



Ph.D. Dissertation

Efficacy of micronutrient powder (MNP) with low-dose of iron  
supplementation in Bangladeshi children living in areas of high level  
of iron in groundwater

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## I. Statement of Originality

This work has not previously been submitted for a degree or diploma in any university. To the best of my knowledge and belief, the document contains no material previously published or written by another person except where due reference is made in the thesis itself.

Signed

Sabuktagin Rahman

## II. Abstract

### Background

Anaemia is a public health concern in Bangladesh, affecting 30-50% of the children under-5 years of age. Iron deficiency (ID) is thought to be the primary cause of anaemia in the country. However, a national micronutrient survey revealed that the prevalence of ID in under-five children is quite low (10.7%) and drinking iron-containing groundwater from tube wells was reported to be linked with low ID.

Despite the low burden of ID, due to the high prevalence of anaemia, the national policy for childhood anaemia prevention recommended iron supplementation through distributing micronutrient powder (MNP) containing 12.5 mg of iron. The MNP programmes have been implemented over the decades to prevent anaemia in children. Physiologically, in an iron-replete state, usage of iron supplement (MNP) might induce side effects such as, diarrhoea, nausea, and vomiting is common. Hence, the present study was conducted in Bangladeshi children drinking from groundwater with high level of iron to assess the effect of the low iron MNP (5 mg iron) against the standard MNP (12.5 mg iron) on haemoglobin concentration and to compare the relative side-effects of the competing MNP treatments.

**Methods:** The RCT was conducted in children 2-5 years old who drank water from groundwater with a high concentration of iron ( $\geq 2$  mg/L) in Belkuchi—a rural district of Bangladesh. A total of 435 children were screened for eligibility, with 327 enrolled in the trial and randomly allocated to receive either the standard MNP (12.5 mg iron per sachet) or the low-iron MNP (5 mg iron per sachet). The trial assessed if low-iron MNP, after consumption of one sachet every day for 60 days, was non-inferior to the standard MNP in regard to haemoglobin concentration of the children. A priori non-inferior margin ( $-0.5$  g/dl) was set; and non-inferiority was concluded if the lower bound of the one-sided 95% CI for the difference in the treatment effect of the low iron MNP was higher than the non-inferiority margin. The treatment effect of the low iron MNP on haemoglobin was examined by Generalized Linear Modelling through controlling for the pertinent baseline covariates. Furthermore, the study assessed the comparative incidence of iron-induced side effect such as diarrhoea, loose stools, nausea, fever, and vomiting between the treatment groups. Incidence Rate Ratio (IRR) which compares incidence rates of an event between two groups was calculated using the poisson regression to assess the incidence of the side effects

in the groups. On a subsample (n=53) of the enrolled children representing both MNP groups, gut microbiome was assessed by sequencing of 16sRNA at baseline and the endpoint. The effects of the intake of MNPs on the composition of gut microbiota were compared between the groups and between endpoint and baseline.

Additionally, to assess the effect of thalassaemia in the background of high groundwater iron and MNP consumption, a sub-sample analysis from the trial was conducted to compare haemoglobin and ferritin status among the thalassaemia carriers and non-carriers. Another sub-study was conducted to examine the haemoglobin status of the children whose drinking groundwater contained low level of iron (0--<2 mg/) for hypothesizing the utility of the low iron MNP in the low groundwater iron setting. Further, the trial was preceded by three sub studies leading up to the preparation of the trial—such as the taste-rating of the groundwater sample for semi quantitative assessment of iron content (annex 1); validation of a semi quantitative food frequency questionnaire (annex 2); and assessment of temporal concentration of groundwater iron (annex 3).

Results: The results of the RCT revealed that the low-dose iron MNP was non-inferior to the standard MNP on haemoglobin outcome ( $\beta = -0.14$ , 95% CI:  $-0.30, 0.013$ ;  $p = 0.07$ ). The lower bound of the 95% CI for the difference in the treatment effect on haemoglobin was higher than  $-0.5$  g/dl, thus confirming the non-inferiority of the low iron MNP. It resulted in a lower incidence of diarrhoea (IRR = 0.29,  $p = 0.01$ , 95% CI: 0.11–0.77), nausea (IRR = 0.24,  $p = 0.002$ , 95% CI: 0.09–0.59) and fever (IRR = 0.26,  $p < 0.001$ , 95% CI: 0.15–0.43) compared to the standard MNP. The 16sRNA sequencing revealed that overall; there was no significant treatment effect of the low-iron MNP on microbiota compared to the standard MNP. However, an apparent treatment effect was observed in children with a relative adult-like microbiota, with a higher relative abundance of potentially pathogenic *Enterobacteriaceae* after receiving the standard MNP compared to the low-iron MNP ( $p=0.07$ ).

The results of the sub-sample of the thalassaemia carriers showed that the haemoglobin concentration of the children with thalassaemia at the end-point remained unchanged relative to the baseline value;  $11.56 \pm 0.59$  (Endpoint) vs.  $11.6 \pm 0.54$  (Baseline),  $p=0.83$ . In the children without thalassaemia haemoglobin tended to increase;  $12.54 \pm 0.72$  (Endpoint) vs.  $12.41 \pm 0.72$  (baseline),  $p=0.06$ . Baseline reserve of body iron was significantly

higher in the thalassaemia carriers compared to their non-carrier peers; 594 mg vs. 558 mg;  $p=0.03$ . The increase of the infection adjusted ferritin level from baseline to the endpoint was 7.37% ( $p=0.7$ ) and 10.17% ( $p=0.009$ ) in the carrier and non-carrier groups respectively.

The sub-study examining the effect of the low iron MNP in a low groundwater iron setting revealed that the combined intake of iron from dietary, groundwater and low-iron MNP in children was  $5.8\pm2.0$  and  $6.9\pm2.5$  mg/day comprising 193% and 169% of the Estimated Average Requirement in the 2-3 year-old and 4-5 year-old subgroups, respectively. The mean concentration of haemoglobin in children exposed to groundwater concentration 0.8-<2.0 mg/L and 0.0-<0.8 mg/L subgroups was  $12.17\pm0.94$  mg/dl and  $11.91\pm0.91$  mg/dl ( $p=0.30$ ) respectively.

Conclusion: The low iron MNP (5 mg iron) was non-inferior to the standard MNP (12.5 mg) in preventing the low level of haemoglobin in Bangladeshi children exposed to high content of iron from drinking groundwater. It caused fewer incidence of side effects, such as diarrhoea, nausea and fever. Overall, there was no treatment effect of the low iron MNP on composition of gut microbiota. Further, a low iron MNP can be potentially beneficial to the thalassaemia carriers. Low iron MNP has the potential to curb the childhood anaemia in settings where groundwater iron is low. The combined findings of the trial and the sub studies demonstrated beneficial role of the low iron MNP in Bangladesh to control childhood anaemia.

Key words: Anaemia, Iron deficiency, Groundwater iron, Low-iron MNP, Gut microbiota, Bangladesh

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## VII. Acronyms

ARI:	Acute Respiratory Infection
BDHS:	Bangladesh Demographic and Health Survey
BGS:	British Geological Survey
DNA:	Deoxyribonucleic acid
DPHE:	Department of Public Health Engineering
EAR:	Estimated Average Requirement
E. coli:	Escherichia coli
EDTA:	Ethylenediaminetetraacetic acid
FAO:	Food and Agricultural Organization
Hb:	Haemoglobin
HbE:	Haemoglobin E
HIV:	Human Immunodeficiency Virus
ID:	Iron Deficiency
IDA:	Iron Deficiency Anaemia
kcal:	Kilocalories
mg:	milligram
MNP:	Micronutrient Powder
NGO:	Non-Government Organisation
NMS:	National Micronutrient Survey
NNS:	National Nutrition Service
PCR:	Polymerase Chain Reaction
qPCR:	quantitative Polymerase Chain Reaction
RDA:	Recommended Dietary Allowance
ROS:	Reactive Oxygen Species
sTfR:	Serum transferrin receptor
SQFFQ:	Semi-Quantitative Food Frequency Questionnaire
SD:	Standard Deviation
WHO:	World Health Organization

# Chapter 1: Publications & Submissions included in the Thesis

The candidate has published four manuscripts thus far during his candidature, all of which were published in international peer-reviewed journals. In addition, three other manuscripts are submitted in journals and under review.

1. The following paper is a preparatory research linked with the RCT. The paper is published in *Groundwater for Sustainable Development* [Impact Factor: 5.2]

*Rahman, S., Khan, M. ur - Rahman, Lee, P., & Ahmed, F. (2020). Development and standardization of taste-rating of the water sample as a semi-quantitative assessment of iron content in groundwater. Groundwater for sustainable development, 11, . doi: 10.1016/j.gsd.2020.100455*

This paper appears in annex 1. The contribution of the authors is outlined below:

Contribution	Contribution statement
Sabuktagin Rahman (candidate)	75% Conceptualisation, design, data collection, analysis, interpretation, and 1 <sup>st</sup> draft
Faruk Ahmed	10% Design, critical review, feedback
Patricia Lee	10% Critical review, feedback
Moududur R. Khan	5% Field supervision

2. The following paper is a preparatory research linking the RCT. The paper is published in the Journal of Nutritional Science [Impact Factor: 6.96]

*Rahman, S., Lee, P., Ireen, S., Khan, M. U., & Ahmed, F. (2021). Validation of an interviewer-administered seven-day semi-quantitative food frequency questionnaire for the dietary assessment of preschool children in rural Bangladesh. Journal of nutritional science, 10, e26. <https://doi.org/10.1017/jns.2021.19>*

This paper appears in the annex 2. The contribution of the authors is outlined below:

Contribution	Contribution statement
Sabuktagin Rahman (candidate)	70% Conceptualisation, design, tool development, data collection, data processing, analysis, interpretation and drafting
Faruk Ahmed	15% Design, data supervision, critical review and editing and feedback
Patricia Lee	12.5% Design, critical review and statistical advice, editing
Santhia Ireen	2.5% Conceptualization and tool development

3. The following paper is a preparatory research linking the RCT.

*Title: “Temporal effect on Iron concentration in the Expressed Groundwater Samples in Bangladesh: Potential Implication for Iron-status in Population”*

This is submitted in the *Journal of Water and Health* (IF: 1.7).

This paper appears in the annex 3. The contribution of the authors is outlined below:

Contribution	Contribution statement
Sabuktagin Rahman	70% Conceptualisation, design, data collection, processing and analysis, interpretation and drafting
Faruk Ahmed	15% Conceptualization, design, critical review and feedback
Patricia Lee	12.5% Critical review and statistical advice
Mordudur R. Khan	2.5% Field supervision

4. The following paper is the first paper from the clinical trial (RCT: study 1). It is published in *Nutrients* [Impact Factor: 5.71]

*Rahman, S., Lee, P., Raqib, R., Roy, A. K., Khan, M. R., & Ahmed, F. (2019). Effect of Micronutrient Powder (MNP) with a Low-Dose of Iron on Hemoglobin and Iron Biomarkers, and Its Effect on Morbidities in Rural Bangladeshi Children Drinking Groundwater with a High-Level of Iron: A Randomized Controlled Trial. Nutrients, 11(11), 2756. MDPI AG. Retrieved from <http://dx.doi.org/10.3390/nu11112756>*

The paper appears in the section 5.2.1.3 of the chapter 5. The contributions of each co-author are highlighted below:

Contribution	Statement of contribution
Sabuktagin Rahman (candidate)	60% Conceptualisation, design, trial preparation & procurement, personnel training, data collection, data processing, data analysis and interpretation and 1st draft
Faruk Ahmed	20% Design, trial preparation, data supervision, critical review, editing and feedback
Patricia Lee	12% Design, critical review and editing
Moududur R. Khan	1% Field administration & supervision
Rubhana Raqib	2% Laboratory data analysis, critical review, editing
Anjan K. Roy	5% Laboratory data analysis

5. The following paper is the second paper from the clinical trial (RCT: study 2). It is published in *European Journal of Nutrition* [Impact Factor: 5.6].

*Rahman, S., Kortman, G., Boekhorst, J., Lee, P., Khan, M. R., & Ahmed, F. (2021). Effect of low-iron micronutrient powder (MNP) on the composition of gut microbiota of Bangladeshi children in a high-iron groundwater setting: a randomized controlled trial. European journal of nutrition, 60(6), 3423–3436. <https://doi.org/10.1007/s00394-021-02523-1>*

This paper appears in the section 5.2.2.3 of the chapter 5. The contribution of the authors is outlined below:

Contribution	Contribution statement
Sabuktagin Rahman (candidate)	50% Study conceptualisation, design, data collection, data analysis, interpretation, and drafting.
Faruk Ahmed	15% Study design, data interpretation, critical review, editing and feedback
Guus Kortman	25% Data processing, analysis, interpretation, critical review, editing
Jos Boekhorst	2.5% Data analysis, critical review
Patricia Lee	5% Design, review and editing
Mordudur R. Khan	2.5% Data collection supervision

6. The following paper is the first supplementary study complementing the RCT.

*Title: “Thalassemia carrier status and groundwater iron: Implication for iron supplementation programme for children in Bangladesh”*

Submitted to *International Journal of Hematology-Oncology and Stem Cell Research* (Citescore: 2.6) and under-review. This paper appears in the section 5.2.3.1.1 of the chapter

5. The contribution of the authors is outlined below:

Contribution	Contribution statement
Sabuktagin Rahman	70% Conceptualisation, design, data collection and processing, analysis and interpretation, 1 <sup>st</sup> draft
Faruk Ahmed	15% Critical review, editing and feedback
Patricia Lee	7.5% Review and editing
Mordudur R. Khan	7.5% Lab data analysis, staff training

7. The following paper is the second supplementary study complementing the RCT.

Title: “Intake of Iron in a Low-iron Groundwater Setting in Rural Bangladeshi Children: Low-iron Micronutrient Powder (MNP) is a potential intervention for prevention of childhood Anaemia”. The paper is submitted for publication in *Anaemia* (IF: 1.7) and under review.

This paper appears in the section 5.2.4.1.1 of the chapter 5. The contribution of the authors is outlined below:

Contribution	Contribution statement
Sabuktagin Rahman	70% Conceptualisation, design, data collection and processing, data analysis, interpretation and 1st draft.
Faruk Ahmed	20% Design, critical review, editing and feedback
Patricia Lee	10% Critical review, editing and feedback

## Chapter 2: Introduction

## 2.1 BACKGROUND

Anaemia is a condition characterised by the relative lack of haemoglobin as per the age, sex, and physiological status of the body. It is one of the largest public health problems in the world affecting 1.6 billion people (McLean et al., 2008). Anaemia affects universally the high-, middle- and, low-income countries, however, the burden are higher in the low and middle-income countries (WHO, 2011). Anaemia is associated with impaired cognitive performance, increased mortality and morbidity, poorer educational attainment in children and decreased work capacity in adults (WHO, 2001; Balaranjan et al., 2011). Iron deficiency (ID) is tipped as the most common cause of anaemia, with the widely held assumption that globally half of all anaemia is caused by ID (Stoltzfus et al, 2003). Other causes of anaemia include other micronutrient deficiencies (vitamin A, folic acid, vitamin B12 and B2), malaria, chronic infections, and haemoglobinopathies (Balaranjan et al., 2011). Globally 42.6% of children aged 6-59 months, and 29.1% of women of reproductive age are affected by anaemia; which translates into 273.2 and 528.7 million of children and women respectively. The respective prevalence of anaemia in under-5 children was 53.8%, 62.3% and 48.6% in South East Asia, Africa, and the Eastern Mediterranean Region, while in women of reproductive age the prevalence was 41.9%, 38.6% and 37.8% respectively (WHO, 2011).

Bangladesh is affected with a high burden of anaemia in vulnerable populations. The Demographic Health Survey 2011 reported the national prevalence in under-5 children as 51%, while the Bangladesh National Micronutrient Survey 2011-12 reported 33% (BDHS, 2011; NMS, 2011-12, Rahman et al., 2016). The causes of anaemia in these populations are inadequate intake of micronutrients, poor dietary diversity, infection, haemoglobinopathy and household food insecurity (Pasricha et al., 2010). However, contrary to the widely held assumption that ID is the most common cause of anaemia, the prevalence of ID (10.7%) and iron deficiency anaemia (IDA, 7.2%) was observed to be low (NMS, 2011-12). A

contemporary study in a north-western district of the country observed a zero prevalence of ID in women while the prevalence of anaemia was high (57%) (Merrill et al., 2011). Several studies attributed the low prevalence of ID in the populations to the high level of iron in groundwater which is the principal source of drinking water in Bangladeshi population (Rahman et al., 2016; Merrill et al., 2011, Ahmed et al., 2018).

Globally, iron supplementation and fortification programmes have been recommended for prevention and control of ID and anaemia (Zimmermann et al., 2007). In-home fortification of micronutrients, where the caregiver adds vitamins and minerals to the weaning foods at home using micronutrient powders (MNPs) containing iron was observed to be reducing the IDA risk in Bangladesh and similar settings (Ip et al., 2009; Mahfuz et al., 2016). The WHO recommended that home fortification of staple foods with MNPs is an effective intervention to control IDA in children 6 to 23 months of age (De Regil et al., 2013).

However, excess of iron may lead to the adverse effects. Recent fortification trials in Pakistan and Ghana showed that the usage of MNP with 12.5 mg iron was associated with the incidence of bloody diarrhoea, respiratory infection (Soofi et al., 2013) and the increased hospitalisations attributed to diarrhoea (Zlotkin et al., 2013). The findings of these studies generated discussion and commentaries on the MNP-induced adversities among the micronutrient researchers and programme personnel. Iron is a growth-limiting nutrient for many gut bacteria which competes for the unabsorbed dietary iron (Andrews et al., 2003). For most enteric Gram-negative bacteria (e.g. *Salmonella*, *Shigella* or pathogenic *Escherichia Coli*), iron acquirement plays a significant role in expressing virulence and colonisation (Naikare et al., 2006). To complement this, the recent studies in Africa have shown that MNP or iron fortification was associated with significant adverse influence in the intestinal microbial composition, leading to the proliferation of pathogenic bacteria (e.g. pathogenic *Enterobacteriaceae*) and decreasing the number of health-promoting bacteria

(*Lactobacillaceae*, *Bifidobacteria*) (Zimmermann et al., 2010; Jaeggi et al., 2014). These researchers have established a biological mechanism of iron induced diarrhoea as a result of iron supplementation or fortification.

## **2.2 RATIONALE AND SIGNIFICANCE**

As stated above, the NMS 2011-12 reported significantly higher mean serum ferritin levels in children and women living in areas with a high level of iron in groundwater than those living in areas of low iron in groundwater. It was concluded that the low prevalence of ID and IDA could be attributed to drinking groundwater from tubewells (Rahman et al., 2016). Merrill et al (2011) reported a strong, positive, dose–response association of the natural iron content in groundwater, intake of iron from such sources, and iron status of women. Another study in the northeast region of the country, where iron content in groundwater is high, reported that the prevalence of anaemia and haemoglobin concentration in women was significantly associated with the iron content in groundwater in a dose-response manner (Wendt et al., 2016). Thus, as per the findings of the above research, the good iron and haemoglobin status in the Bangladeshi population was attributed to the high amount of intake of absorbable iron from drinking groundwater. To complement this, 97% of the country’s population use groundwater for drinking and cooking (DPHE/BGS, 2001).

From a different perspective, phytic acid has a strong binding affinity to important minerals, such as calcium, iron, and zinc. When iron binds to phytic acid they form insoluble precipitates and are far less absorbable in the intestines. Further, phytic acid is abundant in the food matrix of cereal and legume-based complementary foods, especially in Bangladesh. As phytic acid is a potent inhibitor of iron absorption, less than 20% of iron from MNP added to complementary foods is absorbed (Zimmermann et al., 2007).

Additionally, in rural populations of developing countries, such as in Bangladesh, with high levels of inflammation and infection, absorption of iron is likely to be impaired

being induced by hepcidin, a key iron regulator (Saito et al., 2014). Hepcidine reduces the absorption of dietary iron through binding and degradation of ferroportin, the iron efflux protein (Nemeth et al., 2004).

Hence, in under 5 years old Bangladeshi children, factors including rice-based complementary food (rich in phytic acid); high levels of infection, such as acute respiratory infection; diarrhoea (BDHS, 2014); and presumably, a fair amount of iron being consumed from groundwater, render the conditions for building up the unabsorbed iron in the intestines when, concomitantly, iron is provided as supplementation or fortification.

Bangladesh has institutionalised the home fortification of MNP as the national policy for controlling anaemia in children under-5 years old. Currently, Bangladesh has a number of large-scale home fortification programmes for control and prevention of a high burden of anaemia in children under-5-years old, which provides blanket coverage of MNP containing five-component of micronutrients - iron, vitamin A, zinc, vitamin C and folic acid (National Strategy on Prevention and Control of Micronutrient Deficiencies Bangladesh, 2015). A study on the home fortification of MNP in Bangladesh reported that the prevalence of diarrhoea in children was 13% (Angdembe et al., 2015). An evaluation of an ongoing large-scale programme of home fortification of MNP suggested the prevalence of diarrhoea in children of the programme areas ranged from 10-14% (personal communication), which was two to three times the national prevalence of 5.7% (BDHS, 2014). Thus, a prevalence of diarrhoea in the programme areas, higher than the national estimate, was reported, despite only a low effective coverage of the programme (~3%). This low coverage has been attributed to its side effects e.g. diarrhoea (personal communication). Furthermore, qualitative research evaluating the country's largest home fortification - MNP programme reported that diarrhoea, vomiting, and abdominal discomfort were the key factors leading to the discontinuation of MNP in children (Mitra et al., 2015). Therefore, from these observations, it

is plausible that in addition to exposure to a fair amount of natural iron from drinking groundwater, the MNPs might result in the additional load of iron in the intestines. This may adversely affect the gut microbial population leading to adverse health outcomes, e.g. diarrhoea, loose stools. This may partly explain poor coverage of the MNP programme.

On the backdrop of the low prevalence of ID in Bangladesh and its link with the iron taken through drinking ground water, national policymakers have raised the question of using excessive iron, particularly in relation to the MNP programmes which provides blanket MNP supplementation for all young children. The above deliberation clearly justifies the trial to test the effect of the low-iron MNP relative to the standard MNP on the haemoglobin outcome and to compare the side effects in under-5 children living in areas with high level of iron in groundwater.

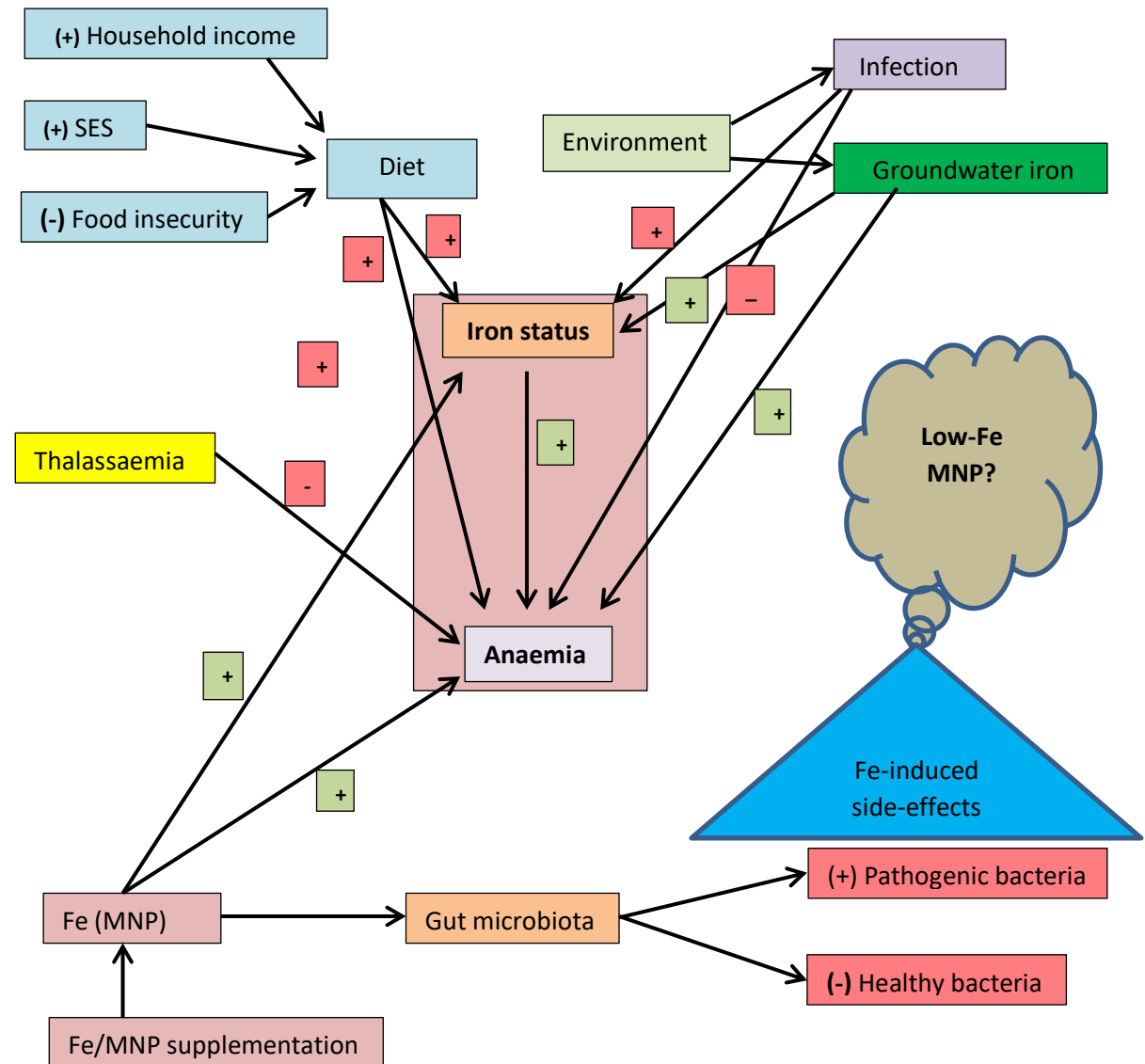
### **2.3 THE PROPOSED RESEARCH**

With a clear justification described above, a double-blind randomized controlled trial was conducted in children living in the areas of predominantly high iron in groundwater to assess the efficacy of low iron MNP relative to the standard MNP on haemoglobin and iron responses, and to compare the effects of these two formulations on the intestinal microbiota profile and morbidity outcomes. We posit that the use of the low iron MNP relative to the standard formulation would result in non-inferior haemoglobin response with minimum adverse effects on gut microbiota and a significant decrease in the incidence of diarrhoea. If these hypotheses were true, the low iron MNP might be considered in national policy and programmes for controlling anaemia in high iron areas (groundwater iron concentration  $\geq 2$  mg/L) which constitute half of the geographical spread of the country. Furthermore, it is likely that the low iron MNP may increase compliance with and coverage of the programme and may positively impact the national anaemia control.

## 2.4 CONCEPTUAL FRAMEWORK

Figure 1 describes the theoretical conceptual framework for the proposed study. Low socioeconomic status, household food insecurity, and low incomes are related to low-quality, poorly diversified diet in low-income countries, such as Bangladesh. Poor diet leads to inferior haemoglobin and iron status. Among environmental factors, infections, such as diarrhoea, respiratory tract infection, dengue, chikungunya are prevalent in the setting. In presence of infection serum ferritin acts as an acute phase reactant resulting in a spurious rise in ferritin levels; at the same time, chronic infection adversely affects anaemia. Among the other important environmental factors, groundwater iron has been observed to be favourably associated with iron and anaemia status in Bangladeshi populations. Of congenital conditions, thalassaemia is positively associated with iron status, but inversely affects the anaemia outcome. All the above variables related to anaemia and iron status were relevant and measured in the study. Micronutrient powders (MNP), which contains iron (12.5 mg), is a conventional intervention to control anaemia and improve iron status in children. However, as discussed in the literature review, the iron in the MNP is associated with adverse changes in the intestinal microbiota, resulting in an increase of pathogenic bacteria and a decrease in the population of health-promoting bacteria. One of the manifestations of this is the increased incidence of clinical morbidities, e.g. diarrhoea, nausea, and vomiting. This has posed scale-up challenges for the MNP-home fortification programmes in Bangladesh, especially in areas where the iron level in groundwater is high. On this backdrop, the present trial is proposed to test the efficacy and adverse consequences of low iron MNP (Fe: 5 mg) vis-à-vis the standard MNP (Fe: 12.5 mg).

Figure 1 Conceptual Framework



#### Legends

(+): Favours (i.e. improves the status)

(-): Unfavours (i.e. worsens the status)

## **2.5 AIM**

The overall aim of the trial was to examine the potential of using low iron MNP containing 5 mg of iron per dose for preventing low level of haemoglobin concentration in Bangladeshi children 2-5 year-old, living in areas with high concentration of iron in groundwater.

### **2.5.1 PRIMARY OBJECTIVES OF THE STUDY**

1. To assess the effect of low iron MNP relative to the standard MNP on haemoglobin response in children aged 2-5 years drinking groundwater with a high concentration of iron ( $\geq 2$  mg/L).
2. To compare the incidence of side effects associated with the intake of the low iron MNP and standard MNP in children participating in the trial.
3. To compare the intestinal microbiota profile associated with the intake of the low iron MNP and standard MNP in children participating in the trial.

### **2.5.2 HYPOTHESES**

1. The low-iron MNP will be non-inferior to the standard MNP in prevention of the low haemoglobin level ( $< 11$  g/dl) in Bangladeshi children aged 2-5 years old exposed to a high concentration of iron from the drinking groundwater source.
2. The low-iron MNP will result in fewer incidence of iron-induced side effects compared to the standard MNP.
3. Low-iron MNP will induce fewer adversaries on the composition of gut microbiota relative to the standard MNP.

### **2.5.3 SECONDARY OBJECTIVES**

1. To develop the taste rating of a groundwater sample as a non-device based tool for a semi-quantitative assessment of the level of iron in tube-well water.

2. To validate an interviewer-administered 7-day semi quantitative food frequency questionnaire for dietary assessment of Bangladeshi children aged 2-5 years old.
3. To assess the temporal effect of time on the concentration of stored groundwater samples.
4. To compare iron status indicators in children with a thalassaemia carrier state and the children without thalassaemia on the backdrop of high groundwater iron and the intake of iron supplements (e.g. MNPs).
5. To assess the potential of the low iron MNP in preventing anaemia in children aged 2-5 years old who drink groundwater with a low content of iron (<2 mg/L).

