have seen, the natural iron hemostatic mechanism ensures that iron-depleted populations absorb more iron than iron replete populations.

An additional problem with the controlled environment is that it does not necessarily reflect the way food is prepared in a natural setting. In this study, the procedures followed in the girls' households for preparing lentils were unknown. In Bangladesh, however, it is common cultural practice to wash vegetables and legumes before cooking them, and lentils are likely no exception. However, washing iron-fortified lentils before cooking them could significantly reduce their iron content because the action could remove the outer layer of the iron fortificants. Personal communication with the research technician revealed that washing practices such as soaking and repeated washing of iron-fortified lentils prior to cooking could reduce the iron in the fortified lentils by 25%, which would pose a great threat to the efficiency of these lentils in increasing body iron status (R. Podder, personal communication, June 18, 2019). One of the major barriers to large-scale implementation of iron-fortified lentil programs is the cost, and this study did not estimate the price increase associated with the production and marketing of iron-fortified lentils. The high price of iron-fortified lentils could become a major issue since an earlier study on nutritionally enhanced food in Kenya and Uganda suggested that although the people were ready to accept new nutritionally enhanced foods in their diet, they would not be willing to pay a higher price for these foods (Wanyama et al., 2019). Furthermore, the study did not measure Fe contents in the served meals in each of the BRAC clubs, which limits the accurate measurement of Fe consumed and relative bioavailability among the adolescent girls. To ensure all cooking methods were adhered to and Fe intake during meals was consistent among clubs

within treatment groups, this would have been an ideal measurement to gather throughout the trial.

As well as these limitations, the two studies that make up this research have several strengths. The major strength of the cross-sectional study is that well-established iron biomarkers such as sFer, sTfR, TBI, and Hb levels were used as the outcome variables to determine the iron profile of adolescent girls in Bangladesh. Furthermore, this cross-sectional study is only the second of its kind to use iron biomarkers to determine the iron status in Bangladesh. The latest country representative report came out seven years ago, and to the best of our knowledge, no study in Bangladesh has calculated iron prevalence since then (ICDDRB et al., 2013). As well, the cross-sectional study used WHO cut-off values for iron biomarkers, and the venous blood samples from the participants were tested in a reliable laboratory setting to determine the iron status of the body. Previous studies have reported that capillary blood has a higher concentration of Hb compared to venous blood samples, and Hb assessment of capillary blood samples using HemoCue has shown poor promise compared with automated hematology analyzers (Hinnouho et al., 2018; Patel et al., 2013). Moreover, the study reported anemia and iron deficiency using both clinical and sub-clinical cut-offs such as anemia (Hb <12.0 g/dL), anemia without ID (Hb <12.0 g/dL, sFer >15 mg/L), clinical IDA (Hb <12.0 g/dL, sFer <15 mg/L), sub-clinical IDA (Hb <12.0 g/dL, sFer 15 to <30 mg/L), clinical ID w/o anemia (Hb >=12.0 g/dL, sFer <15 mg/L), sub-clinical ID w/o anemia (Hb >=12.0 g/dL, sFer 15 to <30 mg/L), clinical ID using a single criterion (sFer <15 mg/L), sub-clinical ID (sFer 15 to <30 mg/L), clinical ID with multiple criteria (sFer <15 mg/L, sTfR >5.0 µg/ml), sub-clinical ID (sFer 15 to <30 mg/L, sTfR >5.0 µg/ml) and iron overload (sFer >150 mg/L). This study, therefore, provides a complete assessment of iron status among adolescent girls in rural Bangladesh.

The major strength of the RCT is the design itself. A detailed protocol was published in the earlier paper (Yunus, Jalal, et al., 2019). The RCT study adopted a double-blind, community-based, clustered randomized controlled trial, which is considered the 'gold standard' of study design. Blinding was carried out for both trial participants and outcome assessors. Furthermore, the study had both comparison (non-iron-fortified lentils) and control groups (usual intake of lentils). This allowed us to defend reasonable criticisms such as *'regular consumption of non-iron-fortified lentils will have a similar effect on body iron status since lentils have a naturally*

higher content of iron' and *'since lentils are a staple food in Bangladesh, natural lentil consumption will have a similar effect on body iron status.'* The study compared groups (comparison and control) with the iron-fortified lentil group and detected the differences among the groups. The study sample size had enough power to establish a causal relationship between the iron-fortified lentil consumption group and body iron status. As in the baseline study, the iron biomarkers used WHO cut-offs for the venous blood samples. Blood samples were analyzed at the beginning of the study (before the intervention), at mid-point (at two months) and at the end of the study (at four months), which allowed us to determine trends in body iron store changes over the intervention period. Moreover, the study's reliance on biological markers rather than only self-reported data strengthened the outcomes. Other strengths of the RCT study include control for confounders' effects and the absence of spill-over effects.

7.5 IMPLICATIONS OF THE STUDY

The two studies in this research have several implications in various domains. The cross-sectional survey updated the iron status of adolescent girls in rural Bangladesh. The results indicated that ID and IDA prevalence have remained static since the last report was published seven years ago. Similarly, the study found 27% anemia prevalence, regardless of the reason, implying that perhaps insufficient steps have been taken to reduce ID, IDA, and anemia in Bangladesh. This study sheds light on the magnitude and prevalence of iron-related health problems among Bangladeshi adolescent girls. Results from the RCT study create a possible opportunity for iron-fortified lentils to become part of the commercial market for lentils. Iron-fortified lentils could be a "new" nutritious food product, that is culturally-familiar, available at regular markets. Lentils are a source of plant-based protein, which is an additional selling point for a cultural staple. They could be labeled and marketed with a slogan such as 'ready to cook.' Another implication of the RCT study is the need for policy assistance in launching regulations for 'mass fortification' of lentils in Bangladesh. Since iron has a high upper tolerable upper intake level (UL) of about 45 mg/day and iron-fortified lentils can provide ~86.3% and ~46% of iron RDA for adolescent girls aged 9–13 (RDA 8 mg/day) and 14–18 years (RDA15 mg/day), the consumption of iron-fortified lentils is unlikely to have adverse effects on health.

Another consideration is that iron-fortified lentils could be used as a major food in crises such as war or a climate catastrophe. These lentils could be beneficial to vulnerable populations

such as adolescent girls, pregnant women, and non-pregnant non-lactating women living in refugee camps due to a crisis. Consumption of iron-fortified lentils would be a 'win-win' situation for both consumers and providers since it would improve consumers' nutritional status (plant protein with high iron and selenium) and profits to providers. Lentils have another advantage as they are considered a staple food in many cultures and are therefore culturally suitable as relief food. Moreover, both national and international NGOs could use iron-fortified lentils as a public health commodity for vulnerable populations. This study has generated new knowledge on lentils as a potential food vehicle for iron fortification, providing evidence on the efficacy of lentils as a food-based, long-term sustainable solution to tackling global micronutrient deficiency.

Overall, the results of the study have important implications for adolescent girls' health, which is linked to the economic growth of Bangladesh. The World Bank has identified investment in adolescent nutrition as a critical component for improved health and economic growth in Bangladesh (Kanti et al., 2019). In 2019, it was estimated that adolescent girls aged 10–19 years comprised 9.3% of the total population of Bangladesh (*Population of Bangladesh*, 2019), the implication being that if the health issues of one-tenth of the population are not addressed, economic growth cannot be achieved. Improving the iron status of the adolescent girls would strengthen their cognitive ability (Lynch et al., 2018; Mesías et al., 2013) and thus their ability to contribute to the economy. It would also improve the health of those who are pregnant. Teenage pregnancy remains a major public health concern in Bangladesh, with studies reporting that about 30.8% of adolescent girls become pregnant, and optimal iron status is linked to improved pregnancy outcomes for both mother and children (Figueiredo et al., 2018; Islam et al., 2017; Stangret et al., 2017; Yi et al., 2013). Unhealthy, pregnant teenagers pose an extra burden to the existing over-burdened health system in Bangladesh (Joarder et al., 2019; Mannan, 2013).

Iron-fortified lentils are beneficial to adolescent girls in two ways: first, they are high in bioavailable iron, and, second, lentils themselves, a staple food in Bangladesh, are a plant-based protein that are rich in macro and micronutrients, dietary fibre, and carbohydrate (Faris et al., 2019; Joshi et al., 2017; Migliozzi et al., 2015; Rathod & Annapure, 2016, 2017). The study used the colorimetric method to fortify lentils with iron (commonly known as 'spray technology'), and in applying this method, the study followed the food fortification policy principles of The United

States Food and Drug Administration (FDA) (Dwyer et al., 2015; Podder et al., 2017). Although iron fortification has complex technological issues, it has a high economic impact (Blanco-Rojo & Vaquero, 2019).

The upshots of the entire project are *(1)* lentils are a plant protein – a staple food rich in fibre, macro and micronutrients and it is a low-cost staple food, familiar to many cultures. *(2)* Iron deficiency is a global micronutrient deficiency problem that impacts arguably, our most important, and most vulnerable population: adolescent girls, non-pregnant non-lactating women, and pregnant women. Since it is a micronutrient deficiency, it requires regular consumption of nutritious foods maintain regular body micronutrient demand. *(3)* Iron-fortified lentil is a food-based approach aimed to maintain body iron status, and it had less chance to pose adverse effect due to iron's high tolerance for high intake levels of iron (45 mg/day) compared to its RDA (8-18 mg/day, depending on age and gender). *(4)* This study concludes the consumption of iron-fortified lentils increases body iron store, particularly those who are iron deficit. *(5)* Iron-fortified lentil remains stable at least for 18 months in the natural environment. (6) Several countries have a food fortification policy in place that creates a window of opportunity to market iron-fortified lentils through mass fortification commercially.

7.6 FUTURE RESEARCH AND STEPS

Future research should focus on finding the underlying cause of high anemia in Bangladesh. Based on the results of the baseline study, anemia prevalence is high in Bangladesh, and iron deficiency is still playing a major role in causing anemia. Other causes of nutritional anemia, including non-nutritional causes, should be considered when studying anemia. Future research should include all vulnerable iron-depleted groups, including pregnant women and women of reproductive age. Generalizability is an issue for future research. When sample sizes for iron-related cross-sectional surveys are calculated, generalizability should be considered. Additionally, well-accepted iron markers and cut-offs should be used when calculating prevalence. Future research on lentil fortification warrants effectiveness trials to understand the natural attitudes and behaviour of consumers towards iron-fortified lentils. To determine if iron-fortified lentils are a worthwhile public health vehicle to reduce iron deficiency at clinical and sub-clinical levels, the next research steps would be to estimate their costs and cost-effectiveness. Additionally, research could consider appropriate marketing and packaging of iron-fortified

lentils, as these decisions could be critical for entry into the commercial market. In addition, iron loss due to washing practices by consumers needs to be considered if this evidence is to be translated into population-level action. Although iron-fortified lentils are found to be efficacious, procedures for avoiding iron loss during washing should be clearly noted on lentil packets. For instance, there should be highlighted messages advising consumers to avoid washing packaged lentils.