## 2.6 MAIN APPROACHES TO STUDIES

Figure 2 depicts the overall organization of the studies under the project. The main trial was preceded by the three preparatory studies—preparatory study 1 (“Taste-rating tool for assessment of groundwater iron content”). The sub-study developed and standardized the methods of taste-rating. The tool was used for identification of the study sites and the initial screening of the tube wells for provisional selection for the trial.

Preparatory study 2 (“Validation of a food frequency questionnaire”). This sub study validated the dietary intake assessment method- a seven-day semi quantitative food frequency questionnaire through comparing against the standard tool (24-hour recalls) to measure the iron and other nutrient intakes in the study children.

Preparatory study 3 (“Temporal effects on the groundwater iron concentration”). The study was undertaken to assess the temporal concentration of iron of the expressed groundwater samples at specified intervals after pumping off the tube wells for up to 6 hours.

The main research under the project (RCT: Study 1) is a randomized controlled trial to investigate the effect of the low iron MNP relative to the standard MNP on the haemoglobin status and to document and compare the side effects in the children who drink the natural groundwater with a richness of iron content.

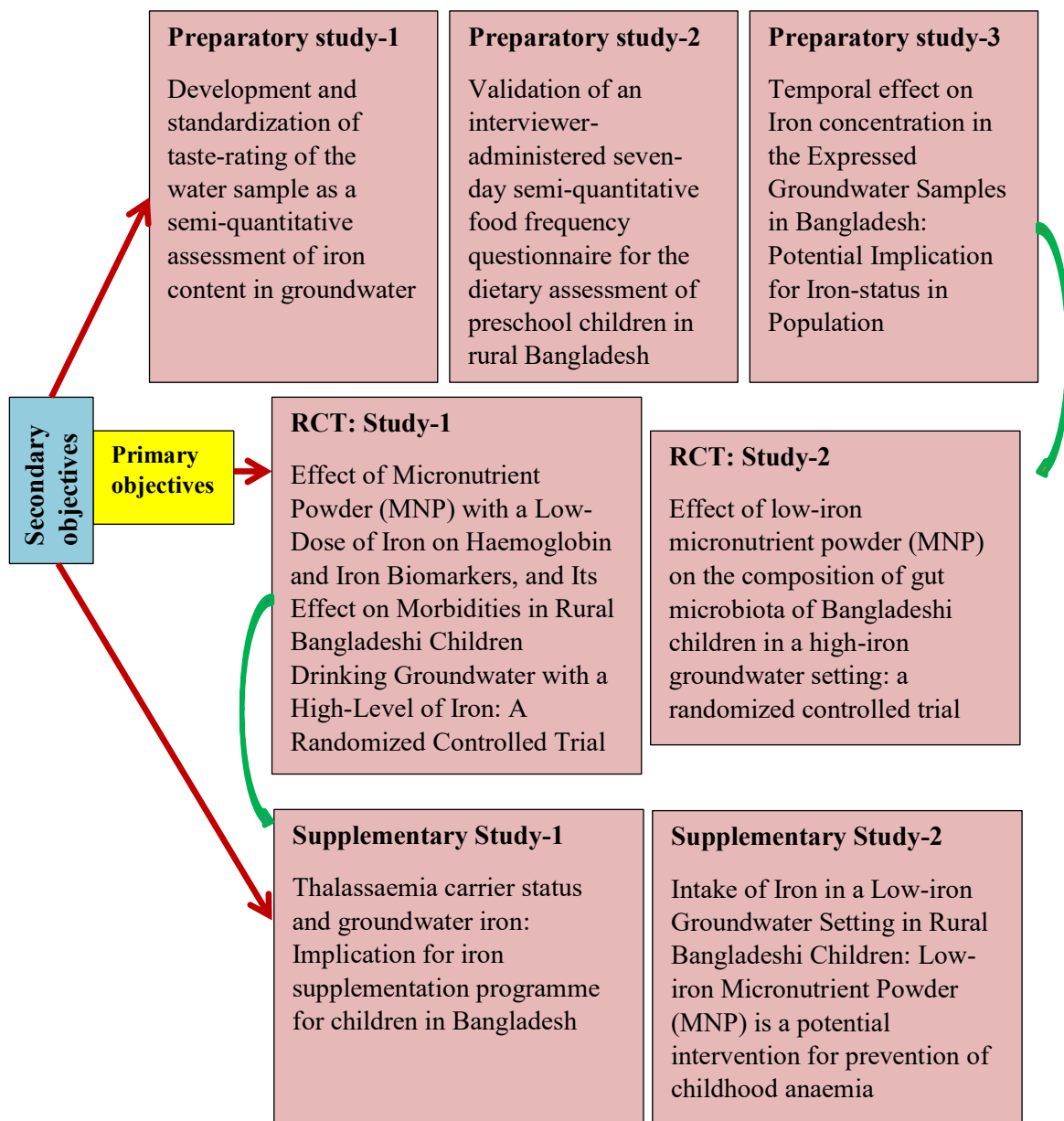
On a subsample from the main trial, intestinal microbiota was examined for the differential effects on its composition between the two MNP groups (RCT: Study 2).

Additionally, two supplementary studies were conducted—“Thalassaemia carrier status and groundwater iron: Implication for iron supplementation programme for children in Bangladesh” (Supplementary study 1). There were roughly 13% of the children diagnosed with the thalassaemia carrier state among the participants of the RCT. Ferritin and haemoglobin status and intake of iron from the various sources-- dietary, groundwater and the

MNPs were compared at baseline and endpoint between the thalassaemia carrier and the non-carrier groups. Key morbidities were compared.

The final sub study examined the potential of the low-iron MNP for prevention of childhood anaemia in the low iron groundwater setting (Supplementary study 2). A cross-sectional study was conducted to assess the intake of iron from the principal sources in children drinking from the wells with a low concentration of iron ( $0 < 2$  mg/L) -- such as the groundwater iron, dietary iron and the hypothetical intake of low iron MNP. The intakes of iron (actual and bioavailable) were compared to the dietary reference intakes and triangulated with the mean haemoglobin level of children. The findings of the supplementary studies complemented the findings of the trial.

Figure 2: Outline of the Studies under the Research Project



## Chapter 3: Literature Review

This chapter provides a review of the global and Bangladeshi literature on the elements pertinent to the research project. The review focuses on the following broad sub sections.

3.1 Anaemia, ID- definition, prevalence, consequences and causes

3.2 Factors affecting iron and/or anaemia status

--Dietary iron, groundwater iron, infection, thalassaemia

3.3 Infection and the adjustment of iron status

3.4 Measures to control iron deficiency

3.5 Adverse effects of iron supplementation and gut microbiota

### **3.1 ANAEMIA, ID- DEFINITION, PREVALENCE, CONSEQUENCES AND CAUSES**

#### **3.1.1 ANAEMIA**

Anaemia is a condition characterised by a reduction in the oxygen-carrying capacity of blood. According to Stedman's medical dictionary, anaemia is defined as a decrease in the total amount of red blood cells (RBCs) or haemoglobin in the blood (Stedman's medical dictionary, 2006). Alternatively, anaemia is defined as the haemoglobin concentration less than -2 standard deviations (SD) of the age and sex-specific normal reference value (Ramakrishnan et al., 2008). Anaemia is a global public health problem affecting both low- and high-income countries with significant consequences on health as well as social and economic development. It occurs at all stages of the life cycle, but pregnant women and young children are more vulnerable (WHO, 2008).

#### **3.1.2 HAEMOGLOBIN THRESHOLD**

Normal haemoglobin distributions vary with age, sex, and physiological status. Most commonly used cut-offs for defining anaemia are the haemoglobin level  $< 110$  gm/l in the

under-5 children and pregnant women, < 120 gm/l in the non- pregnant women and < 130 gm/l in men (WHO, 2004).

### 3.1.3 ID: DEFINITIONS

ID, a condition characterised by exhausted body iron stores and commonly diagnosed with a serum (or plasma) ferritin value <12 µg /L in young children (<5 years) and <15 µg/L in others (≥5 years) (Table 1, WHO, 2004).

Table 1: Ferritin Threshold Defining ID

Age	Serum ferritin (µg/l)	Reference
< 5 years	<12	WHO, 2004
≥5 years	<15	WHO, 2004

### 3.1.4 ANAEMIA AND ID IN BANGLADESH

Prevalence of anaemia is high in Bangladesh. Bangladesh Demographic and Health Survey (BDHS) 2011 reported the prevalence of 51% and 50% in the children 0-59 months and non-pregnant women respectively. The national micronutrient status survey 2011-12 reported the prevalence of 33%, 17-19% and 26% in the preschool age children (6-59 months), school age children (6-14 years) and in the non-pregnant non lactating women of reproductive age (15-49 years) respectively. Prevalence of ID in Bangladesh has been reported to be variable. Some studies have reported relatively high prevalence while the others reported modest estimates. In a recent study in women aged ≥16 years in Pabna, a western district, ID was observed as 17.3% and 19.4% with and without the arsenic-induced skin lesion respectively (Kile et al., 2016). In a study among infants for an iron supplementation trial in Matlab, an eastern sub-district, the baseline ID was found to be 21% (Eneroth et al., 2010). In a study in a north-western district, the prevalence of ID was found

to be zero percent among non-pregnant women (Merrill et al., 2011). In the first-ever national survey of the micronutrients, the prevalence of ID in a nationally representative survey was observed to be 10.7%, 7.1% and 3.9-9.5% (as per age subgroups) in preschool children, non-pregnant non-lactating women and school-age children respectively (Rahman et al., 2016).

### **3.1.5 CONSEQUENCE OF ANAEMIA AND ID**

Globally ID is the most common cause leading to anaemia (Stoltzfus et al., 2010), and consequences of anaemia and ID considerably overlap. Hence it is advantageous to describe the consequences of anaemia and ID in the same place. Anaemia is an indicator of both poor nutrition and poor health. Clinically, patients may suffer from poor mental performance or cold intolerance (Rosenzweig et al., 1999). Tiredness and exercise-induced breathlessness are reported. Rarely, glossitis or dysphagia may be the presenting complaints (Cook et al., 2005; Novacek et al., 2006). In relation to the public health implications, the WHO stated that the consequences of anaemia vary depending on the severity and the population groups affected. It includes decreased work capacity, poor pregnancy outcomes, increased maternal and perinatal mortality and morbidity, reduced cognitive performance, and poorer educational achievement (WHO, 2004). Anaemia unfavourably affects cognitive and motor development and results in the fatigue and decreased productivity (Balaranjan et al., 2011; Hass et al., 2001).

In relation to the adverse consequence of ID, the Global Burden of Disease ranks the ID ninth among 26 risk factors (Lopez et al., 2006); accounting for 841,000 deaths and more than 35 million disability-adjusted life years (DALYs) lost per year (Lopez et al., 2006; Stoltzfus et al., 2003). According to the Lancet series, nutritional ID is attributed to 20,854 deaths in the under-fives and 115,000 maternal deaths; while an estimated 2.2 million and 3.4 million DALYs lost every year, respectively (Black et al., 2008). ID may adversely impact

erythropoiesis and may result in IDA, a combination of ID and anaemia (Cook et al., 1999). Anaemia can result from many conditions but is most commonly caused by ID alone (Sight and Life, 2007).

Iron is vital to life as the enabler of oxygen transport, various metabolic activities, such as oxidative phosphorylation, synthesizing iron-containing enzymes, detoxification of foreign substances in the liver, myelination, metabolism of neurotransmitters, and DNA synthesis. Hence, the consequences of ID is multi-pronged and include impaired cognitive function and workability and increased morbidity and mortality (WHO/FAO, 1998; WHO, 2001; Connor et al., 1996; Stoltzfus et al., 2003; McCann et al., 2007). Following paragraphs further document the evidence in the global context on the consequence of anaemia and ID, especially in young children. Young children (<2 years) with severe anaemia, due to malaria and ID, are at higher risk of mortality (Brabin et al., 2001). A mild form of anaemia, despite being corrected, may result in permanent cognitive damage through decreasing attention span and shortening of memory. Anaemic children have, on average, for every 10 g/L decrease in haemoglobin have two points' lower IQs than other children (Black et al., 2008; Stoltzfus et al., 2004). Case-control studies comparing healthy infants with IDA to infants with adequate iron status, reported that their mental development test scores were lower by average 6 to 15 points (Lozoff et al., 2003). Iron-deficient children were found to have lower motor development scores by 6 to 17 points than their non-deficient peers (Lozoff et al., 2003). Other preventive trials among the well-nourished term infants clearly observed motor developmental benefits of iron supplementation (Moffat et al., 1994; Friel et al., 2003). However, the utility of iron on psychomotor development was not unanimously observed. In Moffat et al study which was a randomized controlled trial comparing the interventions iron-fortified formula milk (12.8 mg/L) vs. the standard infant formula (iron 1.1 mg/L) on the iron status markers and the psychomotor development at various time-points until the children

were 15 months old. There was a significantly higher status of the all measures of iron status in the fortified formula group ( $p < 0.001$ ). To complement this, overall, the psychomotor development patterns differed between groups ( $F_{3, 520, 3.4}$ ;  $p = 0.02$ ) over the time. However, at the time-point comparisons, the mean values were similar at 6 months but differed at 9 and 12 months of age ( $p < 0.001$ ). By 15 months of age the differences were no longer significant ( $p = 0.23$ ). No effect on the mental development and behavior were detected.

The reason for not sustaining the effect on psychomotor development at 15 months is hard to explain. However, the two issues might be implicated—a. sample size was lost by ~20% without any measurement of the parameters; b. the psychometer and the behavioral parameters are distant outcomes (compared with iron status markers) which over the time span and possibly due to sample loss might have failed eliciting an effect. In a few studies (case-controlled) that included the neurophysiologic measures, differences have been reported between iron-deficient and iron-adequate children, in the speed of neuronal transmission in the auditory system (Roncagliolo et al., 1998; Li et al., 1994), rapid eye movement (REM) in active sleep (Algarin et al., 2003), recognition memory (Burden et al., 2007), and in the asymmetry of the frontal lobe EEG (Electro Encephalogram) (Abrams et al., 2005).

### **3.1.6 CAUSES OF ANAEMIA**

The causes of anaemia are multifactorial. In general, 50% of cases of anaemia are assumed to be due to ID, but the proportion probably varies among population groups, in different areas and according to the local conditions (Stevens et al., 2013; WHO, 2001; Stoltzfus et al., 2004). Other common causes of anaemia include other micronutrient deficiencies, such as folate, riboflavin, vitamins A and B12, acute and chronic diseases (e. g. malaria, cancer, tuberculosis, and HIV), and inherited disorders of haemoglobin synthesis

(Balaranjan et al., 2011; Tolentino et al., 2007). On the regional basis, in Sub-Saharan Africa, causes of anaemia include ID, malaria, helminthiasis, schistosomiasis, haemoglobinopathies, and deficiencies of other micronutrients (Kassebaum et al., 2014; Crawley et al., 2004). In the South Asian countries, such as in Bangladesh, the important associations of anaemia identified are – micronutrient deficiencies (e.g. iron, vitamin A, folate and zinc), household food insecurity, poor dietary diversity, infection, and hereditary haemoglobinopathy (Pasricha et al., 2010; Rahman et al., 2016).

As the most common source of iron in humans is food; inadequate food intake and intake of low-quality food are the principal causes of IDA in Bangladesh and similar settings. Bangladesh Demographic Health Survey 2014 reported that only 27% of the 6-23-month-old children consume the acceptable diversity of food ( $\geq 4$  food groups) and only 22% of them fulfilled the three recommended Infant and Young Child Feeding (IYCF) practices- breastfeeding, number of food groups, and frequency of consumption over the day (BDHS, 2014). As a developing country, poverty is potentially an important underlying cause of malnutrition, i.e. anaemia and micronutrient deficiency. The nationally representative data in Bangladesh show that the household asset score and haemoglobin status in preschool-age children is positively correlated (NMS, 2011; BDHS, 2014). In school-age children, being the children representing the bottom two quintiles in terms of the socio-economic status (SES) were associated with the significantly lower haemoglobin level (Rahman et al., 2016). The nationally representative data of Bangladesh suggests that among the other nutritional factors, vitamin A, folate and zinc were found to be associated with haemoglobin (Rahman et al., 2016). Among the non-nutritional contributors to anaemia (i.e. malaria, worm infestation, chronic infections and genetic disorders) the risk of malaria is generally low in Bangladesh (Dreyfuss et al., 2000; Linpisarn et al., 1996). There is no nation-wide data on the prevalence of thalassaemia. One study reporting a 28 % prevalence of thalassaemia in women and an

associated risk of anaemia (Merrill et al, 2012). Therefore, thalassaemia is a likely contributor to anaemia in Bangladesh. Despite the fact that ID is not the predominant cause of anaemia in Bangladesh, it is the most prevalent cause globally and still an important cause in Bangladesh (Rahman et al., 2016).

### **3.2 FACTORS AFFECTING IRON AND/OR ANAEMIA STATUS**

The section provides an overview on the various factors affecting the iron and haemoglobin status.

#### **3.2.1 DIETARY INTAKES**

The section presents a general overview of dietary intake in Bangladesh population. It describes the intake profile of the key micronutrients in Bangladeshi population. Finally, it focuses on the intake of iron in the population with special attention to the under-five children group.

Bangladesh has observed some improvement in the intakes of the major food items. As per the Household Income and Expenditure Survey (HIES) reports, at the national level, per capita daily intake of food has increased by 5.4 % to 1000 grams in 2010 from the 947.8 grams in 2005 (HIES, 2010). Intake of fish rose to 49.5 grams from 42.1 grams between 2005 and 2010. The consumption of eggs rose to 7.2 grams from 5.3 grams between 2005 and 2010.

#### **3.2.2 INTAKE OF THE KEY MICRONUTRIENTS AND TRENDS IN BANGLADESH**

Table 2 depicts a trend in the intakes of the key micronutrient in preschool-age children in 2004, 2011-12 and 2016. The mean consumption of iron was 0.7, 4.1 and 3.7 mg/day respectively. The intake of zinc was 1.2, 2.6 and 2.7 mg/day respectively.

Table 2: The Trend of the Intakes of the Key Micronutrients in Preschool-age Children Over 2004-2016

	Kimmon's (2004)	NMS 2011-12	Epigenetics 2016 <sup>2</sup>
Iron(mg/d)	0.7	4.1	3.7
Zinc(mg/d)	1.2	2.6	2.7
Vitamin A(μg of RE/d)	174	210.6	447.4

<sup>1</sup>Median estimates

<sup>2</sup>Personal communication

### 3.2.3 INTAKE OF DIETARY IRON IN BANGLADESHI POPULATION

As a developing country, Bangladeshi traditional diet is rich in plant-based food which is poor sources of iron. Based on the Household Income and Expenditure Survey 2005, Bermudez et al reported, at the national level 87% of the population was identified as having a high probability of inadequate iron intake (Bermudez et al., 2012). A nationally representative survey on the status of the micronutrient in Bangladeshi population revealed that the proportion of the 2-3 year-old children and 4-5 year old children not meeting the EAR for iron is 42.6% and 52.3% respectively. Proportion of women not meeting the EAR is 65.4% (Bangladesh National Nutrition Council, 2021).

Table 3: Intake of Dietary Iron vs. EAR

	EAR <sup>1</sup> of iron (mg/d)	(%) of population not meeting EAR <sup>2</sup>
Preschool-age children 2-3 y	3	42.6
Preschool-age children 4-5 y	4.1	52.3
Non-pregnant non-lactating women 19-49 y	8.1	65.4

<sup>1</sup>Institute of Medicine (IOM)

<sup>2</sup>Bangladesh National Nutrition Council 2021

### 3.2.4 NON-FOOD FACTORS INFLUENCING IRON AND ANAEMIA STATUS

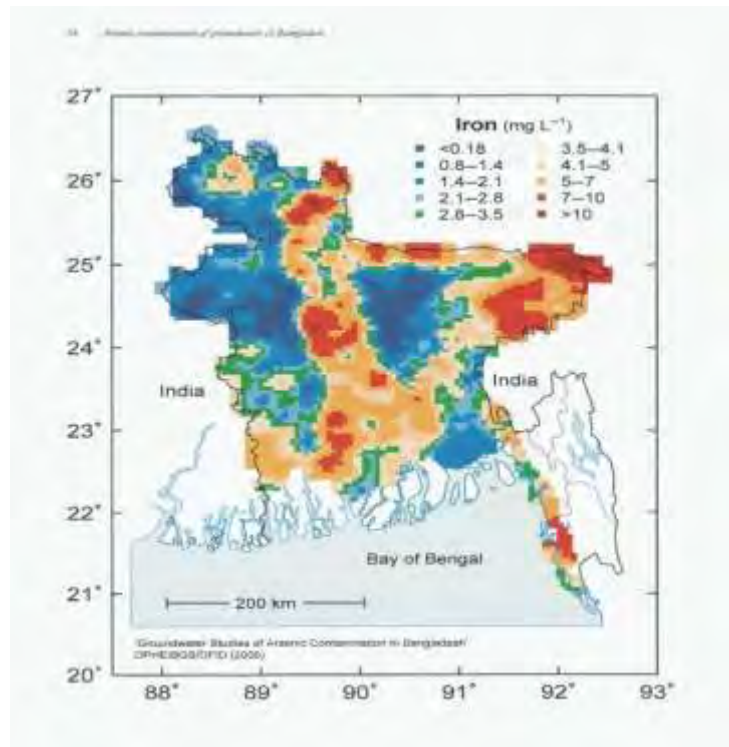
The section provides an overview of the non-food factors which influence iron and anaemia status. There are a few non-food issues which influence the iron and anaemia status in the populations. Among these, the groundwater iron, an emerging environmental phenomenon is observed in Bangladesh to be favourably influencing the iron and anaemia status in population (Merrill et al., 2011; Rahman et al., 2016). The infection is the other important non-food factor influencing the status of iron and other micronutrients which is relevant in Bangladesh and similar settings.

#### 3.2.4.1 GROUNDWATER IRON IN BANGLADESH

Availability of groundwater is plentiful in Bangladesh and the aquifers are highly productive. The sediments are mainly non-indurated and easy to drill by hand, at least to shallow levels. The depth of water tables (i.e. levels) varies across the country but is particularly shallow within ten meters below the ground surface. This made groundwater a natural and easily accessible resource and has led to a rapid expansion in the use of groundwater over the last few decades. Currently, 97% of the population depend on groundwater for potable supplies (DPHE/BGS, 2001).

Groundwater surveys also indicate that the iron is present in high concentrations. Concentrations have been found at up to 61 mg/L (mean: 3 mg/L, median: 1 mg/L). The high values are linked with the anaerobic conditions dominant in the aquifers. The concentration of groundwater iron is high in most of Bangladesh as a result of the predominant presence of reducing conditions in the aquifers. Figure 3 is a map prepared by the Department of Public Health Engineering and the British Geological Survey (DPHE/BGS, 2001) depicting groundwater iron profile of Bangladesh. The concentration is generally high but patchy in the south of the Ganges river and in the north eastern Sylhet regions. The number of wells with a high concentration of iron is particularly high on either side of the Brahmaputra basin with many wells exceeding the 10mg/L mark. Overall, the low concentrations are found in the groundwater from the Barind and Madhupur tracts, deep aquifers of Barisal and the north-western Teesta fan regions. The aquifers of the Barind and Madhupur tracts are from the older Pleistocene era.

Figure 3: Groundwater Iron Concentration in Bangladesh (DPHE/BGS, 2001)



The DPHE/BGS, 2001 further reported that 10% of the country's wells have an iron concentration above 10 mg/L and 23% of the wells have the concentration  $\geq 5$  mg/L. However, according to the Joint FAO/WHO (2004) Expert Committee on Food Additives (JECFA) defined provisional maximum tolerable daily intake (PMTDI) for iron in water, iron contamination limit in drinking water should remain below 2 mg/L to avoid the excess iron storage in the body, and a concentration  $\geq 2$  mg/l is considered "high" level of iron in groundwater (WHO, 1996). A study in a northwestern district of the country showed, 53% of the wells had the iron concentration in water of  $>2$ -22.5 mg/l ("high") and another 18% had the concentration  $>22.5$  mg/l ("very high") (Merrill et al., 2010). By and large, based on the predominant presence of the high and low levels, the country is geographically divided into "predominantly high" and "predominantly low" iron areas (Rahman et al., 2016).

### **3.2.4.2 EFFECT OF IRON CONSUMPTION FROM DRINKING WATER ON IRON STATUS**

A series of studies in Brazil have explored the impact of consuming water fortified with ferrous iron and/or ascorbic acid on iron status. In 1994 Dutra-de-Oliveira et al observed that drinking water fortified with soluble ferrous sulphate resulted in the increase in the haemoglobin and ferritin responses in children 2 to 6 years old. The study by de Almeida et al observed that the interventions - a. ferrous iron and vitamin C and b. vitamin C, provided through drinking water was associated with increased the haemoglobin response in children 12-75 months old (de Almeida et al. 2005). Ferrous iron and vitamin C combined resulted in higher response on haemoglobin than the vitamin C alone; however, the difference between the two intervention outcomes was not statistically significant. Merrill et al (2011) have reported that daily iron intake from groundwater in a rural Bangladeshi district was 42 mg per day in 50% of the studied women. The study further modeled that body stores of iron would be expected to be 0.3 mg/kg of body weight higher for every 10-mg increment in the daily iron intake from water (Merrill et al., 2011). All the above observations point out that groundwater can be a potential source of the environmental iron, especially in Bangladesh setting.

### **3.2.4.3 CONGENITAL HAEMOGLOBIN DISORDERS-THALASSAEMIA**

Thalassaemia, a group of hereditary disorders resulting from genetic mutations involving haemoglobin synthesis, showing a wide range in severity from foetal death to mild anaemia. The hallmark of thalassaemia is the hyperplasia of the erythroid marrow and unproductive erythropoiesis affecting the tetramer structure of haemoglobin (Brock et al., 1994; Marengo-Rowe et al., 2007; Weatherall et al., 2006; Weatherall et al., 2001). This condition can lead to the secondary iron overload, defined as the iron overload which is not the result of direct mutations to proteins involved in iron absorption. Instead, it is a result of

the inefficient erythropoiesis and a higher demand for and absorption of iron (Kattamis et al., 2006; The National Academies Press, 2006). Thalassaemia results in a state of low level of haemoglobin and high iron status. This is confounder in the assessment of haemoglobin and ferritin status in relation to iron supplementation/anaemia control interventions.

### **3.2.5 LOW PREVALENCE OF IDA AMID HIGH PREVALENCE OF ANAEMIA**

As stated earlier that there is a plethora of causes of anaemia—diet and nutritional deficiency, inflammation and infection and congenital haemoglobin disorders (Balaranjan et al., 2011; Tolentino et al., 2007). Iron from the drinking groundwater sources where this is a major source of potable water has emerged as a key determinant of iron status (Merrill et al., 2011, Rahman et al., 2016). However, iron is merely one factor and other factors influence the haemoglobin concentration. On account of these it is often seen that ID or IDA is very low but there is a high prevalence of anaemia. Merrill et al (2011) has shown that in non pregnant women of Bangladesh, the prevalence of ID was zero (due to high amount of iron from drinking groundwater) but the prevalence of anaemia was 57%. The study reported the high prevalence of anaemia attributed to the high burden of congenital haemoglobin disorders in the population. Rahman et al (2016) reported that, ID and IDA were very low in children, 7.9% and 2% respectively, but the anaemia was 33%. Rahman et al further attributed the low intake of other nutrients of the haemopoietic potential such as vitamin A, zinc, B12, folate for high burden of anaemia in Bangladesh population.

Despite all these factors exerting influence on the concentration of haemoglobin, by and large iron remains as one of the prime factors determining the haemoglobin concentration. Earlier, the authors suggested that ID was responsible for 50% causes of anaemia (Stoltzfus et al., 2003); though such a strong attribution of ID is challenged recently (Karakochuk et al., 2017).

On the other hand, the distribution of groundwater iron in Bangladesh or other geological settings is such that, the concentration varies widely. In a predominant high groundwater iron area, there are the tube wells which would contain very low or no iron and the vice versa. Hence, complete avoidance of iron supplementation even in the areas where the population is generally replete with natural iron is untenable and might be counter-productive to some population who do get enough iron from the natural/groundwater sources.

### **3.3 INFECTION AND THE ADJUSTMENT OF IRON STATUS**

#### **3.3.1 INFECTION AND ITS INFLUENCE ON SERUM MICRONUTRIENT/IRON STATUS**

As stated earlier, infection is commonly associated with anaemia and influences iron status. According to a national survey of Bangladesh, the prevalence of childhood diarrhoea and acute respiratory infection (ARI) was 5.6% and 5.4% respectively; and 37% of children aged under- 5, had a fever in the two-weeks preceding the survey (BDHS, 2014). Though, malaria is an important factor for anaemia in many parts of the world, its prevalence is low in Bangladesh. Infection influences the concentration of the micronutrient bio-markers, e.g. ferritin, retinol, serum zinc etc. Biomarkers of nutrition are usually measured by estimating the quantity of a nutrient in blood or other body fluid. Illness impacts directly and indirectly on the level of these biomarkers. Direct effects of illness on nutrient concentrations can result from a decrease in appetite, whereas alteration in immune activity is the indirect effect. During illness, measurements of nutrient concentrations may be misleading and that the effects of disease on the nutritional status of patients could be influenced by both the duration and severity of an illness (Thurnham et al., 2015). After the illness subsides, a period of subclinical inflammation usually follows during which the nutritional biomarkers may continue to be misleading. Thus, in an apparently healthy population, if there is a subclinical inflammation, nutritional biomarkers may not accurately reflect the status. Therefore, to

accurately interpret the micronutrient status, such as iron, zinc, and vitamin A, specific biomarkers of subclinical inflammation must be obtained to quantify the effects of the morbidity on nutritional status (Thurnham et al., 2003; Thurnham et al., 2010).

### **3.3.2 ADJUSTMENT OF FERRITIN BY THE INFECTION BIO-MARKERS**

Ferritin is an acute-phase protein (APP) reactant that is elevated with the concurrent presence of infection or inflammation (Finch et al., 1986; Baynes et al., 1986). The behaviour of ferritin indicates that no single APP (CRP or AGP) can fully reflect the behaviour of nutrient biomarker concentrations during an inflammatory response. Hence, two APP reactants more completely cover the behaviour of ferritin in response to inflammation, and might better suit for adjusting the micronutrient values (i.e. ferritin) for inflammation. Thurnham et al devised a method of correcting plasma ferritin concentrations for the influence of subclinical inflammation by two plasma APP concentrations- CRP and AGP. The 2 APPs are used to define the reference group and 3 inflammation groups (incubation, early convalescence, and late convalescence). The ratios of the respective nutrient concentrations of the reference group to each of the inflammation groups provided the adjustment values (Thurnham et al., 2015; Thurnham et al., 2010). This is a standard method to adjust the micronutrient levels including ferritin for the presence of infection.

## **3.4. MEASURES TO CONTROL IRON DEFICIENCY**

### **3.4.1 DIETARY IMPROVEMENT**

There is a general consensus among the nutritionists and the programme managers, that the sustained consumption of bioavailable iron in food in adequate amounts throughout the life cycle is the most desirable, sustainable, and the safest strategy for the control of the IDA. Influence of food components and meal preparation on the iron bioavailability was identified by the International Nutritional Anaemia Consultative Group (INACG, 1982; Hallberg et al., 1993). The key principles are decreasing the ingestion of inhibitors to iron

absorption and enhancing the intake of the enhancers in a given meal. The strategies include germination of seeds, heat treatment of cereals, fermentation, higher intake of animal source foods, and intake of foods and beverages that contain vitamin C. The intake of the inhibitors of micronutrients can be decreased by reducing the consumption of high-fiber, phytate, and polyphenol-rich foods such as tea, coffee, chocolate etc and isolating the consumption of calcium-rich foods and supplements from iron-rich meals.

### **3.4.2 OTHER STRATEGIES**

There are various methods currently in use for enhancing the level of iron intake. Supplements which are used as capsules, tablets or drops are a conventional intervention for the treatment of severe ID and anaemia in a targeted population. Point-of-use fortification uses micronutrient powders (MNP) containing iron dispensed in the packed, single-dose sachets. The content can be added to the home prepared food to improve its nutritional value. Biofortification employs the breeding of staple food crops to increase the intrinsic content of micronutrients, including iron. Food fortification is an industrial process of addition of micronutrients at the point of manufacture to increase the nutritional content of the particular food. Unlike supplementation, iron fortification at the point of industrial manufacture results in the delivery of small doses of iron in a food vehicle.

Globally, iron supplementation and fortification programmes have been recommended for prevention and control of ID and anaemia in the vulnerable population groups (Zimmermann et al., 2007).

### **3.4.3 EVIDENCE OF ANAEMIA AND ID INTERVENTIONS IN CHILDREN**

#### **3.4.3.1 MICRONUTRIENT POWDER (MNP)**

Home fortification with MNPs containing 5- or 15 micronutrients including iron has been shown to be effective in controlling anaemia and ID in young children (Adu Afawara et

al., 2008). MNPs are single-dose sachets containing the daily required amount (RDA) of multiple vitamins and minerals in powder form that can be sprinkled onto any semi-solid food. The use of MNP for home or point-of-use fortification of complementary foods has been proposed as an intervention for improving micronutrient intakes and anaemia in children under-two. The WHO in a later statement reiterated that in populations where the prevalence of anaemia in children under-2 or under-5 is 20% or higher, point-of-use fortification of complementary foods with the iron-containing MNPs in infants and young children aged 6–23 months should be considered to improve the iron status and reduce anaemia (WHO, 2016, De-Regil 2013). In a prospective cluster randomized trial in low-birth-weight infants aged 6–12 months in Bangladesh, MNP intervention was associated with a reduction of stunting and anaemia and improved language development (Shafique et al., 2014). In Bangladesh, a clinical trial on children 6-23 month reported 4-months of intervention with MNPs resulted in the significant increase of haemoglobin level and a decrease of anaemia prevalence (Mustafa et al., 2016). Another trial in Bangladesh has shown that a flexible regimen of MNP administration was effective in improving the haematological response in children aged 6-24 months (Ip et al., 2009). However, in a large trial in Colombia, the MNP intervention did not result in increased haemoglobin status in children 12 -24 months. The study concluded the difference in the population characteristics from the settings for not having the effect of MNP (Andrews et al., 2016).

#### **3.4.4 WORLD HEALTH ORGANIZATION POLICY TO CONTROL IDA IN YOUNG CHILDREN**

WHO convened an expert consultation which recommended home fortification of complementary foods with MNPs as a measure to improve iron status and reduce anaemia among infants and children 6–23 months of age (WHO, 2008).

The WHO guideline for home fortification with MNPs of foods consumed by infants and children aged 6–23 months is presented in Table 4

Table 4: WHO Guideline of MNP Supplementation for Young Children

Composition per sachet	Iron: 12.5 mg of elemental iron, preferably as encapsulated ferrous fumarate, Vitamin A: 300 µg of retinol,  Zinc: 5 mg of elemental zinc, preferably as zinc gluconate
Frequency	One sachet per day
Duration and time interval between periods of intervention	At minimum, for a period of 2 months, followed by a period of 3–4 months off supplementation, so that use of the MNP is started every 6 months
Target group	Infants and children 6–23 months of age, starting at the same time as weaning foods are introduced into the diet
Settings	Populations where the prevalence of anaemia in children under 2 years or under 5 years of age is 20% or higher

Very recently, the WHO has modified the iron guidelines for the children. The dosing is the elemental from 10-30 mg for the children aged 6 months to 12 years (WHO, 2018).

### 3.4.5 INTERVENTIONS TO CONTROL ID AND ANAEMIA IN CHILDREN IN BANGLADESH

Bangladesh has adopted four complementary policies and strategies that support anaemia prevention and control- the National Strategy for Anaemia Prevention and Control in Bangladesh, 2007, the National Nutrition Policy 2015, and the National Strategy on the Prevention and Control of Micronutrient Deficiencies, Bangladesh 2015-2023, and the National Anaemia Consultation 2016. Children who are moderately or severely anaemic receive treatment through the Integrated Management of Childhood Illness (IMCI) corners placed at the government hospitals and health centres, following the WHO guidelines. Under the MYCNSIA (Maternal and Young Child Nutrition Security Initiative in Asia) activities, supported by UNICEF, the DGHS (Directorate General of Health Service) and the DGFP

(Directorate General of Family Planning) have integrated MNPs with the IYCF (Infant and Young Child Feeding) programme in 16 sub-districts under 7 districts (until 2015), and DGFP is now expanding this effort to 91 sub-districts under 11 districts (National Anaemia Consultation 2016). Under a collaborative partnership of the NNS (National Nutrition Service, MoH), GAIN, and BRAC, the largest programme for the MNP-home fortification has been operating in 160 sub districts which covers one-third of the country. The target population is children aged 6-59 months (personal communication). There was bi-annual deworming for children aged 24-59 months as part of the National Vitamin A-Plus Campaign (NVAC) until 2013; however, presently it is succeeded by the separate month-long campaign using the national immunization platform.

#### **3.4.6 NATIONAL ANAEMIA CONSULTATION, 2016**

National Anaemia Consultation 2016 is the most recent update of the national policy in response to the recent observations that ID is not a major problem in the country and that groundwater iron is a contributor to the good iron status in the population. The policy was developed under the auspices of the NNS, DGHS and the Maternal and Child Health (MCH) Services Unit of the DGFP under the Ministry of Health and Family Welfare (MoHFW) with support from UNICEF and other Development Partners. The consultation reviewed the causes of anaemia and the current and emerging options for anaemia prevention and control programmes.

##### **3.4.6.1 RECOMMENDATIONS FOR YOUNG CHILDREN**

- The national consultation recommended that anaemia prevention and control in the first six months of life should include improving iron stores at birth (e.g., delayed cord clamping), ensuring exclusive breastfeeding, and giving iron for low-birth-weight babies.

- Scale-up complementary feeding promotion for children 6-23 months (adequate amounts and quality of diet), including effective integration of MNP.
- The national anaemia consultation further recommended that additional research was needed to understand the efficacy of MNP for children containing low-dose of iron and document possible side effects of excess iron consumption/supplementation in the high-iron groundwater areas.

### **3.5 ADVERSE EFFECTS OF IRON SUPPLEMENTATION AND GUT MICROBIOTA**

A systematic review reported an association of iron supplementation and the increased risk of diarrhoeal disease (Gera et al., 2002). A WHO consultation, investigating the Pemba study in which the blanket iron supplementation in a malaria endemic area increased the mortality in children (Sazawal et al., 2006), reported that it was unclear whether the risks of iron were specific to malaria or whether they were linked to other infections, including infection from enteric bacteria (WHO., 2006). In 2007, the WHO recommended against universal iron supplementation and fortification in malaria-endemic regions with low quality of health care, taking into consideration the adverse results of the Pemba trial (WHO, 2007). However, the results of a trial in Bangladeshi children showed that MNPs (i.e. containing iron) provided daily for 2 months was efficacious in relation to haemoglobin response; and non-inferior to the placebo for infectious morbidities over a 6-month period (Lamierie et al., 2011).

Nonetheless, the safety of blanket iron provision has been questioned in several large trials (Sazawal et al., 2006; Soofi et al., 2013; Zlotkin et al., 2013). More recent intervention trials have reported that the possible detrimental effects of iron are not limited to a higher risk of malaria and can also result in other adverse effects. It can be affected by administration of iron-containing encapsulated MNPs which is added to foods at the point of use. A large cluster-randomized trial in a non-malarious region of Pakistan showed a significant rise in the

proportion of days with diarrhoea ( $P=0.001$ ), bloody diarrhoea ( $P=0.003$ ) and in-drawing of chest (indicative of lower respiratory tract infection) ( $P=0.03$ ) in children who received an MNP containing encapsulated iron (12.5 mg/d) (Soofi et al., 2013). A trial in Ghana reported no apparent rise in the incidence of malaria but a significant increase in the hospital admissions during the period when sprinkles (containing 12.5 mg of iron) was administered (Zlotkin et al., 2013). Similarly, a study that administered MNPs with a low-iron content (2.5 mg as sodium iron EDTA) to Kenyan infants has reported that infants in the iron group had a significantly higher number of days with a cough and dyspnoea (Barth-Jaeggi et al., 2015). A recent Gambian trial of lipid-based multiple micronutrients (i.e. iron-containing) supplementation (Moore et al., 2012) failed to reduce the frequency of repeat clinic visits; however, on secondary analysis, a rise in the repeat visits in the first 3 weeks of administration was reported in children receiving the supplements (Unger et al., 2017). Stoffel et al in a randomized controlled trial of iron supplementation has shown that, the total incidence of the gastrointestinal side effects that were assessed (epigastric pain/nausea/diarrhea/vomiting) was 40% lower with 100 mg dosing than with 200 mg dosing, however this difference was not statistically significant ( $P=0.105$ ) (Stoffel et al, 2020). A study in Cambodian schoolchildren using 3 different types of micronutrient fortified rice (all the groups containing 8 mg of iron per portion) has revealed that micronutrient-fortified rice was associated with the increased the risk of new hookworm infection (de Gier et al., 2016).

Recent trials have demonstrated that iron supplements adversely affect the human intestinal microbiota which is the basis for many clinical adverse outcomes due to the supplementation. The following two sections describe an overview of the human intestinal microbiota and the adverse consequences on the microbiota as a result of supplementation of iron.

### 3.5.1 GUT MICROBIOTA

Studies have shown that diarrhoea is linked with the intestinal microbiota, which functions to promote the important trophic effects on intestinal epithelia, immune system, and protection of the colonised host against pathogenic microorganisms (Flint et al., 2007; Macfarlane et al., 2012). The healthy gut microbiota is a stable community affected by host metabolism, immunity and environmental factors (Sekirov et al., 2010). Disruptions (e.g. overgrowth of enteric pathogens) of the balance in gut microbiota have been associated with gastrointestinal disorders such as diarrhoea (Sekirov et al., 2010). Oral administration of iron may lead to the disruption of gut microbiota as described below in relation to Figure 4.

Figure 4: Potential Effects of Oral Administration of Iron on Gut Microbiome (FEMS Microbiol Rev 38 (2014) 1202–1234)

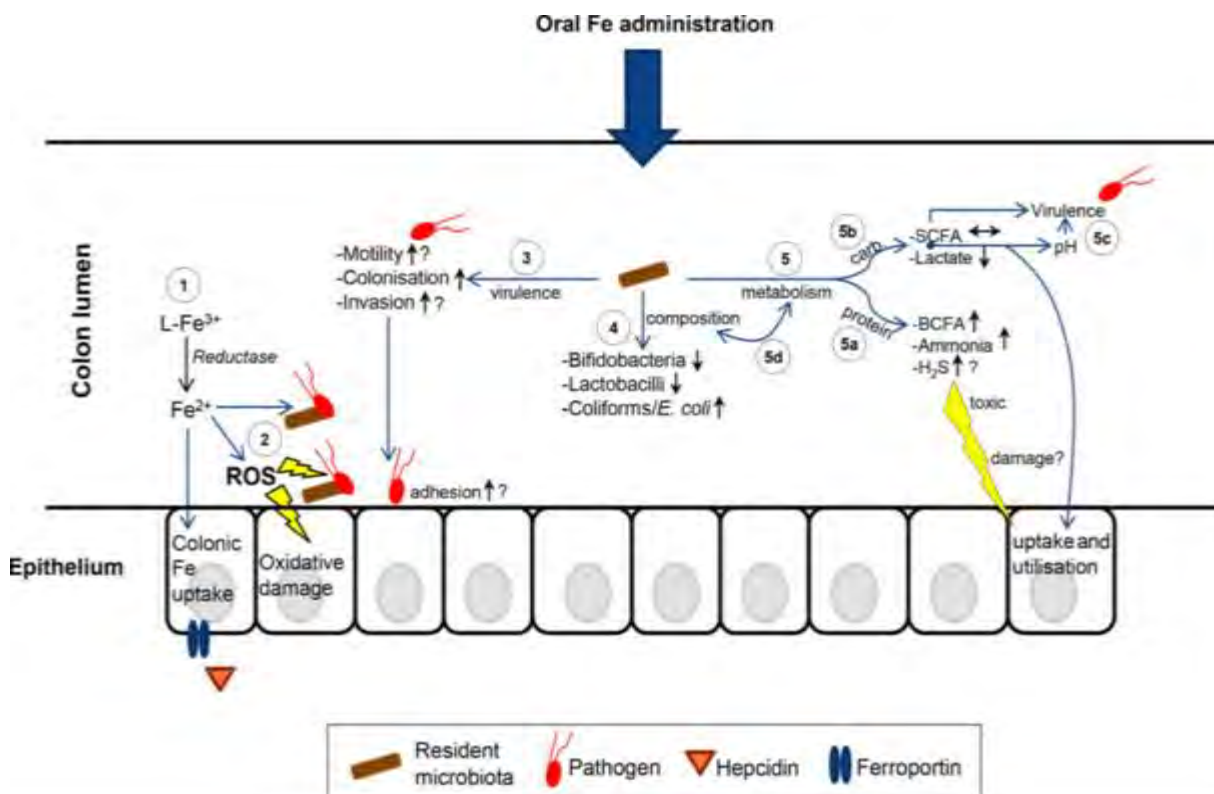


Figure 4: Potential effects of oral iron administration on the microbiota of the non-inflamed colon.

### 3.5.2 ADVERSE EFFECTS OF IRON SUPPLEMENTATION ON GUT MICROBIOTA

Scientific evidence is growing of the iron-associated adverse effects on the intestinal microbiome. Iron is a growth-limiting nutrient for many gut bacteria, which competes for the unabsorbed dietary iron (Andrews et al., 2003). A randomized controlled trial among the Ivory Coast schoolchildren has shown that iron fortification resulted in a potentially pathogenic intestinal microbiota profile (i.e. increased the abundance of pathogenic *Enterobacteriaceae* and a decrease in the beneficial *Lactobacillaceae*), and higher numbers of *Salmonella spp.* in the iron-fortification group (Zimmermann et al., 2010). Similar effects were observed in a recent trial among Kenyan infants where home fortification of MNP resulted in the abundance of pathogenic *E. coli*, decreased the relative abundance of *Bifidobacteriaceae* (the beneficial bacteria) and increased the treatment episodes required for diarrhoea (Jaeggi et al., 2014). For most enteric gram-negative bacteria (e.g. *Salmonella*, *Shigella* or pathogenic *Escherichia coli*), iron acquirement plays a significant role in expressing the virulence and colonization (Naikare et al., 2006). A recent study suggests that in the iron-deficient condition *Bifidobacteria* effectively absorbs iron making it unavailable to enteropathogens, which confers an ecological advantage (Gutierrez et al., 2016).

### 3.6 CONCLUSION

Thus far, the introduction (chapter 2) and this chapter of literature review (chapter 3) provided pertinent description of various factors which are intimately related with iron status and anaemia in children, globally and especially in the settings like Bangladesh. Such as-- the definition, aetiology, consequences of anaemia and iron deficiency; its burden globally and in Bangladesh. Then, the determinants of anaemia and iron status were focused in Bangladesh settings—such as dietary consumption, dietary pattern and sources of the key micronutrients especially iron. Structural/geological issues which are strongly related with iron status and

anaemia in Bangladesh were elaborated. Among these are the issue of groundwater iron, an emerging determinant of anaemia, and quantification of its role in preventing ID. The other structural issue with profound effects on anaemia is the congenital haemoglobin disorders, such as thalassaemia was discussed. The prevention programmes and interventions to control childhood anaemia recommended by the World Health Organization and the Government of Bangladesh was described. Side effects of iron supplementation, such as the diarrhoea, nausea, vomiting; its global and local literature were mentioned. The biological mechanism of the iron induced side effects, such as the disturbances in the composition of gut microbiome was discussed from the global literature.

The introduction and the review of literature addressed the key issues pertaining to Bangladesh, such as high burden of anaemia, low prevalence of ID and its assumed relationship with high amount of iron from the drinking groundwater source, burden of thalassaemia, low coverage of the anaemia prevention programmes (e.g. MNP programmes) and its possible association with side effects clearly opened up the background of the present research. This background deliberation poses the following research questions —

- Is an MNP with a low dose of iron efficacious compared to the standard MNP to prevent childhood anaemia in the background of high content of iron in the drinking groundwater?
- How does the low iron MNP fair compared to the standard MNP in terms of side effects in the background of high content of iron in the drinking groundwater?
- Is the low-iron MNP associated with a favourable effect on the composition of the children's gut microbiota compared to the standard MNP in the background of high content of iron in the drinking groundwater?

## Chapter 4: Methodology

## 4.1 INTRODUCTION

The literature review presented in chapter 3 described the definitions of iron deficiency and anaemia and the factors which influence iron and anaemia status globally and in the Bangladeshi population. It described the context in Bangladesh where the prevalence of anaemia is high despite the low burden of iron deficiency. It further elaborated two key structural issues, intimately linked with iron status and anaemia – groundwater iron and thalassaemia. It identified the interventions to control iron deficiency and anaemia in children and described the iron-associated side effects and its biological mechanism- the disturbance in the composition of the intestinal microbiome. It described the poor coverage of the existing iron supplementation programmes for the children in the form of the standard iron dose micronutrient powder (standard MNP) and documented iron-induced side-effects.

In this background of high childhood anaemia, and low ID with its explicit link with the iron consumed through drinking groundwater, a randomized controlled trial for demonstrating the efficacy of the low iron MNP on haemoglobin outcome and documentation of the side effects was proposed.

To achieve the aim of the project, the thesis is structured into seven studies. The randomized controlled trial consists of two studies. Additionally, there are two supplementary studies in the project, the findings of which complement the findings of the trial. There are three preparatory studies conducted prior to the initiation of the randomized controlled trial.

The chapter 4 provides an overview of the methodological approaches of the various studies. The chapter will outline each study including the study rationale, objectives and a brief description of the methods.

## 4.2 CONTRIBUTION OF THE COLLABORATORS

The funding of the project was awarded by the Nestle Foundation, Switzerland as a Large Research Grant (LRG) in 2018. The project was accomplished through collaboration of Griffith University; Institute of Nutrition and Food Science (INFS), University of Dhaka, Bangladesh; NIZO food research bv, the Netherlands; icddr,b Bangladesh; and Manisha Pharmoplast, Gujarat, India. Griffith University is the host collaborating partner. INFS is chief external partner hosting and supervising the field activities in Bangladesh. icddr,b collaborated with the analysis of blood samples for the measurement of iron and infection status biomarkers. NIZO was involved in the assay of the gut microbiota. Manisha Pharmoplast was involved in the manufacturing of the research grade micronutrient powders (MNPs) as per the prescribed composition.

The field work of the research—the trial and the preparatory and the supplementary studies were operated by a team of 17 staff members, led by the site PI- Sabuktagin Rahman (the Ph.D. candidate). Of the staff members- one was the field coordinator who was responsible for day-to-day coordination, management (administrative and financial) and assignment of tasks for all the studies. There was one field medical technologist who was responsible for blood sample collection and initial processing and preparing prior to dispatch to the laboratory in Dhaka through maintenance of a cold chain. There were three field attendants whose responsibilities were measurement of the water parameters, collection of the children's stool sample from their residence and transporting all the biological samples to the laboratory in Dhaka. There was one data management assistant responsible for data entry and cleaning. There was a strong team of 10 staff members engaged in data collection for the trial and the sub studies.

As the site-PI and as the Ph.D. candidate I spearheaded the research activities. I was involved in selection of the study site, procurement MNPs and logistics, establishing field

office and field laboratory, recruitment of field staff members, conduction of staff trainings, study design and study plan, supervision of data collection, managing the logistics including organising the transfer of biological samples to different laboratories, data analysis plan and analysis and writing the papers. The composition of the field team is provided in the annex 12.

#### **4.3 ETHICAL CONSIDERATIONS AND ETHICS APPROVAL**

The trial was conducted conforming to standard ethical practices. Before enrolment the parents of the eligible children were briefed about the study. They were informed on the kind of information which would be collected, about collection of biological samples and how the samples will be collected by a trained medical technologist; how the pain/discomfort will be managed. They were informed about the free distribution of MNPs and the way the nutrients are fed to the children. They were informed about weekly monitoring of MNP intake data and health checks of their children and free medical services and medicines, should the child experiences any health issue. They were informed about the confidentiality of the data and how that would be kept in a secured place. They were assured that the participation to the trial is voluntary and they are entitled to withdraw from the study at any time during the study. Data collection was conducted only after receiving the signed written informed consent of the parents of the children. One copy of the consent was provided to the parents.

The trial received ethical approval from the Faculty of Biological Science, the University of Dhaka, Bangladesh (Ref# 46 /Biol. Scs. /2017-2018), and the Griffith University Human Ethics Committee, Australia (Ref# 2017/467). The trial was registered with the International Standard Randomized Controlled Trial Register, number ISRCTN60058115.

Trial Preparation: Preparatory Studies [1-3]

#### **4.4 PREPARATORY STUDY I**

Development and standardization of taste-rating of groundwater sample as a tool for a semi-quantitative assessment of iron level in groundwater.

##### **4.4.1 HOW DID THIS STUDY RELATE TO THE MAIN TRIAL?**

The taste-rating tool was developed for a rapid screening of the tube-wells for iron content in groundwater. The tool was used for the site selection.

Further, the main trial required to identify the children with the stipulated age, who drink water from tube-wells with a specified concentration of iron ( $\geq 2$  mg/L). The tube-wells were identified and selected for the trial by measuring the concentration of iron in the water samples by an iron test kit device (HI 3834, Hanna Instruments, USA). However, prior to this measurement, the taste-rating tool was used on the water samples for the level of iron to have an initial idea of the iron content. The idea to develop the taste-rating tool emanated from an earlier small pilot study that reported the taste-rating of the water sample potentially could develop a tool for the semi-quantitative assessment of iron in the water (Rahman et al, 2018). The development of the taste-rating tool and its application had the logistical advantage as the usage of it saved a considerable amount of reagents which is expensive, imported and not readily available.

##### **4.4.2 METHODS**

This sub-study developed and standardized the methods of taste-rating.

##### **4.4.3 STUDY AREA**

The study was conducted in two sub-districts of rural Bangladesh- Belkuchi (24.2917°N 89.7000°E) and Pirganj (25°51.30 N 88°220 E), situated in the north-west and

northern part of Bangladesh respectively. As per the report of the DPHE/BGS, 2001 on the geographical profile and geochemistry of Bangladesh groundwater, Belkuchi and Pirganj represented a predominantly high- and a predominantly low-groundwater-iron area respectively.

#### **4.4.4 PROCEDURE**

The tube wells were selected at random from 8 and 3 villages of Belkuchi and Pirganj respectively. After obtaining the consent of the household head, two external assessors undertook the assessment of the well-water samples separately and recorded their taste-ratings to one of the following categories- (a) no iron, (b) some iron, and (c) heavy iron. Then the assessors asked the respondent of the households, how they perceive the taste for iron of the water of the tube-wells used by them for drinking. The respondents rated their taste-experiences to one of the above categories. This sequence of the taste- assessment was followed, so that the external assessors could avoid the potential information bias for their ratings from the respondents. The proportional magnitude of different taste-ratings and concentration of iron in groundwater by the taste-ratings was studied in both study areas. The association of the taste-ratings of each water sample and its concentration of iron was studied by Spearman rank correlation; and the agreement of the taste-ratings between the various raters were studied in the high groundwater iron area by the Kendall's tau b and Lin's concordance correlation.

The details are presented in the annex 1.

## **4.5 PREPARATORY STUDY 2**

Validation of an interviewer administered Seven-day Semi Quantitative Food Frequency Questionnaire for dietary assessment of young children in rural Bangladesh

### **4.5.1 HOW DID THIS STUDY RELATE TO THE MAIN TRIAL?**

Dietary intake of the children participating in the trial was assessed by an interviewer-administered seven-day, open-ended, semi-quantitative food frequency questionnaire (7-day SQFFQ). Since, dietary intake of some key nutrients, especially iron was important to estimate in the main trial, the tool was required to validate.

### **4.5.2 METHODS: PARTICIPANTS, STUDY SITE AND PROCEDURE**

This study was conducted on 105 children, aged 24-59 months, recruited from 103 households in Belkuchi, a rural sub-district (Belkuchi) in a north-central district of Bangladesh. The children were different from the children who participated in the trial.

The food intake of the children was measured by interviewing the mothers or caregivers, using a seven-day SQFFQ which was adopted from a national survey (National Micronutrient Survey, 2011-12) and a study in Bangladesh. The validity of the nutrient intake measured by the SQFFQ was assessed by comparing to the average intake of the two 24-hour dietary recalls (24-hour DRs) as the reference method, administered on non-consecutive weekdays. The interval between the two 24-hour DRs was  $\geq 1$  week to  $\leq 2$  weeks.

The intakes recorded by the SQFFQ and the average of the 24-hour recalls were compared by standard statistical tests used in the dietary validation studies. Such as, the Spearman Rank correlation, percent difference, cross-quartile assessment, Kappa estimates, Lin's Concordance Agreement and the Bland-Altman Agreements. Details are presented in the annex 2.

#### **4.6 PREPARATORY STUDY 3**

Temporal effect on Iron concentration in the Expressed Groundwater Samples in Bangladesh:  
Potential Implication for Iron-status in Population

##### **4.6.1 HOW DID THIS STUDY RELATE TO THE MAIN TRIAL?**

The iron in groundwater is present in a soluble ferrous state and if consumed immediately or soon after the extraction off the tube well, might not be oxidized by air and thus will be consumed and absorbed in the body. On the other hand, if groundwater after extraction from the well are not consumed immediately and stored in containers for drinking later, it might lose the iron content as the precipitation induced by the ferric iron (oxidized), rendering the prospect of getting iron from groundwater minimal. The main trial required to measure the total intake of iron from various sources, including the drinking groundwater. Therefore, this study was conducted to account for the possible adjustment of iron intake from groundwater in the trial participants in case there was the consumption of the stored water.

##### **4.6.2 METHODS**

The study was undertaken to assess the temporal concentration of iron of the expressed groundwater samples at specified intervals after pumping off the tube wells for up to 6 hours. A portable colorimeter was used to measure the iron concentrations at the specified time intervals—0.0 hrs, 0.5 hrs, 1.0 hr, 2.0 hrs, 3.0 hrs and 6.0 hrs. Mixed-effect multilevel modeling was done to determine the temporal concentrations of the groundwater samples after adjusting for the fixed effects-- area, level of the baseline iron level, pH, temperature and the oxidation-reduction potential of the water samples. Details are presented in the annex 3.

## **4.7 THE SITE SELECTION AND THE MAIN TRIAL [RCT: STUDY 1 AND STUDY 2]**

Efficacy of the Micronutrient Powder (MNP) with a low-dose of iron on selective iron biomarkers, and its effects on morbidities in children of rural Bangladesh drinking from groundwater with a high level of iron: a Randomized Controlled Trial

The main trial (RCT) and the associated nested studies of the project required a scoping assessment for site selection. Before discussing the methods of the main trial, a description on site selection is provided as the following.

### **4.7.1 STUDY SITE SELECTION**

In Bangladesh, iron concentration in groundwater varies between areas and between the tube wells in an area. Even in a predominantly high groundwater iron area, there are tube-wells which contain a low level of iron in groundwater and the vice versa. The present randomized controlled trial required a large number of tube-wells with iron concentration at a specified level. Furthermore, these wells needed to be concentrated in a geographical area which is manageable in terms of field-operations and logistics. Other factors considered included— the distance of the study site from the laboratories (in the capital city, Dhaka); the transportation facilities and the state of local transportation; the presence of preexisting infrastructure; the presence of preexisting MNP programmes in the area; and natural calamities especially the risk of exposure to seasonal flooding.

The site selection activities were conducted in two northern districts (Dinajpur & Rangpur), and in a north-western district (Sirajganj). The initial choice of the districts was guided by the British Geological Survey, 2001 and the data from the Rangpur Dinajpur Nutrition Study (RDNS) project (personal communication).