7.7 RECOMMENDATIONS

Iron deficiency and iron deficiency anemia are prevalent in Bangladesh. This prevalence could be tracked if the national representative micronutrient survey were administered every two years. Additionally, research should focus on nutritional and non-nutritional causes of anemia. Iron-fortified lentils have the potential to substantially increase body iron stores, and, due to iron's high tolerable upper intake level, it is unlikely to have adverse effects on human health; any additional iron is still lower than its RDA. Vulnerable groups such as pregnant women, non-pregnant women non-lactating women, and adolescent girls would benefit most from iron-fortified lentils. To ensure that vulnerable groups are not excluded from the mass fortification, additional community-based nutritional education would be useful. To implement a successful iron-fortified lentil program, understanding cultural food factors–including food perceptions, price, and acceptability of new food–is critical. Furthermore, strong policy advocacy is required to ensure mass fortification is effective.

7.8 CONCLUSION

The focus of the two studies that comprise this research was the body iron status of adolescent girls in Bangladesh and the potential for iron-fortified lentils to improve this status. Other research has shown that lentils are an effective vehicle for iron fortification. They are a low-cost, plant-based food that is rich in protein and other macro and micronutrients. The study found that anemia and ID prevalence is high among adolescent girls in Bangladesh. The results suggest that there are opportunities for public health interventions to reduce iron deficiency and iron deficiency anemia. Of these interventions, a food-based approach could improve the iron stores of the iron-deficient population by providing safe, maintenance doses of iron through a staple food like lentils. It was found that adolescent girls in Bangladesh were willing to consume about 200 g of cooked iron-fortified lentils (from 37.5 g of raw iron-fortified lentils) five days a

week for over about four months if the lentils were cooked using local recipes and dietary practices. The results suggest that the consumption of iron-fortified lentils is an effective intervention strategy for reducing iron deficiency among vulnerable adolescent girls.

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APPENDIX A: ASSENT AND CONSENT FORMS

1. ASSENT FORM (ADOLESCENT GIRLS)

Title: The effect of iron fortified lentils on the iron (Fe) status among the adolescent girls in Bangladesh- a double-blind randomized controlled trial

Introduction:
I want to tell you about a research study that I am doing. Research helps to learn new things. You are invited to participate in a research study about improving iron status in adolescents through iron fortified lentil consumption. Please read this form or either I can read this form for you in front of a witness. Please feel free to ask any questions you may have before taking part in this study or you any ask any question anytime you want even after you decide to participate in this study.

What is the study about?
The purpose of this study is to see if eating iron fortified lentils will affect the iron in your body.

Why are you asked to be in this study?
You are chosen as a possible participant because you are between the ages of 10-17 years, female, and you are a member of BRAC Adolescent Club.

Who is doing the study?
This study is being done by several researchers in Canada, Bangladesh and the United States. The student researcher responsible for collecting information is Dr. Fakir Yunus. He is a medical doctor and a graduate student under supervision of Drs. Henry Carol and Diane DellaValle at the University of Saskatchewan, Canada and Marywood University, USA. The study is being carried out by BRAC Health, Nutrition, and Population Program (HNPP) and The University of Saskatchewan in Canada. This study is funded by Global Institute Food Security (GIFS) and Nutrition International (pending the final funding information) based in Canada.

What will you be asked to do?
If you agree to participate in this study, you will be asked to participate in four different events; (1) For survey, where you will be asked about your family background, housing and school, eating, and health issues. You will have some measurements taken: height, weight, waist size, hip size, and mid-upper arm size. This session will occur twice- beginning of the study (before we serve the cooked lentil) and after end of the study. It would take around 30min in each time. (2) For blood collection, you will have a blood sample taken from her arm (about 1 teaspoons amount) to check iron levels will be collected in BRAC adolescent club- may take around 10min. This blood collection event will occur thrice- beginning of the study (before we serve the cooked lentil), at the middle of the study and after end of the study. (3) Daily-wise data collection on the served cooked lentils will be occurred at the BRAC adolescent club. You will be asked to come in the BRAC Adolescent Club around 4 months (22 weeks) for 5 days a week for this purpose. You along with other adolescent girls will be then served a standard portion of rice with iron fortified lentils prepared as *daal*. The *daal* recipe will be thick in-preparation. This will take about 30 minutes at each visit. (4) Cognitive skill measurement will be taken place at the adolescent clubs- would take

around 45min. There will be participating around 1200+ adolescent girls like you in this study. Please note that there will be three groups of adolescent girls having the same age. However, it will occur randomly, and we don't know which group you will be. The difference among the groups will be that some will iron fortified cooked lentil meal, and some are not.

What are the risks and benefits for participating in this study?

There are minor risks involved in this study. Regards to the venous blood draw, the needle stick may hurt. There is a small risk of bruising and fainting, and a rare risk of infection. Also, there would be a few questions we would ask you such as your date of birth, menstrual information, marital status. We would ask these information in a private setting in front of a witness. However, you can refrain from answering any questions, as you wish. If there is any medical condition related to eating lentils, our staff will refer you to the nearest BRAC health center or government health facilities. There may or may not be direct benefits to you from this study. Please note that others at the club will know about that you are in the study and that everyone is being asked to keep that information confidential. The results of the study may help us to develop a community-based information and service package for nutrition particular for adolescent in future

What are the good things about this study?

While there are no direct benefits to you, the results of the study may help us to measure the effect of iron fortified lentils on your body iron status over 4 months of period.

Is there any payment/rewards for participating in this study?

You will not receive any compensation for taking part in this study. Aggregated results will be shared to both parents and girls in a community meeting in the adolescent club.

How confidentiality will be maintained?

The records of this study will be kept private. Information used in any written or presented report will not make it possible to identify you because we will use a unique identifier instead of your name. Only the investigators will have access to the research records. Paper records will be shipped to Canada and secure in locked cabinet. Blood samples will be stored in the ICDDR,B laboratory (commonly known as Cholera hospital) in Dhaka using a unique identifier instead of your name. Records will be kept for a minimum of five years. Then they will be destroyed (paper records via shredding, computer records will be deleted).

Is taking part in this study voluntary?

You do not have to take part in this study. If you say yes, it should be because you really want to participate as volunteer. If you say no, that is your decision. Your choice to be in the study or not will not affect your membership/relationship with the BRAC Adolescent Club. If you say yes, you can also stop whenever you want. You may withdraw your permission at any time without penalty or loss of benefits to which you are entitled. To stop tell Dr. Fakir Yunus. Research and Evaluation Division, BRAC Centre, 75 Mohakhali, Dhaka 1212. Telephone- +88029881265. If you withdraw your permission from the study, all your records will be destroyed as outlined above.

Who should be contacted and who should be asked questions?

If you have any questions at any time please ask a member of the research team or you can contact Dr. Fakir Yunus, Research and Evaluation Division, BRAC Centre, 75 Mohakhali, Dhaka 1212. Telephone- +88029881265

If you have questions related to the rights of research participants or research-related injuries (where applicable), please contact Research Ethics Office, University of Saskatchewan, Box 5000 RPO University, 1607 – 110 Gymnasium Place, Saskatoon, SK Canada S7N 4J8

I have read what I am asked to do. All of my questions were answered.

☐ YES: I want to be in the study.

☐ NO: I do not want to be in the study.

_____
Your Name

_____ _____
Your Signature Date

_____ _____
Your Thumb Print (if participants cannot write) Date

_____ _____
Name and signature of the witness Date

_____ _____
Name of (Authorized) Person Obtaining Informed Assent Date

A duplicate copy will be given to you for your record

2. CONSENT FORM (PARENTS)

Title: The effect of iron fortified lentils on the iron (Fe) status among adolescent girls in Bangladesh- a double-blind randomized controlled trial

Introduction

Your child is invited to be in a research study about improving iron status in adolescents through iron fortified lentil consumption. Your child was chosen as a possible participant because she is between the ages of 10-17 years, and is female, and is a member of BRAC Adolescent Club. Please read this form or either I can read this form for you in front of a witness. Please feel free to ask any questions you may have before allowing your child to take part in this study or you any ask any question anytime you want even after you allow your child to participate in this study.

Who is doing the study?

This study is being carried out by several researchers in Canada, Bangladesh and the United States. The student researcher responsible for collecting information is Dr. Fakir Yunus. He is a medical doctor and a graduate student under supervision of Drs. Henry Carol and Diane DellaValle at the University of Saskatchewan, Canada and Marywood University, USA. The study is being carried out by BRAC Health, Nutrition, and Population Program (HNPP) and The University of Saskatchewan in Canada. This study is funded by Global Institute Food Security (GIFS) and Nutrition International (pending the final funding information) based in Canada.

What is this study about??

The purpose of this study is to examine the effect of iron fortified lentils on the iron status among adolescent girls in Bangladesh.

What will your child be asked to do?

If you allow your child to participate in this study, she will be first asked to participate in the screening procedure in order to evaluate her eligibility. During screening, she will be asked about her age (in years), menstruation, marital status and if she will be willing to eat cooked lentils (daal) provided by the study team daily, 5 days per week, for the next ~16 weeks (~4 months). Also, we will collect her venous blood sample taken from her arm (about 1 teaspoons amount) to check iron levels will be collected in BRAC adolescent club- may take around 10min. Once she is eligible, she will be asked to participate in four different events; (1) For survey, where she will be asked about family background, housing and school, eating, and health issues. She will have some measurements taken: height, weight, waist size, hip size, and mid-upper arm size. This session will occur twice- beginning of the study (before we serve the cooked lentil) and after end of the study. It would take around 30min in each time. (2) For blood collection, she will have a blood sample taken from her arm (about 1 teaspoons amount) to check iron levels will be collected in BRAC adolescent club- may take around 10min. This blood collection event will occur twice - at the middle of the study and at the end of the study. (3) Daily-wise data collection on the served cooked lentils will be occurred at the BRAC adolescent club. She will be asked to come in the BRAC Adolescent Club around 4 months (22 weeks) for 5 days a week for this purpose. She along with other adolescent girls will be then served a standard portion of rice with iron fortified lentils prepared as daal. The daal recipe will be thick in-preparation. This will take about 30 minutes at each visit. (4) Cognitive skill measurement will be taken place at the adolescent clubs- would take

around 45min. There will be participating around 1200+ adolescent girls in this study. Please note that there will be three groups of adolescent girls having the same age. However, it will occur randomly, and we don't know which group your child will be. The difference among the groups will be that some will iron fortified cooked lentil meal, and some are not.

What are the risks and benefits for participating in this study?