### **4.7.2 OBJECTIVES**

1. To select the site for the trial

2. To identify an “optimum” way of selection of the study children and tube wells

#### 4.7.3 METHODS

The site selection activity was conducted in 13 villages of six sub-districts, namely Chirirbandar and Badarganj (of Dinajpur and Rangpur district respectively), Sirajganj sadar, Belkuchi, Kamarkhanda and Ullahpara (Sirajganj district). Trained field assistants measured the concentration of iron in the tube well waters using commercially available “test-kit” device with reagents (Hach kit, IR 10, Hach USA). The device was a colorimetric test kit using the phenanthroline method to determine concentration of iron in groundwater at the high range (0-10 mg/L). The availability of the high iron ( $\geq 2$  mg/l; Merrill 2011) tube wells and the children aged under-five were explored. Further, the study assessed the optimum strategy for identification of the children and the tube wells. The strategies assessed were:

**A. Tube-well-to-child strategy:** First, the tube wells containing high iron in water were identified by feedback of the local people. Then the water from each of the identified tube well was tested by the colorimeter for iron content. Enquiries were made to determine whether the household had any children under the age of 5 years.

Proportion of the eligible child-tubewell pair =  $[\text{No. of children } < 5 \text{ years} \div \text{No. of tube-wells measured for iron}] \times 100$

**B. Child-to-tube well strategy:** First, the households with children under five years of age were identified with the help of local people. Then the tube wells used by those households for drinking water were measured for concentration of iron.

Proportion of the eligible child-tubewell pair =  $[\text{No. of cases with stipulated iron level in wells } (\geq 2 \text{ mg/l}) \div \text{No. of children } < 5 \text{ years}] \times 100$

In Dinajpur and Rangpur districts, both the approaches were attempted. It was revealed that the “child-to-tube well” strategy was more efficient in detecting the eligible child- tube well pair; and therefore subsequently applied throughout the assessment.

The studied sub districts were graded such as “~~v~~ery high”, “~~h~~igh”, “~~m~~oderate”, and “~~l~~ow” depending on relative magnitude of the favourable status of the particular assessment parameters and ranked accordingly through the assessment of all the parameters. The response to various parameters was reported as percentage. The highest ranked sub district was selected for the trial.

#### **4.7.4 PROCESS OF THE SELECTION AND DISCUSSION**

##### **A. The tube-well to child approach**

The results (not presented) showed that only one-fifth (21.4%) of the iron measurements were turning out to be the eligible study cases, i.e. child-tube-well pair. This method incurred significant loss of logistics. Hence, the “~~t~~ube-well to child” approach was abandoned.

##### **B. Child to tube-well approach**

The results (not presented) showed that the efficiency of detection of the eligible child-tubewell pair was increased from 21.4% (in case of tubewell-to-child strategy) to 45.4% in Chirirbandar. The same approach when applied in Sirajganj district- the detection efficiency was 75%, 78.6% and 93.9% in Sirajganj sadar, Ullahpara and Belkuchi sub districts respectively.

The estimates of concentration of iron in the groundwater samples revealed that all the studied sub districts represented the areas with predominantly high level of iron in groundwater. Among the areas Sirajganj sadar, Ullahpara and Belkuchi had higher levels of iron, with the latter measured the highest concentration (results not shown).

Table 5: Comparison of the Sub districts on the Eligibility Parameters

Traits	Sub-districts			
	Belkuchi (Sirajganj)	Ullahpara (Sirajganj)	Sirajganj sadar (Sirajganj)	Chirirbandar (Dinajpur)
Efficiency of high Fe wells	93.9% ( Very high)	78.6% (High)	75% (High)	45.4% (Low)
Availability of children of stipulated age	Very high	Moderate	High	Low
No. of potential areas visited (Union/Mouza/Villages)	Four (Somospur, Tamai, Pourasobha, Ambaria)	Three (Hatikumrul, Ponchokroshi, Borahor)	Three (Khokshabari, Soidabad, Songacha)	Two (Kochna, Purbo Saintara)
Flood vulnerability	Moderate (Many of the areas were free of risk)	Low	High (Situated on the bank of the river Jamuna)	Low
Local transport	Ample (compressed natural gas-driven taxi, motorized-van, easy bike), Safe, avoids using busy highways	Ample (compressed natural gas-driven taxi, motorized-van, easy bike), however, needs to use busy highways for field movement	Ample (compressed natural gas-driven taxi, motorized-van, easy bike), Safe, avoids using busy highways	Ample (Easy bike, van )
Distance of the sites	All sites are neighboring unions, close and compact.	Moderate distance	Considerable distance between the sites	Considerable distance between the sites and field office
Local people	Medium SES, depends on agriculture, cloth weaving	SES wise more well off, depends on cloth weaving and animal farming	Poorer SES, many are flood refugee.	SES wise more well off, depends on agriculture
Transportation of samples to Dhaka	Very good facilities. Direct bus services (from Belkuchi to Dhaka takes 3-4 hrs). Train takes 3-3.5 hrs.	Medium facilities. First needs to come by CNG taxi to Sirajganj sadar or Belkuchi for the connecting Bus/train to Dhaka. By bus the required time is (3.5-4.5 hrs), by train (3.5-4 hrs)	Good facilities. Direct bus from Sirajganj sadar to Dhaka. For train, first needs to come by CNG taxi/van to near belkuchi. By bus, the time required is 3-4 hrs and by train 3-3.5 hrs.	Inefficient. By bus & train. Bus will take 10-12 hrs. Train will take the similar amount of time or more as trains are often delayed.
Presence of local MNP programmes	BRAC closed its nationwide MNP programme in June 2018. Local people report presently there are no visits by the BRAC's field	-do-	-do-	-do-

	volunteers.			
Local facilities/ infrastructure	Needs to setup field office -cum -staff residence at a convenient point in Belkuchi.	Needs to setup field office-cum-staff residence at a convenient point in Ullahpara.	Needs to setup field office-cum -staff residence at a convenient point in Sirajganj sadar.	RDNS field office at Parbatipur can provide sample storage facility in -20 degree freezers.
Rental charge of field office (3-4 rooms accommodation which can be used as field office-cum-residence of the field staff)	BDT. 8-10K/month	BDT. 10-12 K/month	BDT. 12-15 K/month	BDT. 8-10 K/month
Ranking of recommendations	1 <sup>st</sup>	2 <sup>nd</sup>	3 <sup>rd</sup>	4 <sup>th</sup>

Table 5 provides comparative favourable and unfavourable issues across the sub districts. As per the findings, Badarganj (Rangpur) was not considered for the comparison as it had a predominantly low concentration of iron which did not meet the requirement of the trial (Results not shown). Chirirbandar (Dinajpur) had a few positive factors, such as the presence of infrastructure at the RDNS field office for initial storage of biological sample, good local transport and low flood vulnerability. However, the drawbacks were- a low availability of the study children, a lower availability of the high-iron tube wells and the long distance from Dhaka with around 10 hours travel time, which might pose the difficulties of transporting the samples to the laboratory in Dhaka. Sirajganj sadar had several positive factors, such as the short travel time to Dhaka (~4 hours) and high rate of availability of the high-iron wells. The flip sides were- high vulnerability to flooding as mostly the sites were situated on the bank of the river Jamuna, and a flooding might have caused displacement of many people. Ullahpara had several advantages, such as a low vulnerability to floodings, availability of high-iron wells, a shorter transport time than Chirirbandar. However, the

problem was a relatively low availability of the stipulated age group of children. Belkuchi had advantages on almost all the key issues considered (Table 5). It had the highest rate of the efficiency of the high iron wells, high availability of the under five children, an excellent local transport facility and a short time of transportation to Dhaka. Belkuchi sub district as a whole had a moderate vulnerability to flooding. However, the areas considered for the study (i.e. unions) according to the local residents and local NGO workers were not prone to flooding. The areas are located on the west bank of the embankment and thus protected. Based on the above observations, Belkuchi sub district of Sirajganj appeared as the most suitable to conduct the trial, and therefore was selected.

#### **4.7.5 THE MAIN TRIAL [RCT: STUDY 1]**

To achieve the three primary objectives of this research, a randomized controlled trial was conducted examining the effect of the low-iron MNP relative to the standard MNP on haemoglobin level and to assess the comparative iron-induced side effects in 2-5 years old Bangladeshi children whose drinking water contains a high concentration of natural iron ( $\geq 2$  mg/L). Since, the iron-induced side effects are caused by the changes in the composition of gut microbiota, on a subsample the microbiota profile was compared between the MNP treatment groups (The method of that study is described in the section 4.7.6.2).

##### **4.7.5.1 METHODS**

###### **4.7.5.1.1 MNP FORMULATIONS**

The composition of the MNPs is shown in Table 6. The composition of the standard MNP, containing 12.5 mg iron, was the same as of the Ministry of Health, Government of Bangladesh recommended formulation. For the low iron MNP formulation, the iron dose was reduced to 5 mg while the doses of vitamin A, zinc, folate and vitamin C remained the same as of the standard formulation.

The MNP formulations were manufactured by Manisha Pharmoplast (Gujarat, India), a regionally acclaimed manufacturer of nutritious products. The due diligence was

maintained to ensure that the products passed the stability tests. The nutrient composition of the MNP was cross-checked by an independent laboratory. MNPs were packaged in sachets identical in appearance except the group code marks imprinted indicating different formulations. Each child received 7 MNP sachets/week for 60 days (i.e. 60 MNP sachets in 2 months). The mothers were instructed on how to feed the nutrients. The dosing schedule is compatible with the guidance of the Home Fortification Technical Advisory Group (HFTAG, 2015).

Table 6: Composition of the MNP Formulations

Micronutrients	Standard MNP	Low iron MNP
Iron (microencapsulated ferrous fumarate)	12.5 mg	2.5 mg
Vitamin A (Retinol acetate)	300 µg RE	300 µg RE
Zinc (gluconate)	5 mg	5 mg
Vitamin C (ascorbic acid)	30 mg	30 mg
Folic acid	0.15 mg	0.15 mg

#### 4.7.5.1.2 PROCEDURE

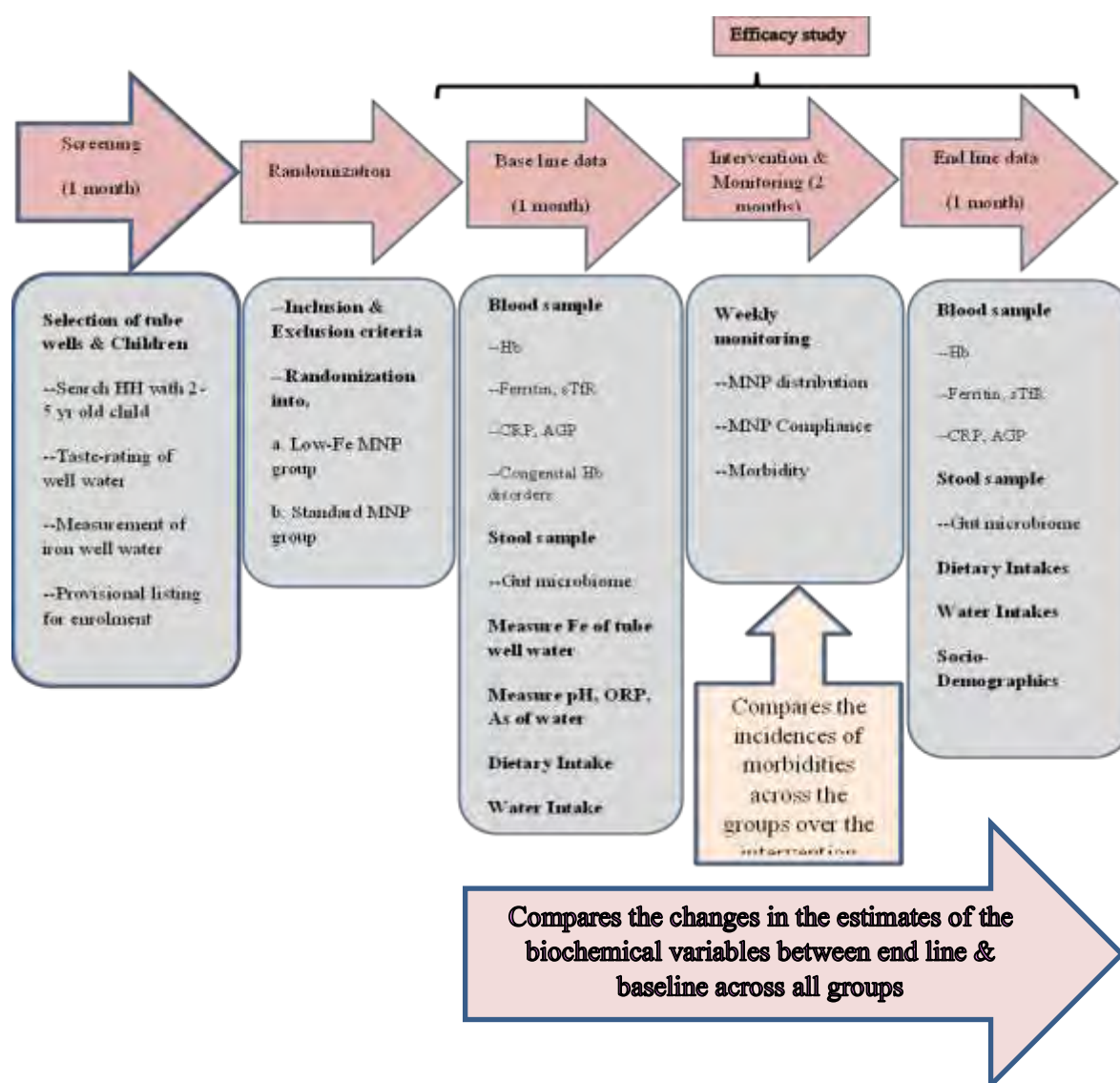
A randomized controlled trial was employed where the enrolled children were randomized to receive any of the two MNP formulations: low iron MNP containing 5 mg iron (i.e., experimental group) and the standard MNP containing 12.5 mg iron. Before enrollment, screening was conducted in three unions (the lowest administrative unit in Bangladesh, consisting of a cluster of villages)—Belkuchi Pourashava, Bhangabari and Daulatpur—of the Belkuchi sub-district to identify the children (2–5 years old), who use the “high-iron” wells ( $\geq 2$  mg/L) for drinking water. During the screening, 436 children from 8 villages were listed as potential participants. The exclusion criteria were children receiving MNPs/iron supplements and/or antibiotics in the preceding two months (Jaeggi et al., 2015); the presence of chronic, congenital debilitating illnesses; and the guardian’s unwillingness to participate.

The child with severe anaemia ( $Hb < 7$  g/l) were excluded from the study for referral to health clinics for treatment of anaemia.

At the time of enrollment (approximately 2 months after the screening) for the trial, 83 children were excluded as they failed to meet the selection criteria. Additionally, 10 children did not show up and another 16 children refused to take part in the trial. Hence, a total of 327 children were enrolled in the trial after obtaining the written informed consent.

Figure 5 shows a diagram of the design of the trial.

Figure 5: The Design of the Trial



#### **4.7.5.1.3 DATA COLLECTION**

Data were collected on—socio-demographics, child morbidities, dietary and water intake assessments. Socio-economic variables included household head's occupation, mother's education, spends on purchasing food, household food insecurity, ownership of assets. Dietary intake of the children was assessed by an interviewer-administered seven-day semi-quantitative food frequency questionnaire (SQFFQ), consisting of a list of commonly consumed local foods. Blood samples were collected from sub-samples for assessing haemoglobin and the iron status parameters. Iron and arsenic levels of the drinking water were assessed (Figure 5).

#### **4.7.5.1.4 STATISTICAL ANALYSIS**

Treatment effect of the low-iron MNP relative to the standard MNP was estimated by general linear modeling (GLM) with adjustment of the baseline values of the pertinent variables—such as age, sex, household food insecurity, household expenses on food, dietary iron, iron concentration of the drinking water, morbidities, status of the thalassaemia carrier state and the baseline serum iron status. Incidence of the morbidities between the MNP treatment groups were compared by calculating the incidence rate ratio (IRR) by using the poisson regression.

Details are given in the section 5.2.1.3

#### **4.7.6 SECOND STUDY UNDER THE MAIN TRIAL [RCT: STUDY 2]**

##### **4.7.6.1 HOW DID THE STUDY RELATE TO THE MAIN TRIAL?**

It is envisaged that the iron-induced clinical side effects are linked with the composition of the gut micorbiota which is disturbed by excess of unabsorbed iron in the distal large intestines (Jaeggi et al., 2015). In Bangladesh, the possibility of iron buildup in the intestines of the children might be accentuated because of the high iron content in the drinking groundwater. On top of this, the children might potentially consume MNP supplements under the policy supported blanket programming to alleviate anaemia. Hence, the present subanalysis of the main trial was planned to examining the comparative effect of the low iron MNP and standard MNP on the composition of the gut micorbiota.

##### **4.7.6.2 METHODS**

On a subsample, the comparative effect of the treatments (standard MNP vs. low iron MNP) on the composition of the gut microbiota was examined. For the gut microbiota study, of the 327 enrolled children, a pool of 100 children was randomly choosen. The sample size requirement for the microbiota study was 50 children in the two treatment groups ( $25 \times 2 = 50$ ). The additional children were considered to buffer for excluding the children consuming MNP fewer than a priori cut-off ( $< 50$  sachets) and/or probable intake of antibiotics over the intervention time. After the intervention, 53 children (26 in the standard MNP and 27 in the low iron MNP groups) were found to be consumed  $\geq 50$  sachets of MNPs and have not taken antibiotics. Paired (baseline and endpoint) assessment of the 53 samples (i.e.stool samples), hence a total of 106 samples ( $53 \times 2$ ) was conducted for the gut microbiota study by analysing the 16sRNA amplicon.

#### **4.7.6.3 STATISTICAL ANALYSIS**

The Mann–Whitney U test with FDR correction for multiple testing was applied to assess differences of the microbiota composition between the two groups. For longitudinal analysis, the change of taxon relative abundance over time, 2log ratios were calculated, in which the relative abundance of a taxon at endpoint was divided by the relative abundance of the same taxon at baseline.

Details are given in the section 5.2.2.3

#### **4.8 SUB-STUDY COMPLEMENTING THE MAIN TRIAL (SUPPLEMENTARY STUDY 1)**

Thalassaemia carrier status and groundwater iron: Implication for iron supplementation programme for children in Bangladesh

##### **4.8.1 HOW DID THIS STUDY RELATE TO THE RESEARCH PROJECT?**

Thalassaemia, a hereditary disease of haemoglobin synthesis is characterised by a high proportion of atypical haemoglobin with a premature breakdown of red blood cells. This typically results in low haemoglobin and a high ferritin level in the carrier population. On this biological background, and in the prevalent practice of drinking of iron-rich groundwater, there is an impetus to examine the effect of iron supplements (MNPs) on iron and haemoglobin status in this population. This would help to indicate the optimum strategy/dosing of iron supplements in the programme setup which might be beneficial to the thalassaemia carriers.

##### **4.8.2 METHODS**

There were roughly 13% of the children diagnosed with the thalassaemia carrier state among the participants of the RCT. Ferritin and haemoglobin status and intake of iron from the various sources-- dietary, groundwater and the MNPs were compared at baseline and

endpoint between the thalassaemia carrier and the non-carrier groups. Key morbidities were compared. Thalassaemia was screened by capillary zone electrophoresis of Hb at pH 9.4 (Capillary 2 system; Sebia, Evry, France). The concentration of iron in groundwater was measured by a hand-held portable colorimeter (HI-721; Hanna Instruments, USA) at baseline. Ferritin was measured by an automated immunoassay analyzer (Cobas C311; Roche Diagnostics, Mannheim, Germany). Ferritin was adjusted for infection by Thurnham's principle (Thurnham et al., 2010).

#### **4.8.3 STATISTICAL ANALYSIS**

Ferritin levels were log-transformed and back-transformed to report the geometric mean of ferritin sorted by the MNP groups and between baseline and end-point. The statistical significance for the difference in ferritin between the groups and between the study points was tested by the Mann Whitney test. For all analyses, the p-value <0.05 was considered statistically significant. Details are presented in the section 5.2.3.1.1

## **4.9 SUB-STUDY COMPLEMENTING THE MAIN TRIAL (SUPPLEMENTARY STUDY 2)**

Intake of Iron in Low-iron Groundwater Settings in 2-5 Year Old Rural Bangladeshi Children: the effect of the Low-iron Micronutrient Powder (MNP) on Prevention of Anaemia in Bangladesh

### **4.9.1 HOW DID THIS STUDY RELATE TO THE RESEARCH PROJECT?**

The findings of the RCT revealed that the low iron MNP was as efficacious as the standard MNP in preventing low haemoglobin concentration and incurred lower incidence of the key iron-induced side effects. The findings recommended the low-iron MNP for controlling childhood anaemia in the predominantly high iron groundwater areas. However, in Bangladesh the iron content in tube wells vary widely even in the adjacent wells and the country is densely populated. Operationally, it is infeasible to run two different doses of MNP in the programme setting. Hence, in this study, the prospect of the low-iron MNP was explored regarding prevention of anaemia in children drinking from the low-iron tube wells (<2 mg/L).

### **4.9.2 METHODS**

A cross-sectional study was conducted to assess the intake of iron from the principal sources in children drinking from the wells with a low concentration of iron (0-<2 mg/L) -- such as the groundwater iron, dietary iron and the hypothetical intake of low iron MNP. The intakes of iron (actual and bioavailable) were compared to the dietary reference intakes and triangulated with the mean haemoglobin level of children.

The study subjects were 105 children aged 2-5 years residing in Belkuchi and who were not the trial participants. The children were selected from a household if the groundwater iron concentration of the tube wells they drink from was low, i.e. 0-<2 mg/L. Low concentration (<2 mg/L) is defined as per the cut-off recommended by the joint

technical committee of the Food and Agricultural Organisation and World Health Organisation FAO/WHO (FAO/WHO, 2004). Intake of dietary iron was measured by a 24-hour recall by asking the mother, the foods the child took over the preceding 24 hours. The concentration of iron in the drinking water sample was measured by a colorimetric test kit device (Hanna 3831, Hanna Instruments, USA). Intake of iron was calculated by multiplying the concentration of iron in the groundwater sample and the volume of intake of water over the 24-hours preceding the survey. The haemoglobin concentration of the children was measured on venous blood samples by a photometer (Hemocue 301, Hemocue AB, Angleholm Sweden).

#### **4.9.3 STATISTICAL ANALYSIS**

Intake of iron was estimated as mean  $\pm$ SD and median (interquartile range). Group difference of the intakes was assessed by the Mann-Whitney tests with p-values < 0.05 considered significant. Spearman rank correlation coefficient ( $\rho$ ) was computed to assess the association of haemoglobin concentration and a) the groundwater iron concentration, b) intake of water iron, and c) dietary iron. Details are presented in the section 5.2.4.1.1

#### **4.10 CONCLUSION**

This chapter (chapter 4) outlined the research methodology of this thesis. First, to answer the research questions identified on the basis of the introduction (chapter 2) and review of literature (chapter 3) the main study was conducted (RCT: study 1 and study 2). To prepare for the RCT three preparatory studies were conducted (preparatory studies: annexes 1, 2, 3). Furthermore, to complement the findings of the RCT, two supplementary studies were done (Supplementary study 1, 2).

For each of the studies a brief description was made on rationale and relation with the RCT. Study design, procedure, data collection, statistical methods were described.

The following chapter (chapter 5) presents findings of the trial and the two supplementary studies. The findings of the three preparatory studies are given as annexes 1-3. Of the seven manuscripts four were published in journals and the other three were submitted. The studies are presented in journal article format. Each manuscript has been written in accordance with specific requirement of the journal, including referencing style and spelling. Before each manuscript, an overview is provided highlighting the publication status, bibliographic details (in case of published papers) and a brief introduction to the manuscript.

## Chapter 5: Results

## 5.1 INTRODUCTION

The key methods pertaining to the trial and various sub studies were described in the previous chapter. The chapter 5 presents the results of the trial and the supplementary studies. The results of the preparatory studies are provided in annexes 1-3. Essentially this chapter and the annexes 1-3 consists of the published and submitted/under review studies belonging to the project.

First, the findings of the Randomized Controlled Trial (RCT) are presented. The first study (RCT Study 1), examined the effect of low-iron MNP on haemoglobin outcome. In RCT Study 2, the trial presents the results of the effect of the low iron MNP on the composition of gut microbiome.

Finally, the findings of the two supplementary studies will be presented. The first study presents the results of the effects of thalassaemia on iron and haemoglobin status of the children who are concurrently exposed to a high amount of iron from drinking groundwater and MNP supplementation.

The second supplementary study assesses the scope of low-iron MNP for prevention of anaemia in children exposed to a low level of iron from drinking groundwater.

Since, the published/submitted studies are inserted in entirety, specific discussions pertaining to the results of the studies will accompany, highlighting the explanations of the results.

## **5.2.1 FINDINGS OF RCT**

### **5.2.1.1 OVERVIEW OF THE MAIN PAPER [RCT: STUDY 1]**

Title: Effect of Micronutrient Powder (MNP) with a Low-Dose of Iron on Hemoglobin and Iron Biomarkers, and Its Effect on Morbidities in Rural Bangladeshi Children Drinking Groundwater with a High-Level of Iron: A Randomized Controlled Trial

Status: Published

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### **5.2.1.2 INTRODUCTION**

Childhood anaemia is one of the key public health problems in Bangladesh and exists at the prevalence (33-51%). This magnitude indicates a public health concern. However, the incidence of ID, the most common cause of anaemia globally, is surprisingly low in the country. This is because people ingest wholesome iron from groundwater, which is the dominant source of drinking water in the population. To control the high burden of childhood anaemia, the national policy supports the blanket supplementation of an MNP programme which contains iron with the standard dose (12.5 mg). But, as ID is low at the population level, such a dose of supplement is unnecessary in many children and is possibly associated with iron-induced side effects and poor compliance and coverage of the programme. Therefore, a policy appraisal (National Anaemia Consultation 2016), advocated for research with MNP with a low dose of iron. Hence, the present trial was conducted to examine the

effect of a low-iron MNP (5 mg) on anaemia outcomes and to document the iron-induced side effects. A total of 327 children who were drinking from *high-iron* wells ( $\geq 2$  mg/L), received either standard (12.5 mg iron) or low-dose iron (5.0 mg) MNP, one sachet per day for 2 months. A generalized linear model was used to determine the treatment effect of the low-dose iron MNP. The low-dose iron MNP was non-inferior to the standard MNP on haemoglobin outcome ( $\beta = -0.14$ , 95% CI:  $-0.30, 0.013$ ;  $p = 0.07$ ). It resulted in fewer incidence of diarrhoea, nausea, and fever.

### 5.2.1.3 PUBLISHED PAPER [MAIN PAPER 1]

Effect of Micronutrient Powder (MNP) with a Low-Dose of Iron on Hemoglobin and Iron Biomarkers, and Its Effect on Morbidities in Rural Bangladeshi Children Drinking Groundwater with a High-Level of Iron: A Randomized Controlled Trial

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## Abstract

Micronutrient Powder (MNP) is beneficial to control Anaemia, but some iron-related side-effects are common. A high level of iron in the groundwater used for drinking may exacerbate the side-effects among MNP users. We conducted a randomized controlled trial examining the effect of a low-dose iron MNP compared with the standard MNP in children aged 2–5 years residing in a high-groundwater-iron area in rural Bangladesh. We randomized 327 children, who were drinking from the “high-iron” wells ( $\geq 2$  mg/L), to receive either standard (12.5 mg iron) or low-dose iron (5.0 mg iron) MNP, one sachet per day for two months. Iron parameters were measured both at baseline and end-point. The children were monitored weekly for morbidities. A generalized linear model was used to determine the treatment effect of the low-dose iron MNP. Poisson regressions were used to determine the incidence rate ratios of the morbidities. The trial was registered at ISRCTN60058115. Changes in the prevalence of Anaemia (defined as a hemoglobin level  $< 11.0$  g/dL) were 5.4% (baseline) to 1.0% (end-point) in the standard MNP; and 5.8% (baseline) to 2.5% (end-point) in the low-dose iron MNP groups. The low-dose iron MNP was non-inferior to the standard MNP on hemoglobin outcome ( $\beta = -0.14$ , 95% CI:  $-0.30, 0.013$ ;  $p = 0.07$ ). It resulted in a lower incidence of diarrhea (IRR = 0.29,  $p = 0.01$ , 95% CI: 0.11–0.77), nausea (IRR = 0.24,  $p = 0.002$ , 95% CI: 0.09–0.59) and fever (IRR = 0.26,  $p < 0.001$ , 95% CI: 0.15–0.43) compared to the standard MNP. Low-dose iron MNP was non-inferior to the standard MNP in preventing Anaemia yet demonstrated an added advantage of lowering the key side-effects.

**Keywords:** [Micronutrient Powder](#); [groundwater iron](#); [hemoglobin](#); [morbidity](#); [children](#); [Bangladesh](#)

## 1. INTRODUCTION

Anaemia is a major public health problem in the low- and middle-income countries [1]. Anaemia in children, defined as a hemoglobin level  $< 11.0$  g/dL, is associated with impaired cognitive performance; increased mortality and morbidity; and poorer educational attainment in children [2]. Iron deficiency (ID) is considered as the most common cause of Anaemia, with the widely held assumption that half of all Anaemia cases are caused by ID [3]. The World Health Organization recommends Micronutrient Powder (MNP), a powdered formulation consisting of key micronutrients, including iron, as an intervention to prevent childhood Anaemia [4]. Accordingly, the Bangladesh Government has also adopted this intervention to prevent childhood Anaemia. However, an increasing number of studies have shown that the supplementation of iron/MNP is associated with side effects, such as diarrhea, nausea, vomiting, bloody stool, malaria, and respiratory tract infections [5,6,7]. Of note, iron is a pro-oxidant and can have deleterious effects if an excessive amount of free iron is present in the body system [8]. Iron in the body is maintained by a tightly controlled regulatory system, and uptake of iron in the body depends on the iron status of the body, and/or the presence of inflammation and infection. In the presence of a sufficient reserve of body iron or systemic inflammation, the intestinal uptake of iron may be limited due to hepcidin-mediated regulation [9,10]. The unabsorbed iron in the gut might affect the composition of the gut microbiome, leading to the side effects [11,12]. In this context, trials have been conducted assessing the efficacy of low-iron MNPs in African settings with a high infection burden. Findings have shown that despite there being efficacy with the low-iron formulations in improving hemoglobin levels, increased side-effects were documented compared to placebo

[13,14]. Groundwater iron has been an evolving area of research in Anaemia science [15,16]. Iron is one of the most abundant metals on Earth, and is ubiquitous in groundwater sources depending on the environment over which the water flows [17,18]. Recent studies have shown a significant association between iron status and daily iron intake from drinking groundwater in different population groups [15,19]. Further, iron status was observed to be good in Bangladeshi populations who are drinking from groundwater with a high level of iron [15,18,19,20]. In the country, the MNP program for the prevention of childhood Anaemia suffers poor coverage (~2%–3%, personal communication), and the side-effects are documented [21]. To date, no study has been conducted to examine the usage of MNPs/iron supplements in iron-replete children, whose potable supplies are iron-rich groundwater. Hence, the present study examined the effect of a low-iron MNP compared with the standard MNP on the hemoglobin concentration, and the associated morbidities, in Bangladeshi children exposed to high-iron groundwater.

## **2. MATERIALS AND METHODS**

### **2.1. STUDY DESIGN, PARTICIPANTS AND RANDOMIZATION**

A randomized controlled trial was conducted among children, 2 to 5 years of age, in the Belkuchi sub-district in north-western Bangladesh. Belkuchi is located within the high-iron groundwater areas [17] and all enrolled children were reported to drink groundwater with a high level of iron. Of note, an iron concentration  $\geq 2$  mg/L was considered as high based on the cut-off for the tolerable upper limit of iron in water defined by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) [18,22]. The exclusion criteria were children receiving MNPs/iron supplements and/or antibiotics in the preceding two months; the presence of chronic, congenital debilitating illnesses; and the guardian's unwillingness to participate. A total of 327 children were randomly allocated to receive either a low-dose-iron MNP (containing 5 mg Fe, 300  $\mu$ g RE vitamin A, 5 mg zinc, 30 mg vitamin C, and 0.15 mg folic acid) or a standard MNP (containing 12.5 mg Fe, 300  $\mu$ g RE vitamin A, 5 mg zinc, 30 mg vitamin C, and 0.15 mg folic acid) to consume 1 sachet every day for 60 days. It is important to note that the standard MNP formulation has been recommended by the Bangladesh Government for the prevention and control of anaemia in children and, accordingly, there is a significant distribution of MNPs by national NGOs. Thus, we did not consider a placebo arm due to ethical concerns. Randomization was done at two levels. At first, roughly 70% of the total enrolled children ( $n = 327$ ) were selected by simple random sampling using a random number generator for collecting blood samples. In the second step, randomization was carried out by an independent researcher and the children were allocated to one of the two letter codes (A and B) using a random number generator, without allowing for duplicate entries and not fixing a seed [23]. The sachets containing MNP preparations (Standard MNP and low-iron MNP) were identical in appearance. The sachets were labelled by the manufacturer (Manisha Pharmoplast Pvt. Ltd, Gujarat, India) with alphabetic codes (A and B) for group identification. The MNP preparations were analyzed for a quality control check by the manufacturer and the amounts of all ingredients were found within required ranges. Except for one (SR), all the investigators, field personnel and participants were blinded to the group assignment. The codes were not disclosed to the researchers until preliminary analysis was completed. The purpose and exact nature of the study were explained to the mothers or caregivers of all prospective participants. We further explained that the project physician would help to manage if their children encounter any common side-effects, such as vomiting, nausea and diarrhea. Besides, all caregivers and mothers were

informed that they can withdraw their children from the study at any time without giving reasons.

The trial received ethical approval from the Faculty of Biological Science, the University of Dhaka, Bangladesh (Ref# 46 /Biol. Scs. /2017-2018), and the Griffith University Human Ethics Committee, Australia (Ref# 2017/467). The trial was registered with the International Standard Randomized Controlled Trial Register, number ISRCTN60058115.

## **2.2. PROCEDURE**

A site selection assessment was conducted in 6 sub-districts of the northern part of the country. The Belkuchi sub-district was selected because of the higher availability of eligible child–tube-well pairs (children of the stipulated age drinking from “high-iron” tube-wells). Screening was carried out in three unions (the lowest administrative division of the country, consisting of a cluster of villages—Belkuchi Pourashava, Bhangabari and Daulatpur—of the Belkuchi sub-district to identify the children (2–5 years old), who use the “high-iron” wells ( $\geq 2$  mg/L) for drinking water. During the screening, 436 children from 8 villages were listed as potential participants. At the time of recruitment (roughly 2 months after the screening) for the study, 83 children were excluded as they did not meet the selection criteria. Besides, 10 subjects did not show up and another 16 subjects refused to take part in the study ([Figure 1](#)). Thus, the overall response rate was 92.6%.

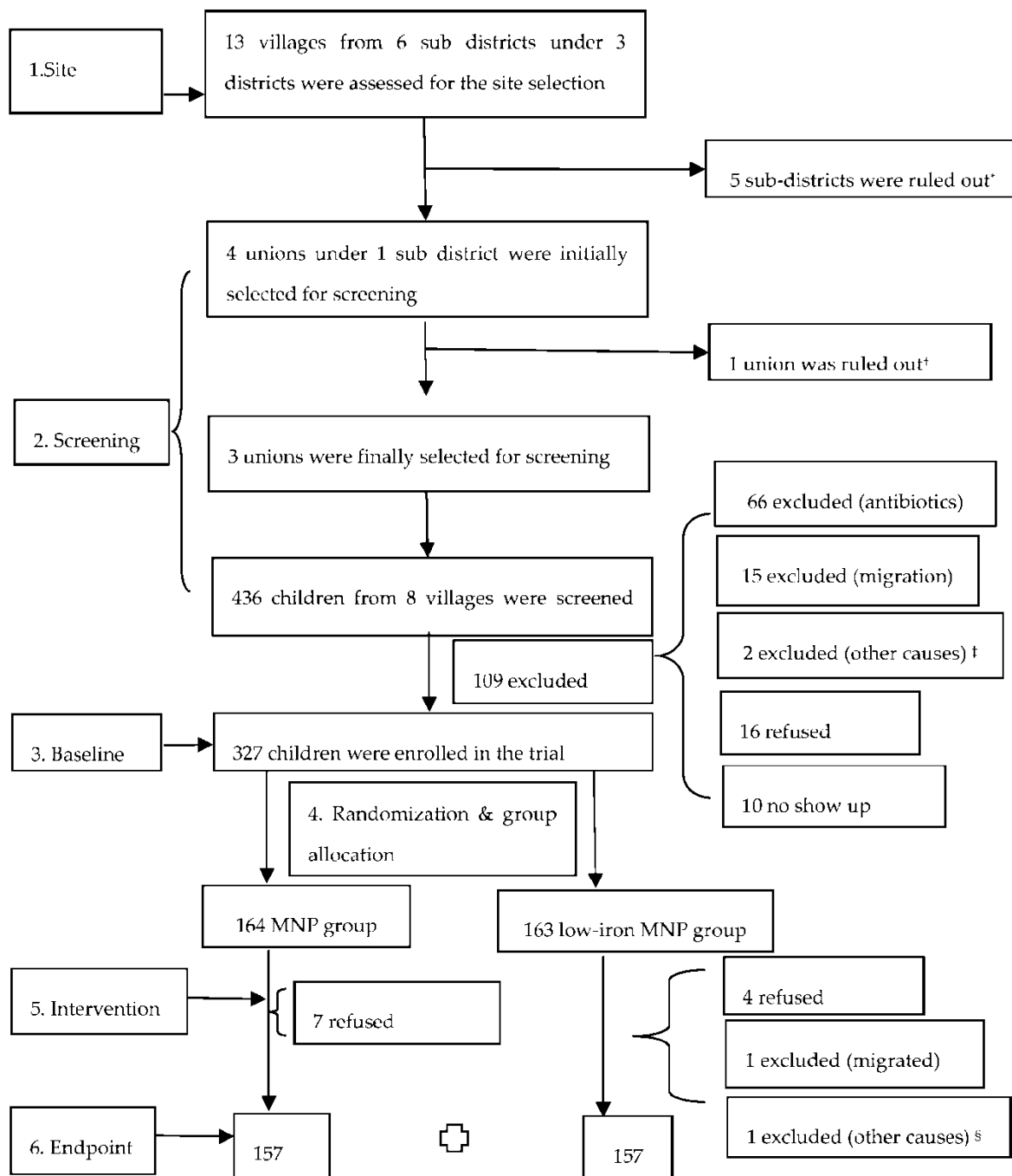


Figure 1. Selection process for the study participants. \* Due to low availability of the child–well pair, and/or the logistical, geographical/natural calamity issues. † Due to an ongoing MNP program, which might have contaminated the study intervention. ‡ Not of the stipulated age ( $n = 1$ ); a tumor in the abdomen ( $n = 1$ ). § Diagnosed with a congenital neurological disease of the colon. As per the required sample size, roughly 70% of the enrolled children were randomly selected for assessment of the blood parameters.

After obtaining either a signature or thumb impression on the written informed consent form of the parents/legal guardians for the participation of their wards in the trial, the mothers of all the enrolled children ( $n = 327$ ) were interviewed using a structured questionnaire for

baseline data collection. Blood samples were collected from sub-samples for assessing hemoglobin and the iron status parameters ([Supplementary Text 1](#)). The questionnaire consisted of several domains—socio-economics, child morbidities, as well as dietary and water intake assessments. Further, iron and arsenic levels of the drinking water were assessed. Socio-economic variables included household head's occupation, mother's education, spends on purchasing food, household food insecurity, ownership of assets, as well as types of household and toilet used. An asset index was constructed considering the socio-economic variables by using a principal component analysis [24,25]. Household food insecurity was assessed by calculating the HFIAS score based on three domains—anxiety of the impending insecurity, qualitative deprivation, and the quantitative deprivation of food intake [26]. We assessed dietary intake of the children using an interviewer-administered seven-day semi-quantitative food frequency questionnaire (SQFFQ), consisting of a list of commonly consumed local foods adapted from the national micronutrient survey 2011–2012 [20,27]. The SQFFQ was modified by adding some food items and validated against two 24 h recalls. The energy-adjusted correlation coefficient for iron was 0.60,  $p < 0.001$ , and the weighted kappa statistic was 0.30, falling within the acceptable range (unpublished). All the food items enlisted were assessed for the daily average intakes. The nutrient intakes were calculated using an updated Food Composition Table (FCT) on Bangladeshi foods [28]. For the foods which were missing in the FCT, the USDA database on the nutrient values was used [29]. Children's body weight was measured using a bathroom scale (Tanita Inc., Japan) with a 100 g precision. The height was taken using a locally made wooden length board with a precision of 1 mm. The measurements were repeated, and the averages were considered.

Venous blood samples (3.5 mL) were collected from the antecubital vein by a trained phlebotomist using a disposable syringe. An aliquot of the whole blood sample was taken in the EDTA tube for the measurement of hemoglobin and hemoglobinopathies. The remainder of the blood sample was dispensed in a centrifuge tube for collection of serum. The serum samples were transported to the laboratory in Dhaka city in an ice-gel cool box and stored at  $-70^{\circ}\text{C}$  until further analysis. Hemoglobin was measured by a Hemocue analyzer (Hemocue 301 Hemocue AB, Angelholm Sweden). Serum ferritin was measured using an electrochemiluminescence immunoassay (ECLIA) on an automated immunoassay analyzer (Cobas C311; Roche Diagnostics, Mannheim, Germany), using a commercial kit according to manufacturer's instruction (Roche Diagnostics, GmbH, 68305 Mannheim, Germany). Serum TfR, serum CRP and AGP were determined by a particle-enhanced immunoturbidimetric assay on an automated, software-controlled clinical chemistry analyzer (Cobas c311, Roche Diagnostics GMBH, Mannheim 68305 Germany) using the commercial kits. The inter-assay coefficient of variations (CVs) for serum ferritin, sTfR, CRP and AGP were 0.32%–1.42%, 0.82%–1.14%, 3.6%–7.4% and 3.7%–6.5%, respectively. In the presence of inflammation or infection, serum ferritin concentration can be in the normal range or elevated despite deficient stores [30]. Thus, serum ferritin was adjusted for infection by using the raised values of CRP ( $>5\text{ mg/L}$ ) and AGP ( $>1\text{ g/L}$ ), by the correction factors calculated following Thurnham's principle [31]. Congenital hemoglobinopathies, which is a potential confounder of hemoglobin status, were identified by capillary zone electrophoresis of Hb at pH 9.4 and a high voltage of 9600 V (Capillary 2 system; Sebia, Evry, France).

Iron concentration in the groundwater was measured using a Handheld Colorimeter (HI721 Checker<sup>®</sup> HC (Hanna Inc. Woonsocket, RI, USA) with a range between 0.0 and 5.0 ppm, a resolution of 0.01 ppm, and an accuracy  $\pm 0.04\text{ ppm} \pm 2\%$  of the readings. Arsenic concentration in the water sample was assessed using an arsenic test kit device (Prerana Laboratories, India). The device was a test strip color-comparator instrument, using the

principle of reduction of the inorganic arsenic compounds (As + 3 and As + 5) present in the groundwater sample [32].

During the two-month-long intervention, the field assistants visited each participant weekly to record compliance. On the first week, the field assistants provided 10 MNP sachets to the mothers of the participants to last until the next visit and explained to them how to consume the MNPs. Thereafter, in each weekly visit, the mothers were asked about the number of MNPs consumed by their children in the previous week. The actual consumption of MNPs was recorded after confirming by counting the returned empty and the intact sachets and replenished with MNPs to last another 10 days. Besides, the portion of the MNP-mixed food that was not consumed by the child was recorded. This information was considered while calculating the total iron consumption from MNP. The interviewers collected data on the episodes of various morbidities, such as diarrhea, loose stools, nausea, vomiting, fever, common cold and acute lower respiratory tract infection (ALRI) by asking mothers of the children every week. Loose stools implied the loose or watery consistency of stool. Diarrhea was defined as three or a higher number of loose, liquid or watery stools over 24 h, separated in time from an earlier or subsequent episode by at least 2 consecutive diarrhea-free days [33,34]. Fever was defined as an axillary temperature higher than 38.3 °C reported by the study worker who measured the temperature using a thermometer. The common cold was defined as cough, sneezing and fever, both without a rapid respiratory rate or chest in-drawing [35]. An acute lower respiratory infection was defined as cough or difficult-breathing, a rapid respiratory rate (>40 breaths per minute in children 12 months of age and older), and either a fever of >38.3 °C or chest retractions [35].

## 2.3. STATISTICAL ANALYSIS

### 2.3.1. NON-INFERIORITY MARGIN AND SAMPLE SIZE

The treatment effects of a low-iron MNP were determined against the standard MNP using a non-inferior design. Typically, the non-inferiority margin is a fraction, a one-half or less of the historical effect size of standard treatment [36,37]. To determine the non-inferiority margin, we considered an earlier trial examining the efficacy of the standard MNP on hemoglobin status in rural anemic children of Bangladesh, which demonstrated an increase in hemoglobin level by 1.61 g/dL following an 8-week-long intervention [38]. Since all the children irrespective of anaemia status were enrolled in the present study, we considered the non-inferiority margin of a modest 0.5 g/dL, which was roughly 30% of the effect size of the earlier study [38]. The non-inferiority of the low-iron MNP compared with the standard MNP for the effect on hemoglobin outcome was concluded if the lower bound of the one-sided 95% confidence interval for the treatment effect was higher than -0.5 g/dL.

We estimated the sample size for the hemoglobin outcome considering the non-inferiority margin. We assumed to establish with 95% confidence that the mean hemoglobin concentration in the low-iron MNP group would be no more than 0.5 g/dL lower than that in the standard MNP group. With the within-subject standard deviation of 1.1 g/dL [20], and using a one-tailed alpha of 5%, with 90% power, the required sample size per group was 83. Considering a 35% attrition due to follow up, 112 children were required in each group, and 224 in the two groups. The mean occurrence of diarrhea in an earlier Bangladeshi trial with the standard MNP was 1.17 cases per child over 3 months [39]. Since a low-dose of iron was used, we assumed that the low-iron MNP would result in a 30% lower magnitude of diarrhea

than that reported elsewhere [39]. To detect a significant change in diarrheal incidence, with a 5% alpha and 80% power, as well as 35% attrition, the required sample size per group was 162; thus, a total of 324 samples was needed for two groups.

### 2.3.2. STATISTICAL ANALYSIS

All variables were checked by visual assessment of histograms and normality was tested by the Kolmogorov–Smirnov normality test. Model assumptions for the treatment effect of the low-iron MNP were tested for linearity by plotting residuals against the covariates. We examined the normality of the model by a kernel density plot of residuals, standardized normal probability (pnorm plots) and the quantiles of a variable against the quantiles of a normal distribution (qnorm plots). We plotted the residuals versus the fitted (predicted) values to assess the heteroskedasticity ([Figure S1](#)). The variance inflation factor was estimated to assess the multicollinearity of the model. The multivariable model seemed linear, had normally distributed residuals, homoskedasticity and showed no evidence of multicollinearity.

Baseline characteristics of the households and study participants and the morbidity data of the children were presented as the mean  $\pm$  SD for continuous variables and as percentages ( $n$  (%)) for categorical variables. Descriptive data were compared between the two MNP groups using the independent sample  $t$ -test for continuous variables and Chi-square test or the Fisher's exact test with a two-sided significance level for categorical variables. Pearson's correlation coefficients were estimated for determining the association between the total intake of iron and the body iron-reserve, after the logarithmic transformation of the relevant variables.

The treatment effects of the low-iron MNP on changes in the concentration of hemoglobin and the iron status markers against the standard MNP was compared by generalized linear modelling (GLM). The dependent variables were the changes (endpoint—baseline) in hemoglobin, ferritin and sTfR concentrations following the intervention; the treatment group was the independent variable. The covariates for adjustment were (i) socio-economic variables (mother's education, possession of cultivable lands, household food insecurity and spending on purchasing of food); and (ii) the child's characteristics (age, gender, thalassemia status, height-for-age Z score, baseline suffering of loose stools, baseline intakes of dietary and groundwater iron, iron intake from MNPs and the baseline values of the corresponding biochemical parameters). We employed the sandwich estimator of variance (i.e., robust standard error) to estimate unbiased standard errors for the effect estimates [40]. The treatment effects of the low-iron MNP against the standard MNP were reported as coefficients with robust standard errors with 95% confidence intervals. Intention-to-treat and per-protocol analyses were done to examine the treatment effects.

The Poisson regression model was used to compare the incidence of various morbidities between the two treatment groups and to report the comparative effect of the treatments as the incidence rate ratio. The dependent variables were the incidence of various morbidities (diarrhea, loose stool, nausea, vomiting, fever, common cold and ALRI) over the two-month-long intervention period. The treatment group was the independent variable, and the length of the exposure time, i.e., person-week for the morbidity conditions, was the exposure variable. We controlled for the socio-economic and child characteristics, which are prognostic to outcomes as covariates, as stated elsewhere. We also controlled for the mother's hand-washing behavior and the duration that the child was breastfed, as the

development of the immune system and breastfeeding are linked [41]. The incidence rate of the morbidities for the low-iron MNP relative to the standard MNP was considered significantly different when the incidence rate ratio (IRR) with 95% CI was estimated with a  $p$ -value  $< 0.05$ . Body iron-reserve was calculated after 2 months of intervention using Cook's method [42] and compared between the treatment groups.

### 3. RESULTS

During the screening in July–August 2018, 436 prospective children were listed, and 327 of them were enrolled for the study (Figure 1). During the intervention, 7 children refused in the standard MNP and 6 discontinued (4 refusals, 1 migration and 1 detected with the abdominal tumor) in the low-iron MNP group (Figure 1). The dropout rate was 4.26% and 3.68% in the groups, respectively.

#### *1.1. Baseline Household and Children Characteristics and Changes in Anaemia, ID, Weight and Height of the Children Over the Intervention*

Table 1 presents the household characteristics, e.g., socio-economics, food insecurity and iron concentration in the groundwater, which did not differ between the treatment groups.

Table 1. Household and children characteristics at baseline by treatment group.

Household Characteristics	Standard MNP		Low-Iron MNP		p-Value
	Data Available	Data	Data Available	Data	
Occupation of household head *¶	164		163		
Business		32 (19.5)		28 (17.1)	0.41
Factory worker		46 (28.0)		56 (34.3)	
Unskilled laborer		23 (14.0)		25 (15.3)	
Farmer		21(12.8)		13 (8.0)	
Mother's education (no of year) †	164	5.3 ± 3.3	163	5.3 ± 13.4	0.91
Possession of cultivable land *	164	53 (32.3)	163	51 (31.3)	0.84
Possession of improved housing *	164	43 (26.2)	163	40 (24.5)	0.08
Usage of unsanitary latrine *§	164	32 (18.9)	163	27 (16.6)	0.26
Expenditure on food (BDT/week) †	164	1833.7 ± 881.4	163	1710.9 ± 739.9	0.17
Household food insecurity *.‡‡	164		163		
Food secure		77 (46.9)		84 (51.8)	0.45
Severe food insecure		17(10.4)		22 (13.6)	
Hand washing behavior of the mother	164		163		
Use soap before feeding child †		28 (17.1)		34 (20.8)	0.71
Use soap after toilet †		96 (58.5)		98 (60.5)	0.73
Iron concentration in groundwater (mg/L) †	164	8.2 ± 7.3	163	7.8 ± 7.5	0.59
Arsenic contamination of water (≥10 ppm)	140	1 (0.7)	140	0 (0.0)	n/a
Child characteristics					
Age (month) †	164	39.5 ± 9.1	163	40.2 ± 9.0	0.49

Household Characteristics	Standard MNP		Low-Iron MNP		<i>p</i> -Value
	Data Available	Data	Data Available	Data	
Gender female *	164	71 (43.3)	163	83 (50.9)	0.16
Breastfeeding	164		163		
Taken colostrum *		155 (95.1)		145 (90.1)	0.08
Exclusive breastfeeding *		7 (4.3)		11 (6.8)	0.31
Daily intake of ASF <sup>‡</sup> (gram raw-weight)	164	35.7 ± 29.3	163	37.3 ± 27.8	0.61

Data are reported as *n* (%) for the categorical variables, and as mean ± SD for the continuous variables. Group differences for the categorical variables were estimated by \* Chi-square test or the <sup>†</sup> Fisher's exact test as appropriate; group differences for the continuous variables were estimated by <sup>‡</sup> student's *t*-tests. <sup>§</sup> Pit latrines without slab/open. <sup>||</sup> ASF includes small fish, large fish, eggs, chicken, beef, mutton and liver. <sup>¶</sup> The main occupations are presented. <sup>##</sup> The severely food insecure and the food secure households are presented

Mean iron concentrations  $\pm$ SD of the groundwater were  $8.22 \pm 7.27$  mg/L and  $7.78 \pm 7.51$  mg/L in the standard MNP and the low-iron MNP groups, respectively. The groundwater in one tube-well contained an arsenic level of 10 ppm in the standard MNP group, while none of the tube-wells was detected with arsenic in the low-iron MNP group. On average, children were roughly 40 months old at baseline with no significant difference between the groups. The proportion of female was slightly higher in the low-iron MNP group, but the difference was not statistically significant ([Table 1](#)).

The prevalence of Anaemia was 5.4% and 5.8% at baseline, and 1.0% and 2.5% after the intervention in the standard MNP and low-iron MNP groups respectively. The differences between the two groups were insignificant both at baseline and the end point ([Table 2](#)). Prevalence of hemoglobinopathies were 13% in each of the groups ([Table 2](#)).

Table 2. Differences in biochemical measures and anthropometry between the treatment groups at baseline and the end of the study period.

Variable	Standard MNP		Low-Iron MNP		p-Value
	Data Available	Data	Data Available	Data	
Anemia (hemoglobin < 11.0 g/dL)					
Baseline <sup>†</sup>	111	6 (5.4)	120	7 (5.8)	1.0
End-point <sup>†</sup>	103	1 (1.0) <sup>‖</sup>	116	3 (2.5) <sup>‖</sup>	0.62
Serum ferritin (ng/mL) <sup>§</sup>					
Baseline <sup>‡</sup>	111	67.0 ± 3.7	119	62.5 ± 2.6	0.73
End-point <sup>‡</sup>	106	72.1 ± 3.2	115	69.7 ± 3.0	0.63
Iron deficiency (serum ferritin < 12.0 ng/mL)					
Baseline <sup>*</sup>	111	2 (1.8)	119	2 (1.7)	1.0
End-point	106	0 (0.0)	115	0 (0.0)	n/a
Serum TfR (µg/mL)					
Baseline <sup>‡</sup>	47	3.99 ± 0.97	59	3.89 ± 1.0	0.59
End-point <sup>‡</sup>	48	3.93 ± 1.02	58	3.64 ± 0.87	0.11
Iron deficiency (serum TfR > 8.3 µg/mL)					
Baseline	47	0 (0.0)	59	0 (0.0)	n/a
End-point	48	0 (0.0)	58	0 (0.0)	n/a
C-reactive protein (mg/L)					
Baseline <sup>‡</sup>	111	1.7 ± 3.9	119	3.1 ± 9.1	0.13
End-point <sup>‡</sup>	106	1.5 ± 3.5	115	1.9 ± 6.6	0.50
Alpha a1-acid glycoprotein (mg/dL)					
Baseline <sup>‡</sup>	111	76.0 ± 29.5	119	75.8 ± 28.1	0.95

Variable	Standard MNP		Low-Iron MNP		p-Value
	Data Available	Data	Data Available	Data	
End-point <sup>‡</sup>	106	72.1 ± 25.8	115	74.1 ± 25.9	0.56
High C-reactive protein (CRP > 5 mg/L)					
Baseline *	111	8 (7.2)	119	16 (13.4)	0.12
End-point *	106	6 (5.6)	115	8(6.9)	0.69
High alpha a1-acid glycoprotein (AGP > 100 mg/dL)					
Baseline *	111	12 (10.8)	119	23 (20.1)	0.05
End-point *	106	14 (13.2)	115	15 (13.1)	0.97
Congenital hemoglobin disorders (any form)					
Present *	107	14 (13.1)	115	15 (13.0)	0.99
Helminth infestation					
Cyst of AL <sup>†</sup>	51	1(1.96)	43	1 (2.32)	0.98
Cyst of Giardia <sup>†</sup>	51	1 (1.96)	43	1 (2.32)	
Ova of AL *	51	7 (13.7)	43	6 (13.9)	0.97
Body weight (kg)					
Baseline <sup>‡</sup>	164	12.38 ± 1.97	163	12.53 ± 2.0	0.49
End-point <sup>‡</sup>	157	12.91 ± 2.05	157	12.9 ± 62.09	0.81
Height (cm)					
Baseline <sup>‡</sup>	164	91.9 ± 6.91	163	92.42 ± 6.61	0.48
End-point <sup>‡</sup>	157	93.29 ± 6.65	157	93.58 ± 6.76	0.70

Data are reported as *n* (%) for the categorical variables, and as mean ± SD for the continuous variables. Group differences for the categorical variables were estimated by \* Chi-square test or the <sup>†</sup> Fisher's two-sided exact test as appropriate; group differences for the continuous variables were estimated by the <sup>‡</sup> student's *t*-tests. <sup>§</sup> Adjusted for high serum CRP and AGP according to Thurnham's principle [31] <sup>||</sup> *p* > 0.05 between baseline and end-point. For

reference body weight, 50<sup>th</sup> percentile at the age of 24 and 59 months are 12.2 kg and 18.2 kg, respectively [43]. For the reference height, the 50<sup>th</sup> percentile at the age of 24 and 59 months are 86.4 and 108.9 cm, respectively [43]. n/a: data not applicable.

The proportion of children with elevated AGP were 20.1% in the low-iron MNP group and 10.8% in the standard MNP group ( $p = 0.05$ ). At baseline, iron deficiency (ID), based on infection-adjusted ferritin concentration, were 1.8% and 1.68%, respectively, and none of the children had an ID at end-point. Mean weight and height in the children increased significantly ( $p < 0.001$ ) following the intervention in both the groups (Table 2).

### 3.2. TREATMENT EFFECT OF THE LOW-IRON MNP ON HEMOGLOBIN AND IRON PARAMETERS

The GLM results showed that the low-iron MNP resulted in a 0.14 g/dL lower effect on the hemoglobin concentration compared with the standard MNP ( $\beta = -0.14$ , 95% CI: -0.30, 0.013;  $p = 0.07$ ). The lower bound (-0.30 g/dL) of the 95% CI for the difference in the effect was higher than the priori non-inferior margin (-0.50 g/dL) (Table 3).

Table 3. Changes in hemoglobin and iron status markers, and comparative treatment effects of

Variable	Standard MNP		Low-iron MNP		$\beta$ (Robust SE)	95% CI	p-value
	Mean	SE	Mean	SE			
Hemoglobin (g/dl)							
Treatment effect** (Reference: standard MNP group)						n=207	
Baseline	12.23 <sup>§</sup>	0.07	12.37	0.07			
End line	12.46 <sup>  </sup>	0.07	12.40	0.07			
Change <sup>†</sup>	0.23	0.07	0.032	0.06	-0.14(0.08)	-0.30, 0.013	0.07
Serum ferritin (ng/ml)							
Treatment effect** (Reference: standard MNP group)						n=210	
Baseline	67.02 <sup>§</sup>	3.70	62.48	2.64			
End line	72.15	3.22	69.68 <sup>  </sup>	2.95			
Change <sup>†</sup>	5.09	2.79	6.81	2.10	0.003(3.22)	-6.31, 6.32	0.99
Serum TfR ( $\mu$ g/ml)							
Treatment effect** (Reference: standard MNP group)						n=104	
Baseline	3.99 <sup>§</sup>	0.14	3.89	0.13			
End line	3.93	0.15	3.64 <sup>  </sup>	0.11			
Change <sup>†</sup>	-0.06	0.07	-0.20	0.09	-0.20(0.12)	-0.44, 0.04	0.09

the low-iron MNP vs. standard MNP.