There are minor risks involved in this study. Regards to the venous blood draw, the needle stick may hurt. There is a small risk of bruising and fainting, and a rare risk of infection. Also, there would be a few questions we would ask your child such as her date of birth, menstrual information, marital status. We would ask these information in a private setting in front of a witness. However, your child can refrain from answering any questions, as they wish. If there is any medical condition related to eating lentils, our staff will refer your child to the nearest BRAC health center or government health facilities. There may or may not be direct benefits to you or your children from this study. Please note that others at the club will know your child is in the study and that everyone is being asked to keep that information confidential. The results of the study may help us to develop a community-based information and service package for nutrition particular for adolescent in future.

Is there any payment/rewards for participating in this study?

You or your child will not receive any compensation for taking part in this study. Aggregated results will be shared to both parents and girls in a community meeting in the adolescent club.

How confidentiality will be maintained?

The records of this study will be kept private. Information used in any written or presented report will not make it possible to identify you because we will use a unique identifier instead of your child's name. Only the investigator will have access to the research records. Paper records will be shipped to Canada and secure in locked cabinet. Blood samples will be stored in the ICDDR,B laboratory (commonly known as Cholera hospital) in Dhaka using a unique identifier instead of your child's name Records will be kept for a minimum of five years. Then they will be destroyed (paper records via shredding, computer records will be deleted).

Is taking part in this study voluntary?

Your child does not have to take part in this study. If you say yes, it should be because you really want your child to participate as volunteer. If you say no, that is your decision. Your choice to be in the study or not will not affect your membership/relationship with the BRAC Adolescent Club. If you say yes, you can also stop whenever you want. You may withdraw your permission for your child at any time without penalty or loss of benefits to which you and/or your child are entitled. To stop tell Dr. Fakir Yunus. Research and Evaluation Division, BRAC Centre, 75 Mohakhali, Dhaka 1212. Telephone- +88029881265. If you withdraw your permission for your child from the study, all your records will be destroyed as outlined above.

Who should be contacted and who should be asked questions?

If you have any questions at any time please ask a member of the research team or you can contact Dr. Fakir Yunus, Research and Evaluation Division, BRAC Centre, 75 Mohakhali, Dhaka 1212. Telephone- +88029881265

If you have questions related to the rights of research participants or research-related injuries (where applicable), please contact Research Ethics Office, University of Saskatchewan, Box 5000 RPO University, 1607 – 110 Gymnasium Place, Saskatoon, SK Canada S7N 4J8

You will be given a copy of this form to keep for your records.

Statement of Consent

I have read the above information. I have asked questions and have received answers. I consent to participate in this study.

_____
Your Name

_____ _____
Your Signature Date

_____ _____
Your Thumb Print (if participants cannot write) Date

_____ _____
Name and signature of the witness Date

_____ _____
Name of (Authorized) Person Obtaining Informed Assent Date

A duplicate copy will be given to you for your record

APPENDIX B: QUESTIONNAIRE

Title: The effect of iron fortified lentils on the iron (Fe) status among the adolescent girls in Bangladesh- a double-blind randomized controlled trial

UNIQUE ID: _ _ _ _ _ _ _ _	CLUB NO: _ _ _ _ _ _ _ _ _ _	Cluster NO: _ _ _ _ _ _ _

CONFIDENTIAL

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

IDENTIFICATION INFORMATION	
Geographic Identification	**Interviewer Record**
Adolescent Club: _____ Upazila: _____ Village: _____ BRAC Branch Office: _____ District: _____ **Unique ID: __ __ __ __ __ __ __ __ __**	Interviewer ID: ___ ___ ___ ___ ___ Name: _____ Signature: _____ Remarks:
Date of Interview: (DD/MM/YYYY)	___ ___ / ___ ___ / ___ ___ ___ ___
Household Land Telephone/Cell Phone No.:	

Written informed consent: ☐ 1=Yes, ☐ 2=No	
Result Code: ☐ Completed=1 Incomplete=2 ☐	
Reason, the data collection was not completed.....	

MODULE A: CONFIRMING INCLUSION CRITERIA		
Question	***Codes***	***Response***
i. Do you regularly attend the BRAC Adolescent Club in your village?	0= No → Do **not** proceed 1= Yes	

ii.	What is your age?	*Calculate Age in years:* _____ 0= **Not** between 12-16 years → Do **not** proceed 1= Between 10-17 years	
iii.	Do you smoke cigarettes?	0= No 1= Yes → Do **not** proceed	
iv.	Have you begun menses?	0= No → Do **not** proceed 1= Yes	
v.	Are you married?	0= No → **skip to vii)** 1= Yes	
vi.	Are you currently pregnant or breastfeeding?	0= No 1= Yes → Do **not** proceed	
vii.	Do you have any chronic illnesses (for example, type 2 diabetes or cancer)?	0= No 1= Yes → Do **not** proceed	
viii.	Are you currently committed to work, either in or outside the home (for example, caring for siblings, working for BRAC), that would limit your ability to attend the Adolescent Club in your village 5 days per week for the next 24 weeks (~6 months)?	0= No 1= Yes → Do **not** proceed	
ix.	Are you willing to eat cooked lentils (daal) provided by the study team daily, 5 days per week, for the next ~16 weeks (~4 months)?	0= No → Do **not** proceed 1= Yes	