\*Generalized Linear Model was used. <sup>†</sup>Changes in hemoglobin, ferritin, and sTfR between end-point and baseline were the dependent variables; treatment group was the independent variable; the covariates for adjustment were: age, gender, thalassemia status, mother's education; possession of cultivable lands; household food insecurity; spends on purchasing food; height-for-age Z score; baseline iron status markers depending on the type of the biomarkers analyzed; baseline morbidities, e.g. suffering from loose stools; baseline intake of dietary and groundwater iron; and the intake of iron from MNP. <sup>§</sup>Intention-to-treat principle was applied. <sup>||</sup>Th

estimates were not statistically significantly different from the corresponding estimates of the other treatment group ( $p > 0.05$ )

¶The estimates were significantly different from the corresponding baseline estimates ( $p < 0.05$ ).  
Unadjusted treatment effects;  $\beta$ : -0.19, 95% CI: -0.38, -0.01,  $p = 0.04$  (hemoglobin);  $\beta$ : 1.71, 95% CI: -5.12, 8.55,  $p = 0.62$  (serum ferritin);  $\beta$ : -0.13, 95% CI: -0.37, 0.10,  $p = 0.26$  (sTfR)

There was no significant difference between the treatment effects of the groups in the infection-adjusted serum ferritin ( $\beta = 0.003$ , 95% CI: -6.31, 6.32;  $p = 0.99$ ) and for serum transferrin receptor levels ( $\beta = -0.20$ , 95% CI: -0.44, 0.04;  $p = 0.09$ ).

### 3.3. BODY-IRON RESERVE, DAILY INTAKE OF IRON FROM ALL-SOURCES AND INTAKE OF TOTAL SUPPLEMENTAL IRON FROM MNP THROUGHOUT THE INTERVENTION PERIOD

After the two months of intervention, body iron reserve increased significantly in both the standard MNP (548.8 to 592.4 mg,  $p < 0.001$ ) and in the low-iron MNP groups (569.8 to 614.5 mg,  $p < 0.001$ ) (Table 4).

Table 4. Differences in the body-iron reserve and intake of iron from MNPs between the two treatment groups during the 2-month intervention.

	All		Standard MNP		Low-iron MNP		<i>p</i> - Value
	Data available	Data	Data available	Data	Data available	Data	
Body-iron reserve*							
Baseline (mg)	106	560.0 $\pm$ 117.4	47	548.8 $\pm$ 111.1	59	569.8 $\pm$ 122.3	0.36
End-point(mg)	106	604.5 $\pm$ 113.9	48	592.4 $\pm$ 102.4	58	614.5 $\pm$ 122.6	0.32
Total iron intake from MNPs (mg)†			164	633.6 $\pm$ 159.8	163	261.1 $\pm$ 55.1	<0.001
Increment‡ of the body-iron reserve (%)	106	7.8 5	47	7.94	59	7.84	0.86

Data are reported as mean  $\pm$  SD or % as appropriate. \*Body iron reserve was estimated using Cook's method [42] †The intake of supplemental iron was calculated as the number of sachets (adjusted for actual intake of MNP-mixed food) consumed over two months (50.68 sachets: standard MNP; 52.21 sachets: low-iron MNP) multiplied by 12.5 (standard MNP) and 5.0 (low-iron MNP) respectively.

‡The increment on the iron reserve (%) = [end line reserve - baseline reserve]/baseline reserve\*100.

Over the 2-month intervention period, the intakes of total supplemental iron were 633.6  $\pm$  159.8 mg and 261.1  $\pm$  55.1 mg in the standard and low-iron MNP groups, respectively ( $p < 0.001$ ). The increase in the body-iron reserve from baseline to end-point were 7.94% and 7.84% in the standard MNP and in the low-iron MNP groups, respectively ( $p = 0.86$ ; Table 4).

The daily total combined intake of iron from all sources (diet, groundwater and MNP) was higher in the standard MNP group than in the low-iron MNP group (18.25 mg vs. 12.37

mg;  $p < 0.001$ ); the difference was largely attributed to the higher amount of iron from MNP (Table S2). During the two months intervention period, after adjusting for actual intake of MNP-mixed food, the mean consumption of MNP sachets were  $50.68 \pm 12.7$  (standard MNP) vs.  $52.22 \pm 11.0$  (low-iron MNP),  $p > 0.05$ , which was 84.46% and 87.0% of the total allocated consumption, respectively (Table S3).

### 3.4. CORRELATION BETWEEN THE TOTAL INTAKE OF IRON FROM ALL SOURCES AND THE BODY-IRON RESERVE

There was a moderate correlation between the total intake of iron from all sources and the body iron reserve in the low-iron MNP group ( $r = 0.28$ ,  $p = 0.03$ ); no such correlation was observed in the standard MNP group (Figure 2).

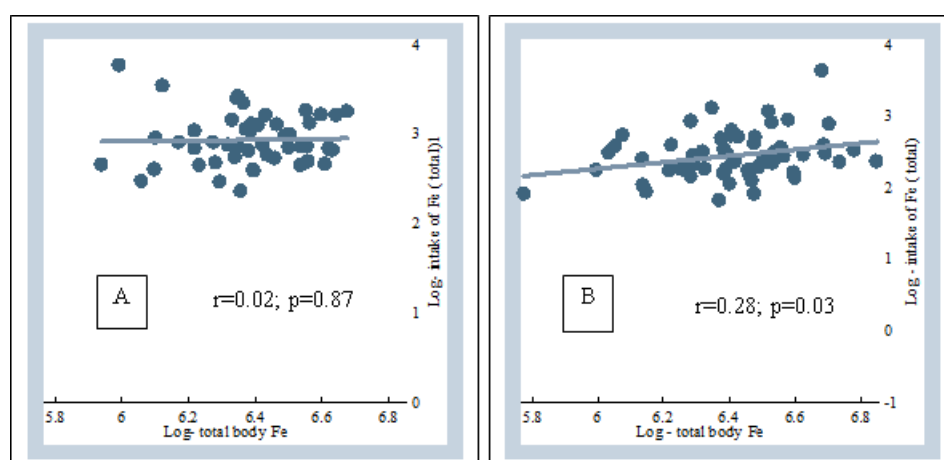


Figure 2. Correlation between the total intake  $^{* \ddagger}$  of iron and the body-iron-reserve  $^{\ddagger \ddagger}$ . \* Total intake of iron was estimated by summing up the dietary iron, iron from MNP and the iron consumed from groundwater.  $^{\ddagger}$  Total body iron was calculated using Cook's method [42].  $^{\ddagger \ddagger}$  Total body iron and total intake of iron were log-transformed. (A) Standard MNP and (B) low-iron MNP.

### 3.5. MORBIDITY PATTERN BY THE TREATMENT GROUP AND THE TREATMENT EFFECT OF THE LOW-IRON MNP ON THE MORBIDITIES

During the 2-month intervention period, there were significantly fewer children in the low-iron MNP group that suffered from diarrhea than in the standard MNP group (14.8% vs. 23.1%;  $p = 0.05$ ). The mean number of diarrhea (0.19 vs. 0.32;  $p = 0.05$ ) and loose stool episodes (1.36 vs. 2.64;  $p = 0.008$ ) were lower in the low-iron group (Table 5). No differences were observed between the groups in the occurrence and number of episodes of other morbidities and usage of medical treatment and consultations.

Table 5. Differences in common morbidities and medical treatment received during the 2-month intervention period between the children receiving the standard MNP and the low-iron MNP.

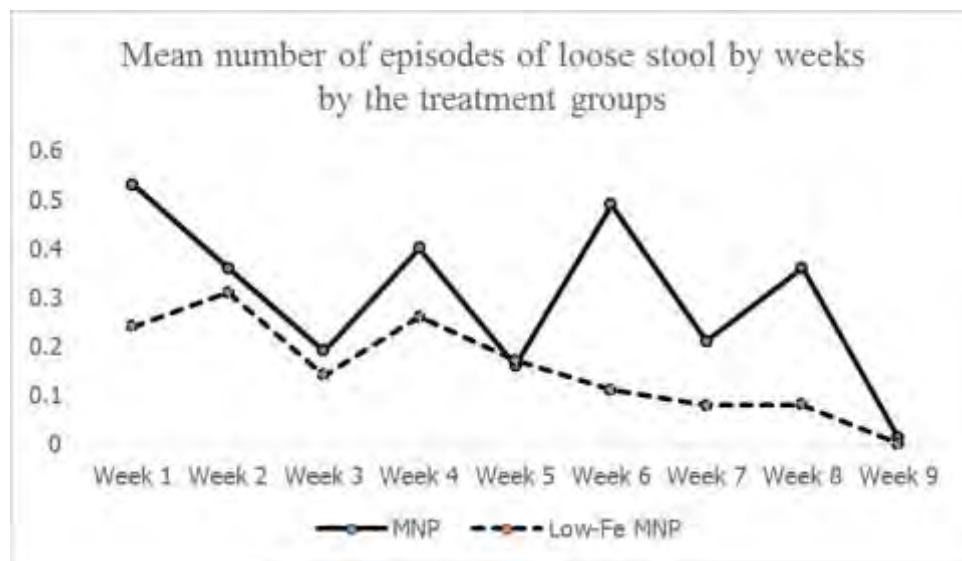
Morbidities	Standard MNP		Low-Iron MNP		<i>p</i> -Value
	Data Available	Data	Data Available	Data	
Suffered from loose stool *	160	48 (30.0)	162	35 (21.6)	0.08
No. of episodes †	160	2.64 ± 5.17	162	1.36 ± 3.21	0.008
Suffered from diarrhea *	160	37 (23.1)	162	24 (14.8)	0.05
No. of episodes †	160	0.32 ± 0.65	162	0.19 ± 0.50	0.05
Suffered from nausea *	160	35 (21.9)	162	32 (19.7)	0.63
No. of episodes †	160	0.65 ± 1.53	162	0.51 ± 1.21	0.37
Suffered from vomiting *	160	51 (31.9)	162	55 (33.9)	0.69
No. of episodes †	160	1.03 ± 2.0	162	0.87 ± 1.53	0.42
Suffered from fever *	160	103 (64.3)	162	99 (61.1)	0.54
No. of days †	160	2.85 ± 3.23	162	2.97 ± 3.56	0.74
Suffered from common cold *	160	126 (78.7)	162	127 (78.3)	0.94
No. of days †	160	7.75 ± 7.0	162	7.74 ± 7.12	0.99
Suffered from Acute Lower Respiratory Infection *	160	71 (44.4)	162	65 (40.1)	0.44
No. of days †	160	2.02 ± 2.95	162	2.38 ± 4.02	0.35
Medical treatment					
Used Oral Rehydration Salt *	160	32 (20.0)	162	28 (17.2)	0.53
Used zinc *	160	8 (4.97)	162	7 (4.32)	0.78
Used antibiotics *	160	33 (20.6)		27 (16.6)	0.36
Consulted doctor *	160	94 (58.7)	162	91 (56.1)	0.64
No. of times consulted doctor †	160	0.96 ± 1.07	162	0.99 ± 1.23	0.80

Morbidities	Standard MNP		Low-Iron MNP		<i>p</i> -Value
	Data Available	Data	Data Available	Data	
Needed referral to study physician *	160	55 (34.37)	162	52 (32.1)	0.66
No. of times needed referral to study physician †	160	0.49 ± 0.77	162	0.41 ± 0.67	0.32
Needed hospital admission *	160	1 (0.63)	162	2 (1.2)	0.56

Data are reported as *n* (%) for the categorical variables, and as mean ± SD for the continuous variables. Group differences were estimated by \* Chi-square test for the categorical variables, and by † student's *t*-tests for the continuous variables. An episode of diarrhea was defined as three or more loose/watery stools over 24 h. To define another episode, at least a 48 h symptom-free interval was considered [33,34]. A fever was defined as an axillary temperature higher than 38.3 °C [35], measured by the field worker using a thermometer. A common cold was defined as cough, sneezing and fever (implying pharyngitis or rhinitis), without a rapid respiratory rate or the chest in-drawing [35]. Acute lower respiratory infection was defined as cough or difficulty breathing, a rapid respiratory rate (>40 breaths per minute in children 12 months of age and older) and either a fever of >38.3 °C or chest retractions [35].

The trends of the weekly occurrences of loose stools remained higher in children receiving the standard MNP than in the children receiving the low-iron MNP (Figure 3).

Figure 3. Mean number of episodes of loose stool by weeks\* by the treatment group.



\*8 weeks and 4 days are required to complete the 60-day intervention, and thus it closes around the mid-point of the week 9.

The results of the Poisson regression model indicated a significantly lower incidence rate of diarrhea in the low-iron MNP group compared with the standard MNP (IRR = 0.29, 95% CI: 0.11–0.77,  $p = 0.01$ ). The incidence rate for loose stool was lower, approaching statistical significance in the low-iron MNP group (IRR = 0.46, 95%CI: 0.19–1.09,  $p = 0.08$ ). We observed a significantly lower incidence of nausea (IRR = 0.24, 95% CI: 0.09–0.59,  $p = 0.002$ ) and fever (IRR = 0.26, 95% CI: 0.15–0.43,  $p < 0.001$ ) in the low-iron MNP group compared to the standard MNP group (Table 6). No differences in the incidence of other morbidities were observed between the groups.

Table 6. Poisson regression modeling <sup>\*,†,‡</sup> to estimate the incidence rate ratio (IRR) of various morbidities in children for the usage of the low-iron MNP to the standard MNP over the intervention period.

Morbidities (Ref: standard MNP)	IRR	Robust SE	95%CI	p-value
Diarrhea	0.29	0.14	0.11-0.77	0.01
Loose stool	0.46	0.20	0.19-1.09	0.08
Nausea	0.24	0.11	0.09-0.59	0.002
Vomiting	0.63	0.32	0.23-1.71	0.36
Fever	0.26	0.06	0.15-0.43	<0.001
Common cold	0.77	0.28	0.37-1.61	0.49
ALRI	0.64	0.30	0.25-1.62	0.35

\*Poisson regression was used to estimate the IRR of the morbidities in children receiving the low-iron-MNP and the standard MNP, considering the first occurrence of the event and the total exposure time of the condition (person-week). †Intention-to-treat analysis was applied. ‡Adjusted for the baseline covariates (household and child characteristics). Unadjusted treatment effects IRR:0.61, 95%CI:0.36,1.03, $p=0.06$  [diarrhea]; IRR:0.70, 95%CI:0.45,1.08, $p=0.11$  (loose stool); IRR:0.86,95%CI:0.55,1.35, $p=0.53$  [nausea];IRR: 10, 95%CI:0.75,1.61, $p=0.61$ (vomiting);

IRR:0.91,95%CI:0.75,1.10,p=0.33 [fever]; IRR: 1.05, 95%CI:0.81,1.36, p=0.69 (common cold);  
IRR: 0.91,95%CI:0.65,1.27, p=0.60 (ALRI)

#### 4. DISCUSSION

This randomized controlled trial examined the effect of a low-dose iron MNP against the standard MNP on hemoglobin and iron status in rural Bangladeshi children (2–5 years old), who drink from the “high-iron” groundwater. Using the intention-to-treat analysis, we observed, the lower bound of the 95% CI for the difference of the treatment effect of the low-iron MNP with the standard MNP was  $-0.3$  g/dL. This was above the priori non-inferior margin of the acceptable difference of  $-0.5$  g/dL, thus establishing the non-inferiority of the low-iron MNP against the standard treatment. The per-protocol analysis also yielded similar findings (Table S1). The finding of the low baseline (5.4%–5.8%) and end-point (1%–2.5%) prevalence of Anaemia warrants discussion. Our study site resides in the areas with a very high concentration of iron in groundwater [17]. Of note, in the present study samples, the median value of iron concentration in groundwater was 4.54 mg/L (mean:  $\sim 8$  mg/L), which was much higher than the cut-off for defining the “high” level of iron in groundwater [18]. There were hardly any children who were iron deficient (baseline  $< 2\%$ , end-point 0%). Taking these into considerations, the low prevalence of Anaemia was not surprising. Further, we used a venous blood sample to measure hemoglobin concentration. Studies have shown that the capillary blood sampling, which is commonly employed for measuring hemoglobin concentration in surveys and studies, tend to overestimate Anaemia estimates [44,45,46]. The reasons for the difference between the methods are the measurement errors (mostly happens with capillary sampling) or the biological variability, which is difficult to minimize [44,46].

We observed the usage of low-iron MNP (5 mg iron) resulted in significantly fewer incidence of side-effects, such as diarrhea, nausea and fever, compared with the usage of the standard MNP (12.5 mg iron). The lower incidence of side-effects from a low-iron MNP is expected since these morbidities commonly occur with iron supplementation [5,6]. The findings of the low incidence of side-effects with the low-iron formulation are promising for the MNP programs in Bangladesh that suffers from suboptimum coverage, and side-effects were identified as an important underlying cause of the poor coverage [21].

Studies examining the efficacy and morbidities of the low-iron MNP are scarce. Samuel et al. [13], in Ethiopian children, have shown that a low-iron MNP containing 6 mg of iron in combination with an infant and young child feeding (IYCF) intervention effected in a marginal improvement of hemoglobin compared with the non-intervention group (no-iron), but caused a higher incidence of diarrhea [13]. This was relatively consistent with our finding, as we observed fewer incidence of diarrhea with the low-iron formulation compared with the standard MNP, which contain a higher amount of iron. Paganini et al. observed in young Kenyan infants that the MNP with 5 mg of highly bioavailable iron resulted in a 50% reduction in Anaemia over a 4-month intervention when compared with the control (no iron) [14]. These trials, e.g., Samuel et al. and Paganini et al., demonstrated the superior efficacy of the low-iron MNP (5–6 mg of iron) against the control (0.0 mg of iron) on hemoglobin concentration, while the present study showed a non-inferior efficacy of the low-iron MNP (5 mg of iron) against the standard MNP (12.5 mg of iron), which is a logical outcome.

Paganini et al. employed a highly bioavailable iron in their low-iron formulation (containing 2.5 mg ferrous fumarate + 2.5 mg NaFeEDTA + 190 FTU phytase), and they observed an 18.8% absorption of iron [14]. In the present study, the low-iron MNP contained 5 mg of iron as ferrous fumarate. Tondeur et al. showed that ferrous fumarate in MNP, mixed

in a cereal-based diet, had an absorption rate of 4.65% in the iron-replete children [47]. Using the ferrous fumarate and presumably with a much lower rate of absorption of iron than in Paganini et al.'s trial, the present study demonstrated the efficacy of low-iron MNP in preventing low hemoglobin levels, which could be explained by the consumption of iron from groundwater. Iron in groundwater remains mostly in a reducing and bioavailable (ferrous) state [15,48], and is reported to have a high absorption rate [49]. We considered the intake of iron from all sources—diet, groundwater and MNP—and calculated the amount of potentially bioavailable iron, considering the differential absorption potentials for different sources. Based on a study of the absorption of iron from iron-rich natural water [49], we assumed an estimated absorption potential for iron from groundwater. Accordingly, the estimated lowest amount of potentially bioavailable iron from all sources combined in children taking the low-iron MNP was 0.85 mg/day (Supplementary Text 2), which is sufficient to meet the daily requirement in this group of children [50].

The body iron reserve was sufficient, with >550 mg of baseline values in all groups. There was a similar magnitude of the increment of the iron reserve from baseline to end-point in both treatment groups, though the intake of supplemental iron in the standard MNP group was ~2.5 times (633.6 vs. 261.1 mg) higher than that in the low-iron MNP group. This suggests that, relative to the dose of iron, the amount of absorption of iron was smaller in the standard MNP group compared to its counterpart. This might have led to a higher amount of unabsorbed iron in the intestinal tract for the standard MNP group, which might have contributed to a significantly higher number of diarrheal and loose stool episodes observed in that group than in the low-iron MNP group. Further research is needed in this setting to examine the iron-induced adversities on the composition of gut microbiota, which is linked with iron supplementation and the occurrence of diarrhea and loose stool, to support the present findings.

The combined intake of iron from all sources (diet + groundwater + MNP) were 18.25 and 12.37 mg in the standard MNP and the low-iron MNP groups, respectively (Table S2). There was no group difference for intakes from dietary and groundwater sources; the difference was attributed to the intake of iron from the different MNPs. An intake of 18.25 mg iron from all sources did not show any association with the body-iron reserve in the standard MNP group ( $r = 0.02$ ;  $p = 0.87$ ). However, the intake of 12.37 mg iron in the low-iron MNP group showed a significant association ( $r = 0.28$ ,  $p = 0.03$ ). One possible explanation for the differential outcome between the groups is that the higher amount of iron in the standard MNP group might have initiated the stimulation of hepcidin at some point, through the iron-transferrin transportation complex [51]. This might have led to the subsequent inhibition of the absorption of further iron from the intestine [51], thus limiting the buildup of an iron reserve in the standard MNP group. This was reflected in the similarities of the levels of body-iron reserves between the groups at the end-point. However, for the low-iron MNP, a moderate degree of association indicates that the amount of iron (i.e., from low-iron MNP and other sources) present in the duodenum maintained a positive gradient of absorption of iron with minimal/no inhibition of absorption. This suggests that the dose of iron (5 mg) in the low-iron formulation was optimum in Bangladeshi children exposed to a high level of iron from groundwater. As the absorption was efficient, there might be less iron remaining unabsorbed, leading to lower incidence of side-effects (e.g., diarrhea, loose stool, nausea and fever) compared with the standard MNP group. This was further complemented by the findings of the mean number of loose stools by weeks during the 2-months intervention, which after initial occurrences in both the groups, declined and

stabilized in the low-iron MNP group from the 4th week onwards. However, it continued to occur in higher numbers in the standard MNP group.

A baseline prevalence of ~5.5% anemia in a high iron groundwater area may question the relevance of the iron supplementation program for the prevention of anemia in children. However, the iron level in groundwater is considerably variable in the tube-wells [15,17]. In a predominantly high iron groundwater area, there are the wells that contain either no iron or a negligible level of iron (<0.3 mg/L, the WHO aesthetic limit) [52]. Hence, in the context of a less diversified traditional diet with suboptimum dietary iron [27], the absence of the supplementation program might be counterproductive to some children even in the high iron groundwater areas. In this setting, the low-iron MNP with a reduced risk of side-effects can be an optimum measure.

A limitation of the study was that one of the main investigators, who did the preliminary analyses, could not be blinded to the treatment group coding. This might have introduced some risk of bias. Unfortunately, this could not be avoided as the MNP preparations were imported from India and the customs clearance required the declaration of the composition of the different MNP preparations. However, all field personnel engaged in the distribution and recording of the compliance of MNP consumption and morbidity data, and parents of the children remained blind to the treatment group coding. Morbidity data were collected on the weekly recalls. The method, though widely practiced, is subject to recall bias. However, we provided extensive training to the monitoring staffs to collect data objectively. Among the strengths of the study, the uptake of the interventions was satisfactory (~86% MNPs were consumed) (Table S3). Dropouts were fewer (<5% in the groups), which improved the precision of the findings.

## 5. CONCLUSIONS

In conclusion, in Bangladeshi children, who are largely iron-replete from the source of drinking water, the low-iron MNP was efficacious in preventing low levels of hemoglobin compared with the standard MNP treatment. It resulted in a lower incidence of morbidities—diarrhea, nausea and fever—than the standard MNP. The low-iron MNP, being efficacious and safer, has a potential policy consideration for prevention of childhood anemia in Bangladesh, where groundwater iron level is predominantly high in many parts [17,20] and the coverage of the MNP program is suboptimum. The formulation can be evaluated for effectiveness and compliance in a program context operated in the high iron groundwater areas. Further research is needed to examine the efficacy and side-effects of the low-iron MNP in the predominantly low groundwater iron areas. Globally, in similar environmental settings, the findings may generate interests to assess the groundwater iron profile and exploring the optimum iron/MNP supplements for prevention of childhood anaemia, as some 2 billion people, mostly in the low- and middle-income countries, rely on groundwater as potable supplies [53].

## Supplementary Materials

The following are available online at <https://www.mdpi.com/2072-6643/11/11/2756/s1>, Supplementary Text 1: The sample profile of hemoglobin and iron status parameters; Supplementary Text 2: An assessment of potential bioavailable iron from all sources in children taking the low-iron MNP; Figure S1: Assessing normality and homoskedasticity of the multivariable model for the treatment effect of the low-iron MNP; Table S1: Changes in hemoglobin and iron status markers, and comparative treatment effects of the low-iron MNP

vs. standard MNP (per-protocol analysis); Table S2: Contribution of iron from dietary, groundwater and MNP by the treatment groups; Table S3: Consumption profile of MNPs.

### Author Contributions

S.R. conceived the idea, designed the study, trained field staff, and supervised data collection, conducted the data analysis and wrote the first draft of the manuscript; F.A. took the lead in study planning and design, guided data collection and data analysis, and critically reviewed the manuscript; P.L. contributed to study design, data analysis and interpretation; M.R.K. was responsible for field work including training of staff for biological sample collection; R.R. supervised laboratory sample processing and analysis; A.K.R. contributed to staff training for collecting the biological sample and conducted the laboratory analysis; F.A. had primary responsibility for the final content; all authors contributed to writing and approved the final version of the paper.

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### CONFLICTS OF INTEREST

The authors declare no competing interests. The funder was not involved in the study design, data collection, analysis and interpretation of results, report writing, or the decision to submit for publication.

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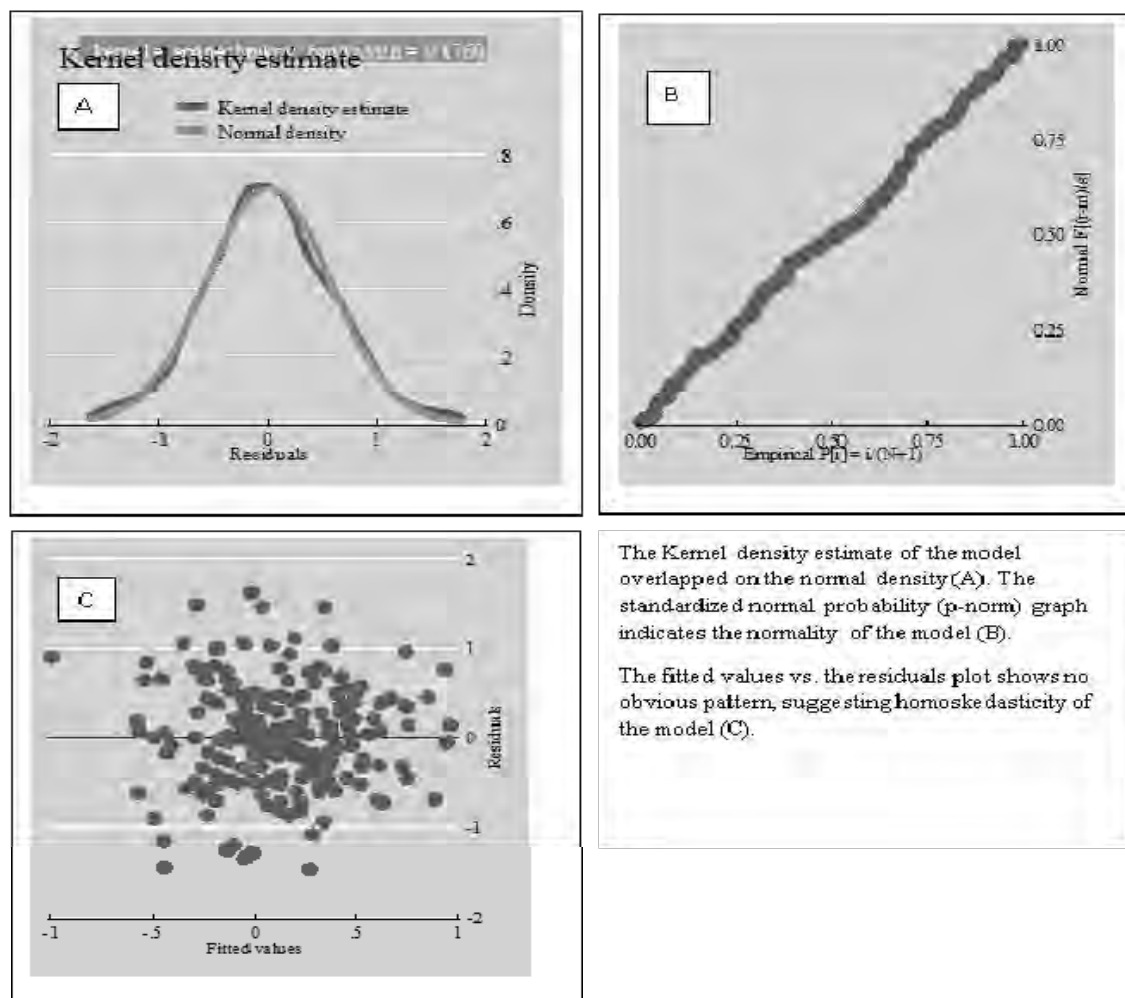
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## Supplementary Text 1: The sample profile of hemoglobin and iron status parameters

As per the requirement of the sample size to detect a non-inferior difference in the hemoglobin estimates between the groups, approximately 70% of the enrolled ( $n = 327$ ) children were randomly selected for blood sample collection. This translated in 231 for hemoglobin and 230 for ferritin, CRP, and AGP. Out of 231 samples of hemoglobin, 111 and 120 samples were in the standard MNP and the lowiron MNP groups respectively. Out of 230 samples for ferritin, CRP and AGP, 111 and 119 samples were in the respective treatment groups. Over the intervention period, 9 samples were excluded (7 refused, 1 migrated, and 1 diagnosed with a congenital disorder of colon), leaving 221 samples at end-point. For hemoglobin, ferritin, CRP, and AGP, out of these 221 samples, 106 and 115 samples belonged to the standard MNP and the low-iron MNP groups respectively. Serum transferrin receptor (sTfR) was sub sampled in 106 samples, paired over baseline and end-point. Of this, 47 and 59 samples belonged to the standard MNP and the low iron MNP groups, respectively. At baseline, 222 samples were tested for congenital hemoglobin disorders; 107 in the standard and 115 in the low-iron MNP groups, respectively.



**Figure S1:** Assessing normality and homoskedasticity of the multivariable model for the treatment effect of the low-iron MNP.

Table S1: Changes in hemoglobin and iron status markers, and comparative treatment effect of the low- iron MNP.

Variable	Standard MNP		Low-Iron MNP		$\beta$	95% CI	<i>p</i> -Value
	Mean	SE	Mean	SE	(Robust SE)		
	Hemoglobin (g/dL)				Treatment effect <sup>*,‡</sup> (Reference: standard MNP group)		
Baseline	12.25 <sup>§</sup>	0.07	12.38	0.07			
End line	12.46 <sup>§   </sup>	0.07	12.39	0.07			
Change <sup>†</sup>	0.23	0.06	0.03	0.06	−0.14 (0.08)	-0.30, 0.018	0.08
	Serum ferritin (ng/mL)				Treatment effect <sup>*,‡</sup> (Reference: standard MNP group)		
Baseline	68.8 <sup>§</sup>	3.75	62.42	2.70			
End line	72.04 <sup>§</sup>	3.25	69.68 <sup>  </sup>	2.95			
Change <sup>†</sup>	5.09	2.79	6.80	2.10	−0.4 (3.25)	−6.77, 5.94	0.90
	Serum TfR (μg/mL)				Treatment effect <sup>*,‡</sup> (Reference: standard MNP group)		
Baseline	3.99 <sup>§</sup>	0.14	3.89	0.13			
End line	3.93 <sup>§</sup>	0.14	3.64 <sup>  </sup>	0.11			
Change <sup>†</sup>	−0.06	0.08	−0.20	0.09	−0.20 (0.12)	−0.44, 0.03	0.09

\* Generalized Linear Model was used. <sup>†</sup> Changes in hemoglobin, ferritin, and sTfR between end-point and baseline were the dependent variables; treatment group was the independent variable; the covariates for adjustment were: age, gender, thalassemia status, mother's education; possession of cultivable lands; household food insecurity; spends on purchasing food; height-for-age Z score; baseline iron status markers depending on the type of the biomarkers analyzed; baseline morbidities, e.g., suffering from loose stools; baseline intake of dietary and groundwater iron; and the intake of iron from MNP. <sup>‡</sup> Per-protocol principle is applied. <sup>§</sup> The estimates were not statistically different from the corresponding estimates of the other

treatment group ( $p > 0.05$ ). <sup>||</sup> The estimates were significantly different from the corresponding baseline estimates ( $p < 0.05$ )

Table S2: Contribution of iron from dietary, groundwater and MNP by the treatment groups.

Treatment Group	Source of Fe (mg/day)			
	Diet <sup>†</sup>	Groundwater <sup>‡</sup>	MNP <sup>§</sup>	Total <sup>  </sup>
	Mean $\pm$ SD			
MNP	3.11 $\pm$ 1.74	4.78 $\pm$ 5.07	10.56 $\pm$ 2.66	18.25 $\pm$ 6.8 *
Low-iron MNP	3.39 $\pm$ 2.49	4.80 $\pm$ 5.86	4.3 $\pm$ 50.9	12.37 $\pm$ 6.3

\*  $p < 0.001$  for group difference. <sup>†</sup> The daily dietary intake of Fe was measured by a seven-day SQFFQ. <sup>‡</sup> The intake of iron from groundwater was estimated by multiplying the amount of water intake in the preceding 24 h with the concentration of iron in the groundwater (i.e., tube well) used for drinking. <sup>§</sup> The daily average intake of iron from the MNP was calculated as the number of sachets of MNP consumed over the

two months of the intervention, times the dose of the iron present in the sachet, divided by 60. The daily total intake of iron was calculated by combining the intakes from all the sources.

Table S3: Consumption profile of MNPs

Standard MNP ( <i>n</i> = 164)			Low-Iron MNP ( <i>n</i> = 163)			All ( <i>n</i> = 327)		
Mean ± SD*	Median	% <sup>†</sup>	Mean ± SD	Median	% <sup>†</sup>	Mean ± SD	Median	% <sup>†</sup>
50.68 ± 12.7	54.0	84.46	52.22 ± 11.0	55.0	87.0	51.45 ± 11.9	54.1	85.75

\*  $p > 0.05$  for the group difference. <sup>†</sup>Proportion to the allocated number of sachets ( $n = 60$ ).

## Supplementary Text 2: An assessment of potential bioavailable iron from all sources in children taking the low-iron MNP

Worwood [49] et al. has calculated the absorption of iron from iron-rich natural water. Estimated absorptions were 40% for the iron-depleted subjects (ferritin < 10 ng/ml); ~10% for the subjects with ferritin ~200ng/mL. The average rate of absorption was 23%. The rate of absorption and serum ferritin had a high negative correlation ( $r = -0.78$ ). We did not study the absorption of iron from groundwater. The iron in groundwater in Bangladesh largely exists as the ferrous form (99%) when freshly extracted, which is readily bioavailable [47]. Since, the infection-adjusted ferritin concentration in our participants was ~65 ng/mL, which was neither deficient nor very high, we conservatively assumed the absorption rate of iron could be within the lowest (10%) and the average values (23%) reported in Worwood's study.

At 10% absorption of iron from groundwater (considering the actual intakes of iron as presented in Table S2), the potential absorption of iron from groundwater =  $4.8 \text{ mg} * 0.1 = 0.48 \text{ mg/day}$ . From diet =  $3.39 \text{ mg} * 0.05 = 0.17 \text{ mg/day}$  (considering 5% absorption as the diet is cereal based). From the low-iron MNP =  $4.35 \text{ mg} * 0.046 = 0.20 \text{ mg/day}$  (considering 4.6% absorption from MNP [47]). Thus, the total amount of potentially bioavailable iron from all sources =  $0.48 + 0.17 + 0.20 \text{ mg} = 0.85 \text{ mg/day}$ .

At 23% absorption of iron from groundwater (considering the actual intakes of iron as presented in Table S2), the potential absorption of iron from groundwater =  $4.8 \text{ mg} * 0.23 = 1.1 \text{ mg/day}$ . From diet =  $3.39 \text{ mg} * 0.05 = 0.17 \text{ mg/day}$  (considering 5% absorption as the diet is cereal based). From the low-iron MNP =  $4.35 \text{ mg} * 0.046 = 0.20 \text{ mg/day}$  (Considering 4.6% absorption from MNP [47]). Thus, the total amount of potentially bioavailable iron from all sources =  $1.1 + 0.17 + 0.20 = 1.47 \text{ mg/day}$ .

## 5.2.2 FINDING OF RCT

### 5.2.2.1 OVERVIEW OF THE MAIN PAPER [RCT: STUDY 2]

Title: Effect of low-iron micronutrient powder (MNP) on the composition of gut microbiota of Bangladeshi children in a high-iron groundwater setting: a randomized controlled trial

Status: Published

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#### 5.2.2.2 INTRODUCTION

This study is a subanalysis of the parent trial. Iron induced clinical side effects, such as diarrhoea, loose stool, nausea, and vomiting are rooted in the adverse composition of the gut microbiota. Unabsorbed iron in the intestine adversely affects the composition of the microbiota, i.e., promoting disease-causing bacteria and decreasing the population of health-promoting bacteria. Thus, in this subanalysis of the main trial, the treatment effect of the low-iron MNP on the composition of the gut microbiota was examined. A subsample of 53 children was considered for paired assessment of the gut microbiome by 16S rRNA amplicon sequencing. Overall, there was no significant treatment effect of the low-iron MNP compared to the standard MNP. However, an apparent treatment effect was observed in children with a relatively adult-like microbiota, with a higher relative abundance of potentially pathogenic *Enterobacteriaceae* after receiving the standard MNP compared to the recipients of low-iron MNP. The results revealed that a low-iron MNP supplementation did not have a significant impact on their gut microbiota profile/composition compared to the standard MNP.

### 5.2.2.3 PUBLISHED PAPER [MAIN PAPER 2]

Original Contribution; [Open Access](#), [Published: 25 February 2021](#)

Effect of low-iron micronutrient powder (MNP) on the composition of gut microbiota of Bangladeshi children in a high-iron groundwater setting: a randomized controlled trial

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[European Journal of Nutrition](#) (2021)

## Abstract

### Purpose

Adverse effects of iron fortification/supplements such as Micronutrient Powder (MNP) on gut microbiota have previously been found in infection-prone African settings. This study examined the adversaries of a low-iron MNP compared with the standard MNP on the composition of gut microbiota in Bangladeshi children exposed to a high concentration of iron from potable groundwater.

### Methods

A randomized controlled trial was conducted in 2- to 5-year-old children, drinking groundwater with a high concentration of iron ( $\geq 2$  mg/L). Children were randomized to receive one sachet per day of either standard MNP (12.5 mg iron) or low-iron MNP (5 mg iron), for 2 months. A sub-sample of 53 children was considered for paired assessment of the gut microbiome by 16S rRNA amplicon sequencing.

### Results

At baseline, the gut microbiota consisted of *Bifidobacteriaceae* (15.6%), *Prevotellaceae* (12.2%), *Lactobacillaceae* (3.6%), *Clostridiaceae* (4.1%) and *Enterobacteriaceae* (2.8%). Overall, there was no significant treatment effect of the low-iron MNP compared to the standard MNP. However, an apparent treatment effect was observed in children with a relative adult-like microbiota, with a higher relative abundance of potentially pathogenic *Enterobacteriaceae* after receiving the standard MNP compared to the low-iron MNP. This effect, however, was statistically non-significant ( $p = 0.07$ ).

### Conclusion

In Bangladeshi children drinking iron-rich groundwater, a low-iron MNP supplementation did not have a significant impact on their gut microbiota profile/composition compared to the standard MNP.

The trial registration number is ISRCTN60058115; Date of registration 03/07/2019; retrospectively registered.

## Introduction

The Bangladeshi population is affected by a high burden of Anaemia. Two nationally representative surveys have reported the prevalence of Anaemia in 51% and 33% of preschool-age children [1,2,3]. However, contrary to the widely held assumption that iron deficiency (ID) is the most common cause of Anaemia, the prevalence of ID (10.7%) and iron deficiency Anaemia (IDA, 7.2%) was low [2]. A contemporary study in a north-western district of Bangladesh observed a zero prevalence of ID in women, while the prevalence of Anaemia was high (57%) [4]. These studies attributed the low prevalence of ID in the populations to the high level of iron in groundwater, which is the principal source of drinking water for the Bangladeshi population [3, 4].

Globally, iron supplementation and fortification programs have been recommended for the prevention and control of ID and Anaemia [5]. In-home fortification of micronutrients, where a caregiver adds vitamins and minerals to the weaning foods at home using micronutrient powders (MNPs) containing iron, has demonstrated a significant reduction in the risk of IDA in Bangladesh and other settings [6, 7]. The WHO recommended MNPs as an effective intervention to control IDA in children 6–23 months of age [8]. However, an excess of iron may lead to adverse effects. Recent fortification trials in Pakistan and Ghana showed that the use of MNP with 12.5 mg iron was associated with higher incidence of bloody diarrhea, respiratory infection [9] and hospitalizations attributed to diarrhea [10]. The findings of these studies generated discussion and commentary on MNP-induced adversities. Iron is a growth-limiting nutrient for many gut bacteria; and as such these bacteria compete for unabsorbed colonic iron [11]. For most enteric Gram-negative bacteria (e.g., *Salmonella*, *Shigella* or pathogenic *Escherichia coli*), iron acquirement plays a significant role in expressing virulence and colonization [12]. In contrast, *Bifidobacteriaceae*, *Lactobacillaceae* and other beneficial bacteria in the colon provide an important ‘barrier effect’ against colonization and invasion by pathogens [13]. To complement this, recent studies in Africa have shown that MNP or iron fortification was associated with significant adverse influence in the intestinal microbial composition, leading to the proliferation of pathogenic bacteria (e.g., pathogenic *Enterobacteriaceae*) and a decrease in the number of health-promoting bacteria (*Lactobacillaceae*, *Bifidobacteriaceae*) [14,15,16]. Hence, a biological mechanism of iron-induced diarrhea as a result of iron supplementation or fortification has been established. Studies have yet to establish an optimum level of iron in supplements that would be efficacious in preventing Anaemia without increasing the risk of adverse effects. In African infection-prone settings, the use of MNP with 12.5 mg of iron in Kenyan infants induced an adverse effect on gut microbiota, i.e., an increase of potential pathogens such as *Enterobacteriaceae*, particularly *Escherichia/Shigella*, the *Enterobacteriaceae/Bifidobacteriaceae* ratio, and *Clostridium* [15]. The same study employing a low-iron MNP formulation containing 2.5 mg iron as NaFeEDTA failed to demonstrate an improvement of iron status. Moreover, compared to a placebo, it resulted in significant adverse changes in the gut microbiota [15]. Bangladesh, however, presents a different context. Since Anaemia in Bangladeshi children has been high [1, 2], the government has endorsed in-home fortification of MNP containing key micronutrients including iron (12.5 mg) in the national policy for the prevention of childhood Anaemia [17, 18]. For over a decade, the MNP program has been run by national Non-government organizations (NGOs). However, as stated above, the iron status in the populations is generally sufficient, and iron in the drinking groundwater plays a key role [2, 3]. This implies that iron deficiency has a modest role at best on the causation of Anaemia and there are other reasons for the condition in this setting, such as inadequate intake of other pertinent nutrients

[3]. In the context where the population is exposed to a fair level of iron acquired naturally through drinking water, the existing programs of MNPs suffer from suboptimum coverage (2–3%, personal communication); and gastrointestinal side effects are reported to be important underlying factors [19]. In the Bangladeshi context of the high background level of groundwater iron, Rahman et al. examined the efficacy of an MNP with a low dose of iron (5 mg) in preventing childhood Anaemia against the standard MNP (12.5 mg iron), and assessed the comparative side effects. The results found a significantly lower incidence of side effects (e.g., diarrhea, nausea and fever) in the children who received the low-iron MNP [20]. To date, no study has been conducted to examine the effect of iron supplements on the gut microbiota in the Bangladeshi population. The present trial as a part of the Rahman et al. trial [20] examined the effect of an MNP with a low dose of iron (5 mg) compared with the standard MNP (12.5 mg iron) on the composition of the gut microbiota in Bangladeshi children exposed to a high concentration of iron from potable groundwater.

## Methodology

The study was conducted among children aged between 2 and 5 years, living in Belkuchi—a sub-district in north-western Bangladesh, approximately 125 km from the capital city, Dhaka. Belkuchi is an area where iron concentration in groundwater is predominantly high ( $\geq 2$  mg/L) [21]. As ubiquitous in rural Bangladesh, people in Belkuchi rely on groundwater for the drinking purpose [21].

A total of 327 children were enrolled in the trial and were randomized to receive the standard MNP (containing 12.5 mg Fe as ferrous fumarate, 300  $\mu$ g RE vitamin A, 5 mg zinc, 30 mg vitamin C, and 0.15 mg folic acid) and the low-iron MNP (identical except for 5 mg Fe as ferrous fumarate). The MNPs, manufactured by Manisha Pharmoplast Ltd, Gujarat, India, were packed in group-coded silver-colored identical sachets. Further information on randomization and group allocation is provided in Rahman et al. [20]. Children who took antibiotics within 2 months before enrollment and during the intervention period, children who took MNP/iron supplement within 2 months before enrolment, and children consuming MNPs below a specified amount ( $< 50$  sachets) during the intervention were excluded from consideration in the study.

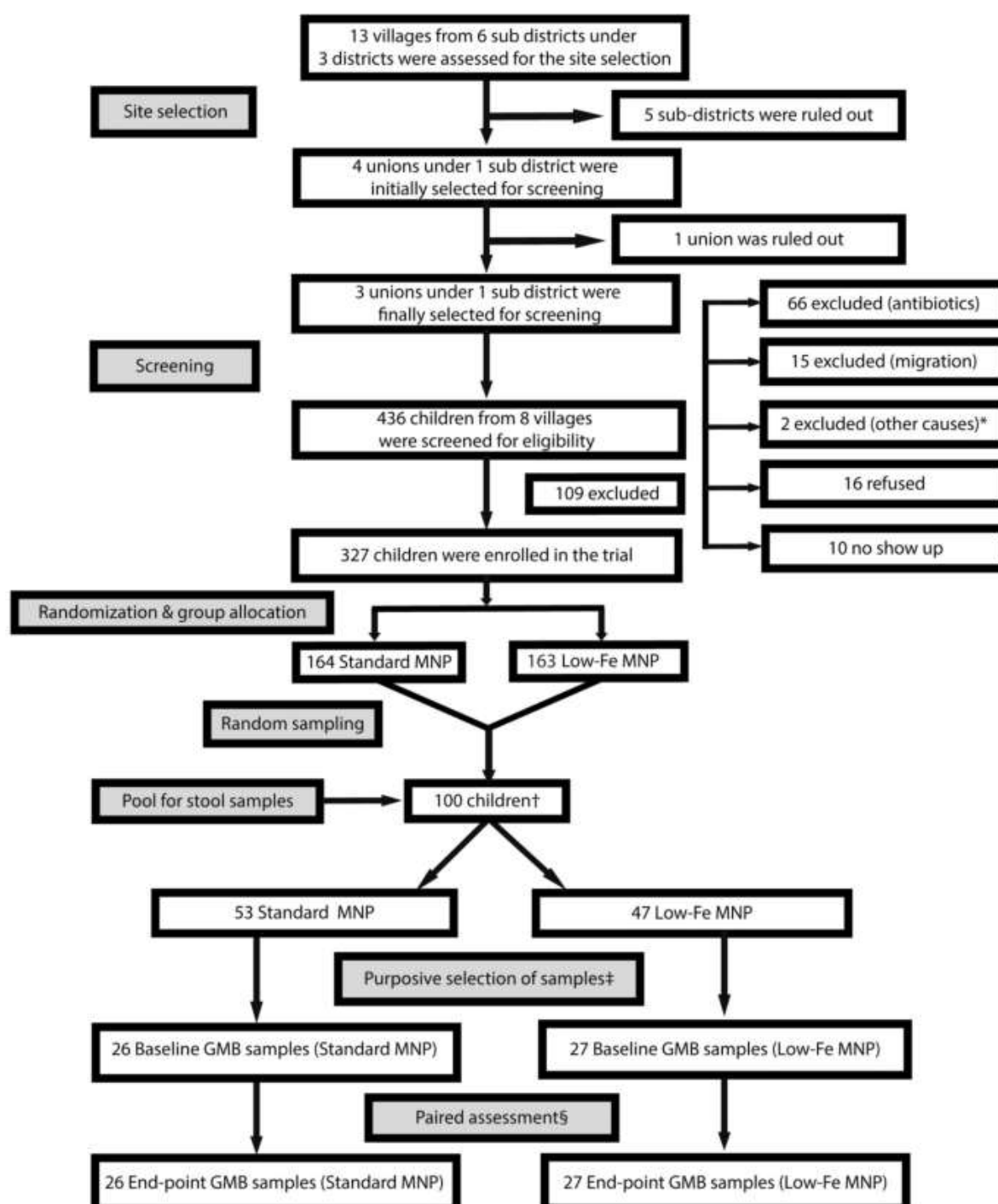
Written informed consent for the children's participation was provided by their parents.

## Sample size, sampling and the procedure

We considered 25–30 subjects per group would be adequate for comparison of the dominant bacteria based on previous studies [14, 22]. Hence, the required sample size was 50 for the two groups (standard MNP and the low-iron MNP). One hundred children were selected randomly from the enrolled children ( $n = 327$ ) before the start of the intervention to form a pool of the stool sample for gut microbiota assessment. A higher number of stool samples than required ( $n = 50$ ) was done to buffer for the subsequent exclusion of cases (children) who took antibiotics and/or consumed  $< 50$  sachets of MNP over the 60-day intervention period. Fifty sachets, which translated to a compliance rate of  $\sim 84\%$ , was determined based on a trial in Bangladeshi children that studied the efficacy and side effects of MNPs, reporting a  $\sim 85\%$  adherence [7]. Following the intervention at the endpoint, it was found that 53 children had consumed  $\geq 50$  MNP sachets (26 standard MNP and 27 low-iron MNP). The remaining 47 had either consumed antibiotics or had consumed fewer than 50 sachets, rendering them ineligible for gut microbiota assessment. The 53 children who had not taken antibiotics during the intervention period and had consumed  $\geq 50$  sachets of MNPs were

considered for gut microbiota assessment. Paired (baseline and endpoint) assessment of the samples resulted in a total of 106 stool samples being analyzed for gut microbiota (Fig. 1).

Fig. 1



Selection of the children for gut microbiota assessment. \* Out of the stipulated age ( $n = 1$ ); a tumor was found the abdomen ( $n = 1$ ). †One hundred children were selected from the enrolled children ( $n = 327$ ) randomly prior to the initiation of the MNP intervention as a pool to collect the stool samples from. This number was higher than the number required for the gut

microbiota assessment. The priori consideration of higher number of the samples was done to buffer for the subsequent exclusion of cases in the cases of antibiotic would be taken by the children; and that they would consume MNPs at and above a specified level. <sup>‡</sup>Purposive selection of samples was done after the endpoint data collection for the children not taking antibiotic during MNP intervention and consuming  $\geq 50$  sachets of MNP for microbiota assessment. <sup>§</sup>Baseline samples are paired to the endpoint samples. *MNP* Micronutrient Powder, *GMB* Gut Microbiota

Before the intervention, mothers were shown how to mix rice with MNPs to feed to their children. Mothers were also instructed to feed their children one sachet of MNP every day for 60 days. During the 2-month intervention period, the children were visited each week by field personnel to record the occurrence of any illness including diarrhea, loose stools, nausea, vomiting, fever and acute respiratory infection over the preceding week. Compliance was assessed each week by the field personnel, who recorded the intake of the previous week's MNPs by counting returned empty and intact sachets. During each visit, 10 sachets of MNP were provided to the mother to last until the next visit. Detailed procedures of the study are described elsewhere [20]. The high consumption (i.e.,  $\geq 50$  sachets) of MNPs was considered for the analysis to enable that a fair amount of the iron supplement was consumed to induce an effect on the gut microbiota, since the duration of the intervention was relatively short, i.e., 2 months.

Iron concentration in the groundwater sample was measured using a Handheld Colorimeter (HI721 Checker<sup>®</sup> HC (Hanna Instruments, USA), with a range between 0.0 and 5.0 ppm; a resolution of 0.01 ppm, and an accuracy  $\pm 0.04$  ppm  $\pm 2\%$  of the readings. Serum ferritin was measured by electrochemiluminescence immunoassay (ECLIA) on an automated immunoassay analyzer (Cobas C311; Roche Diagnostics, Mannheim, Germany), using a commercial kit according to the manufacturer's instruction (Roche Diagnostics, GmbH, 68,305 Mannheim, Germany). Serum C-Reactive Protein (CRP) and 1- $\alpha$  Acetylated Glycoprotein (AGP) were determined by the particle enhance immunoturbidimetric assay on an automated, software-controlled clinical chemistry analyzer (Cobas c311, Roche Diagnostics GMBH, Mannheim 68,305 Germany) using commercial kits.

### Stool sample collection

The mothers of the selected children were briefed on how to collect stool samples, and were advised to have each child defecate on a square piece of paper that was provided for the purpose. Mothers were cautioned that the stool sample should not be mixed with urine. Immediately after the child had defecated, the mother folded the paper to cover the sample and then phoned a field attendant who collected the sample within 30–40 min. After discarding the top layer, the attendant used a sterile swab stick to collect  $\sim 5$  g of the stool sample from the mid-layers of the mass in a sterile stool pot. The pot was capped and labelled with the sample ID and returned to the field laboratory in an ice box. The left-over stool sample was disposed of in the household's toilet. At the field laboratory, the samples were refrigerated overnight at 3–4 °C, and dispatched in an ice-gel cool box to the laboratory in Dhaka early next morning. At the laboratory, the samples were homogenized on the same day, and aliquots were prepared with  $\sim 0.5$  g of the homogenized sample in a cryovial,

labelled and stored in a  $-80^{\circ}\text{C}$  freezer. For DNA separation and sequencing, the homogenized stool samples were sent in dry ice to NIZO in the Netherlands.

#### Bacterial DNA extraction, PCR amplification and 16S rRNA gene Illumina sequencing

Fecal samples were first thawed at  $4^{\circ}\text{C}$ . Then, in a 2.0-mL screw-cap tube containing 0.5 g of 0.1-mm sterilized zirconia beads, 250 ( $\pm 10\%$ ) mg of feces and 700  $\mu\text{L}$  S.T.A.R. buffer (Roche, Indianapolis, IN, USA) were added. The FastPrep instrument (MP Biomedicals, Santa Ana, CA, USA) was used for lysis at 5.5 ms for 3 times 1 min at room temperature. Thereafter, the samples were incubated while shaking at 100 rpm and  $95^{\circ}\text{C}$  for 15 min. The samples were then centrifuged at  $16,000\times g$  for 5 min at  $4^{\circ}\text{C}$ . The collected supernatant was kept on ice, and the lysis round was repeated once more as described above, except that only 350  $\mu\text{L}$  S.T.A.R. buffer was added, with the remaining stool pellet. The supernatant kept on ice was then pooled with the supernatant from the second lysis round. Purification of DNA was performed on the automated Maxwell instrument (Promega, Madison, WI, USA) by applying the Maxwell 16 Tissue LEV Total RNA Purification Kit (Promega) according to the manufacturer's instructions. To the first well of the Maxwell cartridge 250  $\mu\text{L}$  of the supernatant was added and finally, DNA was eluted with 50  $\mu\text{L}$  of RNase/DNase free water.

Using a 2-step PCR, barcoded amplicons from the V3–V4 region of 16S rRNA genes were generated (see library PCR below for a description of the second PCR step). For initial amplification of the V3–V4 part of the 16S rRNA universal primers with the following sequences were used: forward primer, *5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGAGGCAGCAG'* (broadly conserved bacterial primer 357F in bold and underlined); reverse primer, *5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTACNVGGGTATCTAAKCC'* (broadly conserved bacterial primer 802R (with adaptations) in bold and underlined), appended with Illumina adaptor sequences (in italics). The PCR amplification mixture contained: 1  $\mu\text{L}$  fecal sample DNA and 49  $\mu\text{L}$  master mix (1  $\mu\text{L}$  KOD Hot Start DNA Polymerase (1 U/ $\mu\text{L}$ ; Novagen, Madison, WI, USA), 5  $\mu\text{L}$  KOD-buffer (10 $\times$ ), 3  $\mu\text{L}$   $\text{MgSO}_4$  (25 mM), 5  $\mu\text{L}$  dNTP mix (2 mM each)), 1  $\mu\text{L}$  forward primer (10  $\mu\text{M}$ ), 1  $\mu\text{L}$  reverse primer (10  $\mu\text{M}$ ) and 33  $\mu\text{L}$  sterile water (total volume 50  $\mu\text{L}$ ). PCR conditions were:  $95^{\circ}\text{C}$  for 2 min followed by 30 cycles of  $95^{\circ}\text{C}$  for 20 s,  $55^{\circ}\text{C}$  for 10 s, and  $70^{\circ}\text{C}$  for 15 s. The approximately 500 bp PCR amplicons were then purified using the MSB Spin PCRapace kit (Invitek, Berlin, Germany). For the second PCR in combination with sample-specific barcoded primers, purified PCR products were shipped to BaseClear BV (Leiden, The Netherlands). PCR products were checked on a Bioanalyzer (Agilent) and quantified. This was followed by multiplexing, clustering and sequencing on an Illumina MiSeq with the paired-end (2 $\times$ ) 300 bp protocol and indexing. FASTQ read sequence files were generated using bcl2fastq2 version 2.18. Initial quality assessment was based on data passing the Illumina Chastity filtering. From the raw sequencing data, the sequence reads of too low quality (only “passing filter” reads were selected) were discarded and reads containing adaptor sequences or PhiX control were removed with an in-house filtering protocol. On the remaining reads, quality assessment was performed using the FASTQC tool version 0.11.5.

#### 16S rRNA gene sequence analysis and statistics

16S rRNA gene sequences were analyzed using a workflow based on Qiime 1.8 [23]. We performed operational taxonomic unit (OTU) clustering (open reference), taxonomic assignment and reference alignment with the `pick_open_reference_otus.py` workflow script of Qiime, using `uclust` as clustering method (97% identity) and GreenGenes v13.8 as the reference database for taxonomic assignment. Reference-based chimera removal was done with `Uchime` [24]. The RDP classifier version 2.2 was performed for taxonomic classification [25]. Statistical tests were performed as implemented in SciPy (<https://www.scipy.org/>), downstream of the Qiime-based workflow.

### Statistical analysis

Characteristics of the participants, e.g., (age, ferritin status, CRP, AGP, and total intake of iron) and the concentration of iron in drinking water were presented as mean  $\pm$  SD and median with interquartile ranges. The mean estimates were compared between the study groups by student's *t* test. We tested for between-group differences in alpha-diversity (PD whole tree metric), phylogenetic distance (weighted UniFrac), and abundance of the taxa of primary interest (*Bifidobacterium*, *Enterobacteriaceae*, *Lactobacillus* and *Clostridiales*) without correction for multiple testing. In the bivariate explorative analysis of all taxa, the Mann–Whitney *U* test with FDR correction for multiple testing was applied to assess differences between the two groups. For comparisons of more than two groups, the non-parametric Kruskal–Wallis test with Dunn's post hoc test was applied. For longitudinal analysis, the change of taxon relative abundance over time, 2log ratios were calculated, in which the relative abundance of a taxon at endpoint was divided by the relative abundance of the same taxon at baseline. Ratios were compared between groups by Mann–Whitney *U* tests with FDR correction for multiple testing.

We performed redundancy analyses (RDAs) on the gut microbiota composition as assessed by 16S rRNA gene sequencing in Canoco version 5.11 using default settings of the analysis type “Constrained” [26]. Relative abundance values of genera or OTUs were used as response data and metadata as the explanatory variable. For visualization purposes, families (and not OTUs) were plotted as supplementary variables. The microbiome age of a child at baseline was determined by RDA in which genera were response variables and calendar age was an explanatory variable. The x-coordinates of the cases (baseline samples) reflected the microbiome age (i.e., older children would have a more adult-like microbiota profile). The participants were divided by age group using the median value of the age distribution. The lower 50% of the cases were grouped in the “young” microbiome category and the higher 50% of the cases were grouped in the “older” microbiome category. Longitudinal effects of the intervention were assessed by calculating 2log ratios in which the relative abundance of an OTU or genus at endpoint was divided by the relative abundance of the same OTU or genus at baseline. These ratios were used as response variables in RDAs and were weighted based on the average relative abundance of each OTU in all infants. RDA calculates *p* values by permutating (Monte Carlo) the sample status. Partial RDA was employed to account for covariance attributable to age (always); age was first fitted in the regression modeling and then partialled out (removed) from the ordination as described in the Canoco 5 manual [26]. In all analyses, *p* values  $< 0.1$  were considered modest statistical evidence [27]; *p* values  $< 0.05$  were considered statistically significant.