MODULE B: HOUSEHOLD GENERAL INFORMATION					
Unique ID: __ __ __ __ __ __ __ __ __ __ __					
LINE NO.	**RELATIONSHIP with HH member**	**Male/ Female**	**RESIDENCE**		**AGE**
	What is the relationship of (NAME) with each HH member starting with the head of the household *	Is (NAME) male or female?	Does (NAME) Usually sleep here?	Does (NAME) usually eat here?	How old is (NAME)? IF AGE IS LESS THAN 1 YEAR, WRITE '00'
(1)	(2)	(3) Response	(4a) Response	(4b) Response	(5) Response
1.					In years ____. ____ . ___
2.					In years ____. ____ . ___
3.					In years ____. ____ . ___
4.					In years ____. ____ . ___
5.					In years ____. ____ . ___
6.					In years ____. ____ . ___
7.					In years ____. ____ . ___
8.					In years ____. ____ . ___
9.					In years ____. ____ . ___

10.					In years _____ . _____ . ____
11.					In years _____ . _____ . ____
12.					In years _____ . _____ . ____

*CODES FOR Col. 2 and 6 Relationship to head of household	1= Self 2= Husband/wife 3= Son, 4= Daughter, 5= Son/Daughter-in-law, 6= Grand-son/grand-daughter, 7= Brother/sister, 8= Brother/Sister-in-law, 9= Nephew/niece, 10= Father/mother, 11= Father-in-law/Mother-in-law, 77= OTHER (specify)

MODULE C: DEMOGRAPHIC INFORMATION		
Question	***Codes/instructions***	***Response***
13. What is the religion practiced by most of the people who live in this household?	1. Islam 2. Hinduism 3. Christianity 4. Buddhism 5. Other…….. (Specify) *6.* Don't know	
14. Which ethnic group do you belong to?	1. Bangali 2. Chakma 3. Marma 4. Garo 5. Saotal *6.* Other…….. (Specify)	
15. What is the main occupation of the head of the household?	1. Professional/technical 2. Small Business 3. Large Business 4. Factory worker 5. Service 6. Skilled labour/service 7. Unskilled labour 8. Farmer/agricultural worker 9. Poultry/cattle raising 10. Home based manufacturing 11. Domestic help 12. House wife 13. Other…….(Specify)	
16. How much formal education did the (Household Head) attain?	1. No education 2. Primary incomplete 3. Primary complete 4. Secondary incomplete 5. Secondary complete or higher 6. Don't know	
17. What is your date of birth?	*DD-MM-YYYY*	__ __ - __ __ - __ __ __ __
18. What is your current marital status?	1= Never married → **skip to 23**	

	2= Married, 3= Divorced, separated, deserted, widowed	
19. Are you currently taking oral contraceptives?	0= No 1= Yes	
20. Have you ever given birth?	0= No → **skip to q22** 1= Yes	
21. How many children have you given birth to?	*Write the numbers*	
22. What is the date of birth of your youngest child?	*DD-MM-YYYY*	__ __ - __ __ - __ __ __
23. Are you currently, or have you ever, attended school or madrasha?	0= No → **skip to q25** 1= Yes	
24. What is the highest level of school/madrasha you are currently attending, or have completed: primary, secondary, college, or higher?	1= Primary 2= Secondary 3= College/University 4= Higher	
25. Are you currently working outside of the home?	0= No → **skip to q27** 1= Yes	
26. What is your occupation, that is the kind of work you **mainly** do?	1. Agriculture 2= Labourer (Skilled/unskilled) 3= Housewife 4= Homestead task 5= Service/Professional 6= Business 7= Student 8= Unemployed 9= Others	
27. What **main** material is used to build the walls of your main living house?	1= No walls 2= Clay or mud 3= Brick or cement 4= Bamboo or wood 5= Tin 6= Jute stick, polythene, straw, or dry leaves 7= Other: _____	
28. What are the **main** materials used to build the roof of your main living house?	1= Tally 2= Tin 3= Rcc 4= Wood, Straw, Leaves 5= Chatai-Polithin 6= Other: _____	
29. What are the **main** materials used to build the floor of your main living house?	1= Mud 2= Bamboo Or Wood 3= Brick Or Cement 4= Tally Or Mosaic 5= Other: _____	
30. Which household assets do you have?	***Read list*** *to participant.*	Electricity Solar Panel

	Check and put tick (√) all that apply: 0 = No 1 = Yes	Radio	
		Television	
		Telephone	
		Mobile Phone	
		Khat/Chawki	
		Almirah	
		Refrigerator	
		Table/Chair	
		Watch/Clock	
		Bicycle	
		Motorcycle/Scooter/ Tempo	
		Animal Drawn Cart	
		Car/Truck	
		Rickshaw/Bicycle Trolley	
		Power Tiller	
		Shallow Machine	
		Fishing Equipment	
		Other: _____	
31. What type of fuel does your household usually use for cooking?	***Read list*** *to participant.* *Check and put tick (√) all that apply:* 0 = No 1 = Yes	Electricity	
		LPG	
		Natural gas	
		Biogas	
		Kerosine	
		Coal	
		Wood	
		Straw/Grass	
		Agriculture crop	
		Animal dung	
		Others	
32. **Right now,** what is the main source of drinking water for members of your household? *(Please write down only one code)*	1= Piped water 2= Tube well or borehole 3= Dug well - protected 4= Dug well – unprotected 5= Water from spring 6= Rainwater 7= Taker truck 8= Cart with small tank 9= Surface water (river/lake/canal/hawar/pond) 10= Bottled water 11= Other: _____		
33. **Most of the time,** do you do anything to the water to make it safer to drink?	0= No → **skip to q35** 1= Yes		

34. If yes, **most of the time,** what do you usually do to make the water safer to drink?	1= Boil 2= Add Bleach/Chlorine 3= Use Water Filter 4= Solar Disinfection 5= Sit And Settle 6= Other:		
35. **Right now,** what is the main source of water for household washing, bathing, cleaning, and cooking for members of your household?	1= Piped water 2= Tube well or borehole 3= Dug well - protected 4= Dug well – unprotected 5= Water from spring 6= Rainwater 7= Taker truck 8= Cart with small tank 9= Surface water (river/dam/lake /stream/canal/hawar/pond) 9= Bottled water 10= Other:		
36. **Right now,** what kind of toilet facility do members of your household usually use?	1= Sanitary latrine 2= Flush or pour flush toilet 3= Pit latrine 4= Composting toilet 5= Bucket toilet 6= Hanging toilet/latrine 7= No facility/bush/field 8= Other:		
37. **Most of the time,** do you wash your hands with soap?	0= No → **skip to q39** 1= Yes		
38. If yes, **most of the time** when do you wash your hands with soap?	***Do not read list***. *Check and put tick (√) all that apply:* 0 = No 1 = Yes	Before preparing/ handling food	
		Before Feeding Children	
		Before Eating	
		After Preparing Food	
		After Field Work/Cleaning	
		After Changing Babies/ Cleaning Child	
		After Eating	
		After Defecating /Using Toilet Facility	
		Other:_____	
39. How many meals and snacks did you consume yesterday?	(in last 24 hours)	Meals	
		Snacks	
40. Was yesterday's food intake typical/usual for you?	0= No 1= Yes → **Skip To Q42**		
41. If no, why was it not typical/usual?	1= I was ill 2= I was not hungry 3= There was not enough food		

	4= It was a celebration (ate more or differently) 5= Other (specify):_____	
42. Who is primarily responsible for food preparation in your household?	1= Self 2= Mother 3= Father 4= Grandmother 5= Grandfather 6= Male Children 7= Other Female Children 8= Other Specify:_____	

MODULE D: KNOWLEDGE OF NUTRITION, IRON DEFICIENY AND ANEMIA			
Question	***Codes/instructions***	***Response***	
43. Have you ever heard of anemia or iron deficiency anemia?	0= No → **skip to Q49** 1= Yes		
44. Can you identify some signs that a person has anemia or iron deficiency anemia?	0= No → **skip to Q46** 1= Yes		
45. If yes, please tell me some of the signs that a person has anemia.	***Do NOT read list***. *Check and put tick (√) all that apply:* 0 = No 1 = Yes	White/pale eyes	
		While/pale nails or hands	
		Feeling faint	
		Tiredness	
		Dizziness	
		Apathy	
		Weight loss	
		Hair changes colour (goes blonde or red)	
		Edema	
		Other: _____	
46. How can you treat or prevent anemia or iron deficiency anemia?	***Do NOT read list***. *Check and put tick (√) all that apply:* 0 = No 1 = Yes	Eat more iron-rich foods	
		Eat iron-fortified foods	
		Take iron supplements	
		Prevent infection (malaria, hookworm, etc)	
		Eat foods high in vitamins and minerals ***other than iron***	
		Other: _____	
47. Do you know of any foods that contain iron?	0= No → **skip to Q51** 1= Yes		

48. If yes, what foods do you know that contain iron?	***Do NOT read list***. *Check all that apply:* 0 = No 1 = Yes	Meat (eg. Beef, goat)	
		Poultry	
		Dried or fresh fish	
		Organ meats (eg. Liver, kidney)	
		Green leafy vegetables	
		Other vegetables/fruits	
		Eggs	
		Milk	
		Pulses	
		Other:	
49. Have you heard about foods you can buy that have vitamins and minerals such as iron already added to them?	0= No → **skip to Q51** 1= Yes		
50. If yes, what are some examples of foods with vitamins and minerals such as iron already added to them?	***Do NOT read list***. *Check and put tick (√) all that apply:* 0 = No 1 = Yes	Milk	
		Salt	
		Flour	
		Pulses	

MODULE E: HOUSEHOLD FOOD SECURITY **I would now like to ask you some questions about the amount of food available for members of your household.**		
Question	***Codes/instructions***	***Response***
51. In the past four weeks, did you worry that your household would not have enough food?	0= No → **skip to Q52** 1= Yes	
51a. How often did this happen?	1= Once or twice in the past four weeks (Rarely) 2= Three to ten times in the past four weeks (Sometimes) 3= More than ten times in the past four weeks (Often)	
52. In the past four weeks, were you or any household member not able to eat the kinds of foods you usually have because of a lack of resources?	0= No → **skip to Q53** 1= Yes	
52a. How often did this happen?	1= Once or twice in the past four weeks (Rarely) 2= Three to ten times in the past four weeks (Sometimes) 3= More than ten times in the past four weeks (Often)	
53. In the past four weeks, did you or any household member have to eat a limited variety of foods due to a lack of resources?	0= No → **skip to Q54** 1= Yes	

53a. How often did this happen?	1= Once or twice in the past four weeks (Rarely) 2= Three to ten times in the past four weeks (Sometimes) 3= More than ten times in the past four weeks (Often)	
54. In the past four weeks, did you or any household member have to eat some foods that you really did not want to eat because of a lack of resources to obtain other types of food?	0= No → **skip to Q55** 1= Yes	
54a. How often did this happen?	1= Once or twice in the past four weeks (Rarely) 2= Three to ten times in the past four weeks (Sometimes) 3= More than ten times in the past four weeks (Often)	
55. In the past four weeks, did you or any household member have to eat a smaller quantity of food in a meal than you felt you needed because there was not enough food?	0= No → **skip to Q56** 1= Yes	
55a. How often did this happen?	1= Once or twice in the past four weeks (Rarely) 2= Three to ten times in the past four weeks (Sometimes) 3= More than ten times in the past four weeks (Often)	
56. In the past four weeks, did you or any other household member have to eat fewer meals in a day because there was not enough food?	0= No → **skip to Q57** 1= Yes	
56a. How often did this happen?	1= Once or twice in the past four weeks (Rarely) 2= Three to ten times in the past four weeks (Sometimes) 3= More than ten times in the past four weeks (Often)	
57. In the past four weeks, was there ever no food to eat of any kind in your household because of lack of resources to get food?	0= No → **skip to Q58** 1= Yes	
57a. How often did this happen?	1= Once or twice in the past four weeks (Rarely) 2= Three to ten times in the past four weeks (Sometimes) 3= More than ten times in the past four weeks (Often)	
58. In the past four weeks, did you or any household member go to sleep at night hungry because there was not enough food?	0= No → **skip to Q59** 1= Yes	
58a. How often did this happen?	1= Once or twice in the past four weeks (Rarely) 2= Three to ten times in the past four weeks (Sometimes) 3= More than ten times in the past four weeks (Often)	

59. In the past four weeks, did you or any household member go a whole day and night without eating anything because there was not enough food?	0= No 1= Yes	
59a. How often did this happen?	1= Once or twice in the past four weeks (Rarely) 2= Three to ten times in the past four weeks (Sometimes) 3= More than ten times in the past four weeks (Often)	

MODULE F: FOOD CONSUMPTION DATA											
Now I would like to ask you some questions about the food eaten by you/your child. I know this is sometimes hard to remember, but please give me the best answer you can.											
During the past 7 days, on how many days did you/ your child (Name) eat the following foods?											
Foods	**Serving size**	**# of days in last 7 days**	**# of servings in last 7 days**	**gm/ml [Calculate the amount]**							
1. Rice?	1 cup	___ ___	___ ___								
Breads											
2. Chapatti?	2 pieces	___ ___	___ ___								
3. Bread?	2 slices	___ ___	___ ___								
4. Parata?	1 piece	___ ___	___ ___								
Fish											
5. Small fish (with bones)?	60 gram	___ ___	___ ___								
6. Big fish (boneless)?	30 gram	___ ___	___ ___								
7. Egg?	One	___ ___	___ ___								
8. Dal?	½ cup	___ ___	___ ___								
Green Leafy Vegetables (Shak)											
9. Pui shak?	½ cup	___ ___	___ ___								
10. Palong shak?	½ cup	___ ___	___ ___								
11. Lal skak	½ cup	___ ___	___ ___								
12. Kalmi shak	½ cup	___ ___	___ ___								
13. Paat shak?	½ cup	___ ___	___ ___								
14. Kochu shak?	½ cup	___ ___	___ ___								
15. Shorisha shak?	½ cup	___ ___	___ ___								
16. Moola shak?	½ cup	___ ___	___ ___								
17. Others (specify.....)	½ cup	___ ___	___ ___								
Yellow/orange vegetables/fruit				**Record the amount**							
18. Carrots	½ cup	___ ___	___ ___	_____							

19. Ripe mango	½ cup	___ ___	___ ___	_____						
20. Weet Pumpkin	½ cup	___ ___	___ ___	_____						
21. Ripe jackfruit	½ cup	___ ___	___ ___	_____						
22. Ripe papaya	½ cup	___ ___	___ ___	_____						
23. Tomato	½ cup	___ ___	___ ___	_____						
24. Sweet potato	½ cup	___ ___	___ ___	_____						
25. Orange	½ cup	___ ___	___ ___	_____						
26. Water melon	½ cup	___ ___	___ ___	_____						
27. Banana	½ cup	___ ___	___ ___	_____						
28. Others(Specify.....)		___ ___	___ ___	_____						
Meats										
29. Chicken	60 gram	___ ___	___ ___							
30. Beef	60 gram	___ ___	___ ___							
31. Mutton	60 gram	___ ___	___ ___							
32. Liver	60 gram	___ ___	___ ___							
Milk and milk products										
33. Milk	1 cup	___ ___	___ ___							
34. Yogurt	½ cup	___ ___	___ ___							
35. Cheese	Measure of a thumb		___ ___							
			___ ___							
36. Sugar, honey, molasses	1 tbsf	___ ___	___ ___							
37. Beans, nuts	½ cup	___ ___	___ ___							

MODULE G: ANTHROPOMETRIC MEASUREMENTS	
60. Height of Participant	1. __ __ __ . __ cm 2. __ __ __ . __ cm 3. __ __ __ . __ cm
61. Weight of Participant	1. __ __ . __ __ kg 2. __ __ . __ __ kg 3. __ __ . __ __ kg
62. Waist Circumference of Participant	1. __ __ __ . __ cm 2. __ __ __ . __ cm 3. __ __ __ . __ cm
63. Hip Circumference of Participant	1. __ __ __ . __ cm 2. __ __ __ . __ cm 3. __ __ __ . __ cm
64. Mid-upper Arm Circumference (MUAC) of Participant	1. __ __ . __ cm 2. __ __ . __ cm

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