Children's age, biochemical and morbidity characteristics were compared between the groups by the independent sample *t* test.

## Results

At baseline, the treatment groups were similar with regard to the children's age, mean concentrations of serum ferritin, CRP and iron concentration of groundwater (Table 1).

Table 1 Comparison of the groups with regard to children, biochemical characteristics, water iron concentration, total iron intake and morbidities between the treatment groups at baseline and over the intervention period

	Standard MNP (n, 26)		Low-iron MNP (n, 27)		<i>p</i> -value**
	Mean $\pm$ SD	Median (IQR)	Mean $\pm$ SD	Median (IQR)	
Baseline					
Age (months)	42.8 $\pm$ 7.7		42.1 $\pm$ 7.8		0.74
Iron concentration in groundwater (mg/L)	14.9 $\pm$ 3.4	4.4(3.7,12.6)	13.1 $\pm$ 3.1	4.2(3.5,8.2)	0.07
Serum ferritin (ng/ml)	82.9 $\pm$ 36.9	81.9(56.3,96.8)	71.4 $\pm$ 32.7	69.3(54.2,94.6)	0.30
CRP (mg/L)	2.0 $\pm$ 2.6	1.0(0.3,2.8)	1.9 $\pm$ 5.5	0.3(0.3,0.5)	0.97
AGP (mg/dl)	83.6 $\pm$ 31.0	75.0(62.0,74.0)	65.4 $\pm$ 17.1	62.5(55.0,73.0)	0.02
Over the Intervention					
Total Fe intake* (mg/day)	20.9 $\pm$ 5.6	18.7(17.2,22.4)	12.5 $\pm$ 5.1	11.5(9.5,14.6)	<0.001
Mean episodes of loose stool	1.65 $\pm$ 5.7	0(0,0)	1.48 $\pm$ 3.3	0(0,0)	0.89

\* Intake of iron from the combined sources of diet, groundwater and MNPs

\*\**p*-values in relation to the mean difference

Serum AGP at baseline was higher in the standard MNP group; 83.6  $\pm$  31.0 mg/dL vs. 65.4  $\pm$  17.1 mg/dL (*p* = 0.02), but most values were below the threshold of 100 mg/dL (values > 100 mg/dL indicate infection). The intake of iron from all sources (Fe from the diet,

MNP and groundwater) over the intervention period was  $20.9 \pm 5.6$  mg/d and  $12.5 \pm 5.1$  mg/d in the standard and the low-iron MNP groups, respectively ( $p < 0.001$ , Table [1](#)). The mean number of episodes of loose stool over the intervention period was  $1.65 \pm 5.7$  and  $1.48 \pm 3.3$ , respectively ( $p = 0.89$ ).

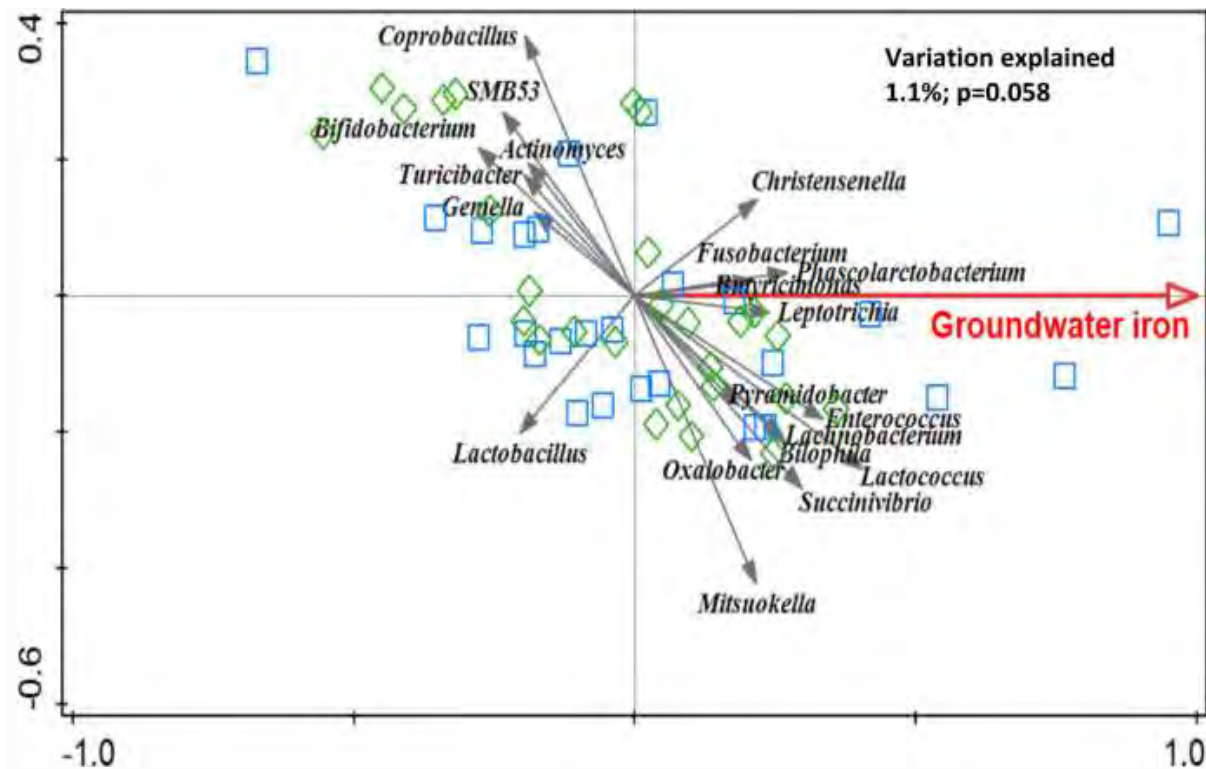
#### General attributes of the microbiota samples

The average number of sequencing reads count per sample was 40,832. At baseline, there was a significant association between microbiota composition and calendar age (RDA; variation explained 1.3%,  $p = 0.05$ ). Age was, therefore, considered as a covariate in subsequent multivariate analysis. Within-sample diversity, i.e., alpha-diversity, was not significantly different between the standard and the low-iron MNP groups at baseline and endpoint [Supplementary Fig. 1].

#### Association of iron concentration of tube-well water with gut microbiota composition

RDA at baseline showed that iron concentration of tube-well water (groundwater) was associated with the gut microbiota composition; variation explained 1.1% with a modest statistical evidence ( $p = 0.058$ , Fig. [2](#)). *Bifidobacterium* and *Lactobacillus* were negatively associated with iron concentration.

Fig. 2

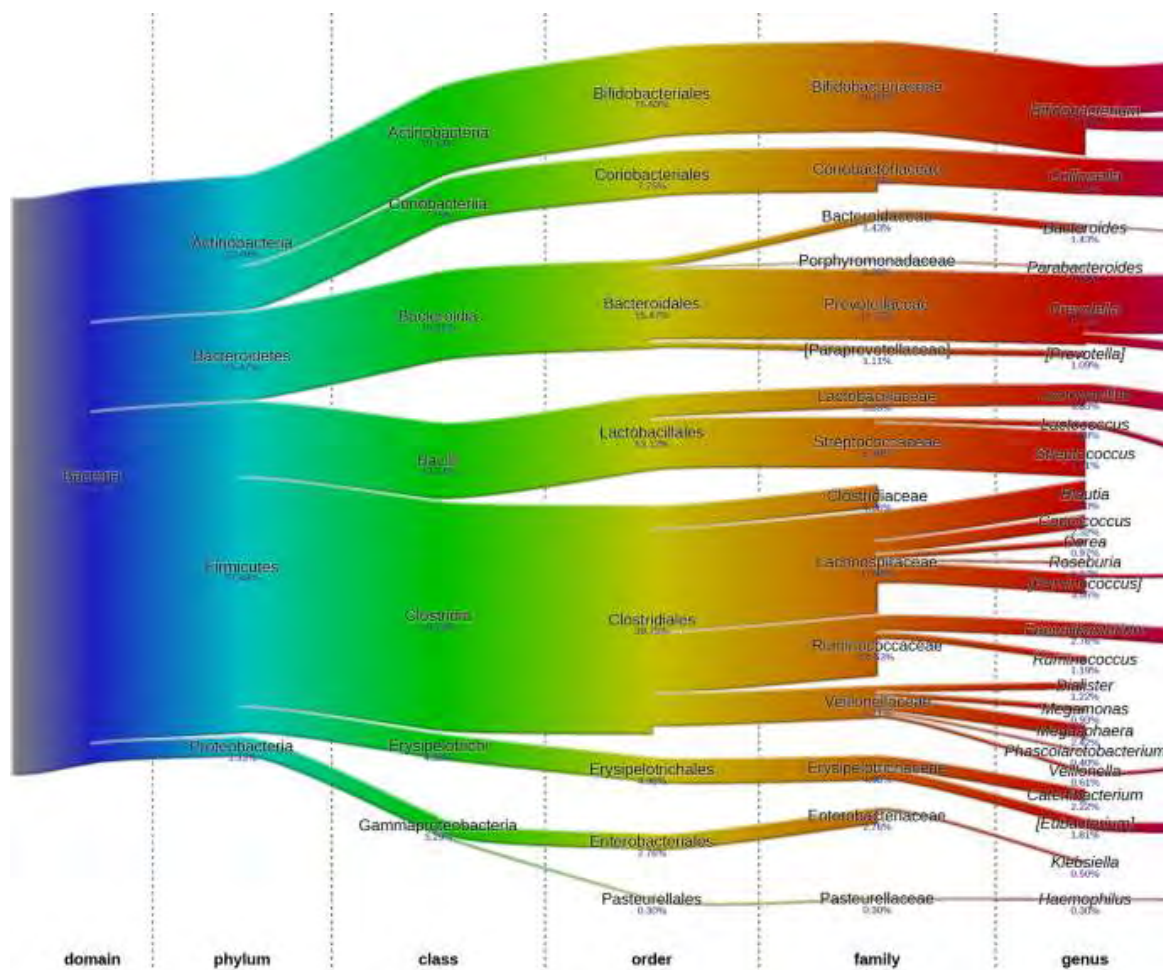


Redundancy analysis (RDA) on the genus level, assessing the effect of the concentration of iron in groundwater on gut microbiota composition at baseline. Genera were used as response data and groundwater iron concentration was explanatory data. Variation explained by groundwater iron concentration was 1.1%,  $p = 0.058$ . Blue squares indicate samples from the standard MNP group and green diamond samples represent children assigned to the low-iron MNP group

#### Baseline profile of gut microbiota and the overall treatment effect

Average microbiota composition at baseline consisted of, among others: *Lachnospiraceae* 17.9%, *Bifidobacteriaceae* 15.6%, *Prevotellaceae* 12.2%, *Streptococcaceae* 8.8%, *Clostridiaceae* 4.1%, *Lactobacillaceae* 3.8%, and *Enterobacteriaceae* 2.8% (Fig. 3). There was no significant treatment effect on the overall microbiota composition as assessed by cross-sectional RDA and longitudinal RDA. Particularly, there was no effect on the relative abundance of *Enterobacteriaceae* and *Bifidobacteriaceae*. The relative abundance of *Enterobacteriaceae* at baseline, endpoint (Fig. 4a) and its relative changes over time (2log ratio) was not different between the treatment groups (Fig. 4c). Similarly, the relative abundance of *Bifidobacteriaceae* at baseline and endpoint (Fig. 4b) and its change over time (2log ratio) were not significantly different between the groups (Fig. 4d). Besides, there was no significant treatment effect on the 2log ratio of *Enterobacteriaceae/Bifidobacteriaceae* at endpoint (Fig. 4e). In the absence of a placebo group, microbiota composition was also compared between baseline and endpoint; there were no significant differences for the whole study population (both MNPs) and also not within the low- and standard-dose groups separately.

Average composition of the gut microbiota in Bangladesh rural children aged 2–5 years at baseline, exposed to a high-level iron acquired from drinking groundwater. The fraction of 16S rRNA reads (in %) attributed to specific taxonomic level is given below the taxon name. Figure was generated using software described in Sandquist et al. [28]



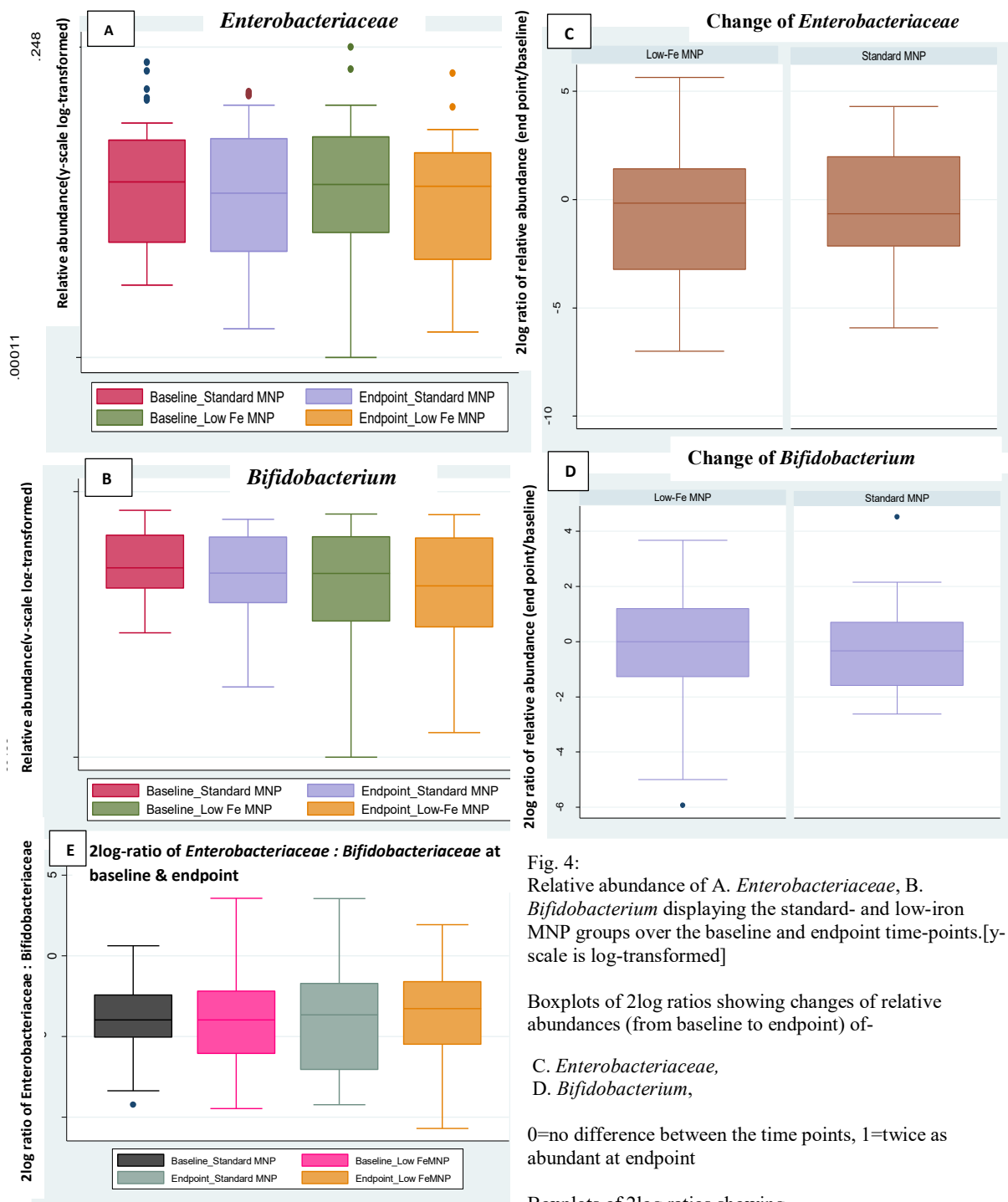


Fig. 4: Relative abundance of A. *Enterobacteriaceae*, B. *Bifidobacterium* displaying the standard- and low-iron MNP groups over the baseline and endpoint time-points.[y-scale is log-transformed]

Boxplots of 2log ratios showing changes of relative abundances (from baseline to endpoint) of-

C. *Enterobacteriaceae*,  
 D. *Bifidobacterium*,

0=no difference between the time points, 1=twice as abundant at endpoint

Boxplots of 2log ratios showing,  
 E. *Enterobacteriaceae* : *Bifidobacteriaceae* at baseline & endpoint

### Treatment effect on the gut microbiota at the subgroup level based on microbiome age

In the present study, the age of children varied between 24 and 59 months; the microbiota of younger children might respond differently compared to the microbiota of older children. However, RDA showed no difference in microbiota composition between the low-iron MNP and the standard-iron MNP at the endpoint in the younger age group or in the older age group (age groups were determined by dividing the children into two equal groups based on calendar age). As some younger children might have a relatively adult-like microbiota profile and some older children might have young microbiota, a microbiome age of each child was determined at baseline. Subsequently, children were categorized as either having a relatively young microbiota or a relatively old (adult-like) microbiota. RDA showed that MNP treatment was associated with gut microbiota composition in the old-microbiome group at the endpoint (variation explained 3.49%,  $p = 0.014$ ) [Supplementary Fig. 3], but not in the young-microbiome group (0.0%,  $p > 0.05$ ).

Using bivariate analysis on the old-microbiome group, the relative abundance of *Bifidobacterium* and *Lactobacillus* in the standard MNP group at endpoint appeared to be higher compared with the low-iron MNP group; however, the difference was not statistically significant (*Bifidobacterium*,  $p = 0.116$ ; *Lactobacillus*,  $p = 0.13$ ) (Fig. 5). The relative abundance of *Enterobacteriaceae* at endpoint appeared higher in the standard MNP group compared to the low-iron MNP group [ $p = 0.076$ , (Fig. 6a)]. The relative abundance of *Clostridiales* was higher at endpoint in low-iron MNP group compared to the standard MNP group [ $p = 0.028$ , (Fig. 6b)]. In contrast, within the young microbiota group, there were no statistically significant treatment effects on these taxa of primary interest.

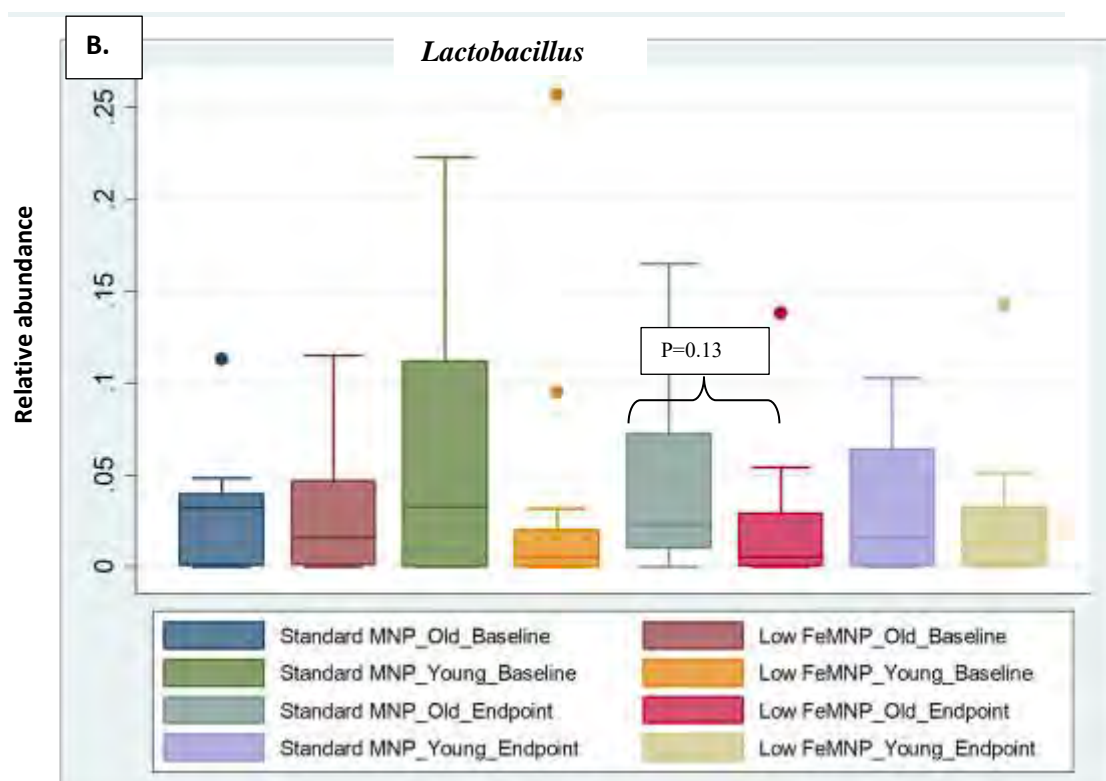
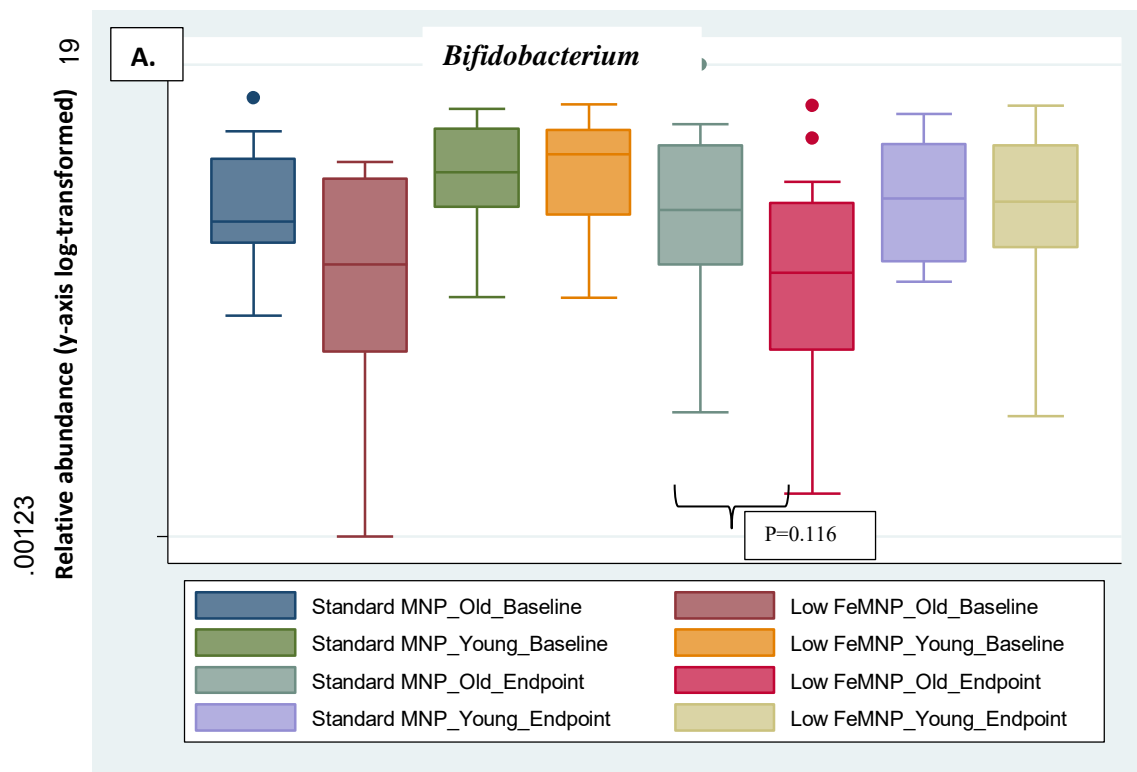


Fig. 5: Relative abundance of A. *Bifidobacteriaceae*, B. *Lactobacillus* sorted by old- and young-age microbiota, comparing standard MNP and the low-iron MNP over baseline and endpoint time-points. Relative abundance was slightly higher at endpoint in standard MNP-Old microbiome compared to low-iron MNP-Old microbiome groups, both for *Bifidobacteriaceae* ( $p=0.116$ ) and *Lactobacillus* ( $p=0.13$ ); but statistically non-significant

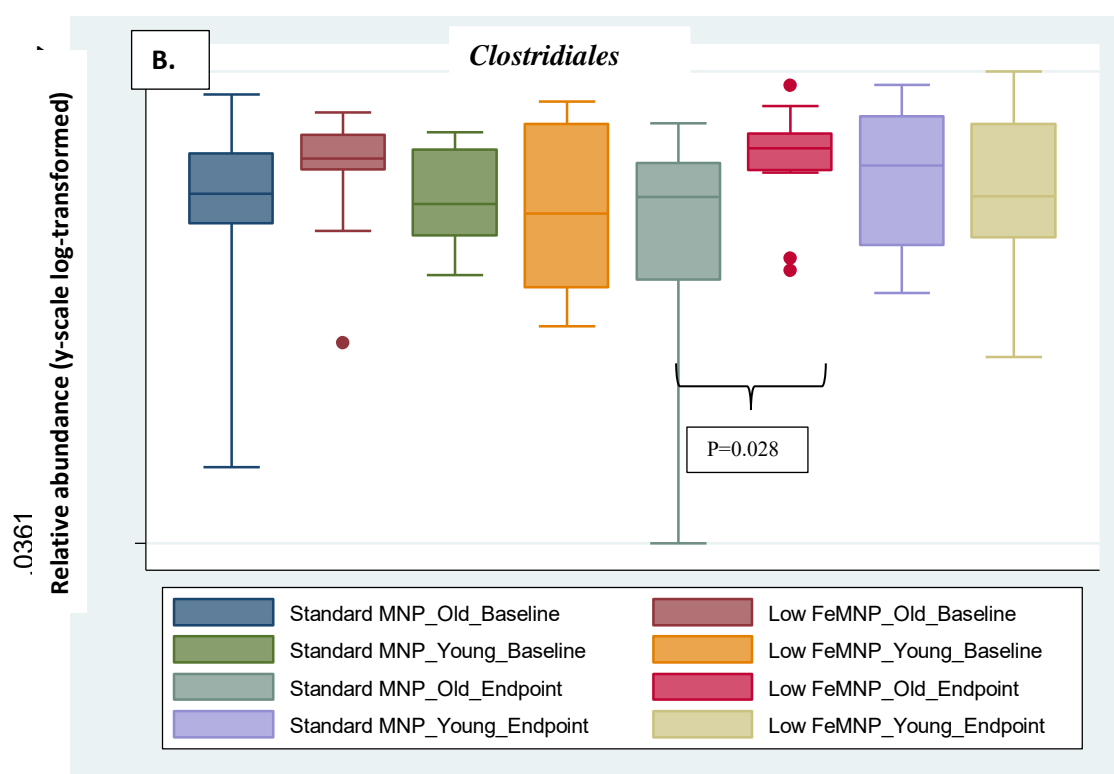
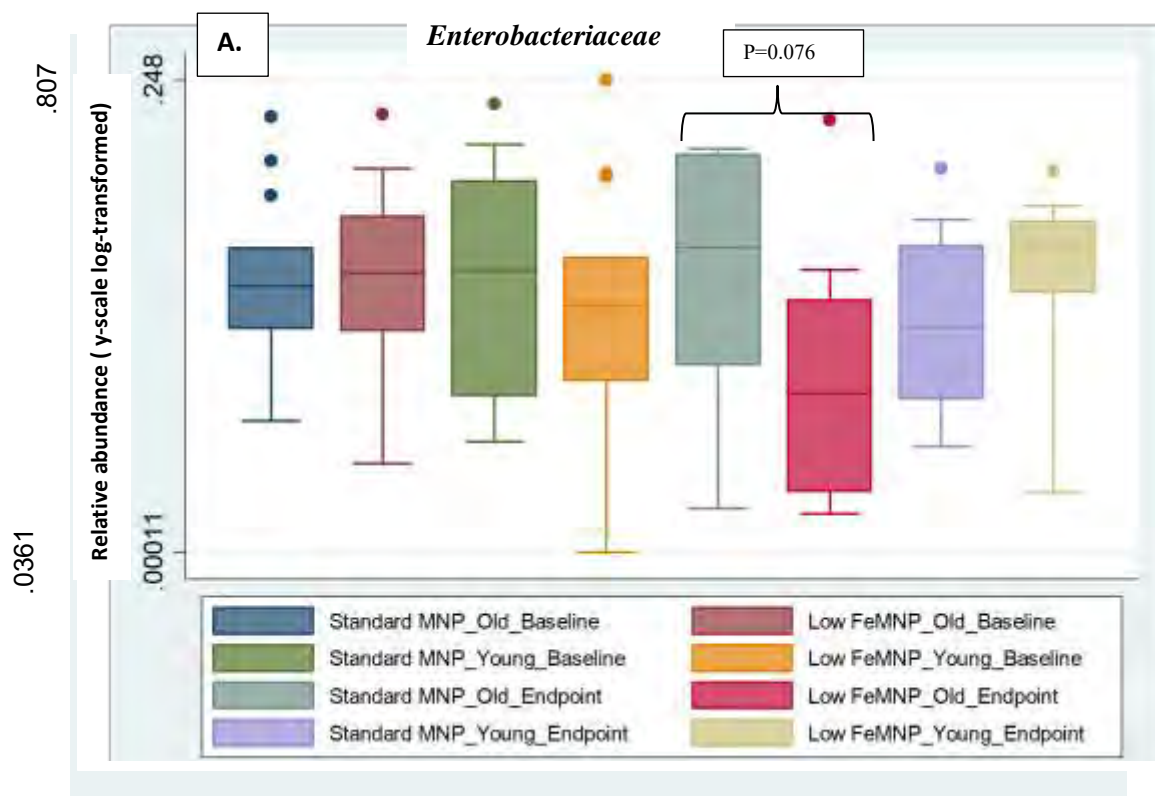


Fig 6: Relative abundance of a *Enterobacteriaceae*, b *Clostridiales* sorted by old- and young-age microbiota, comparing standard MNP and the low-iron MNP over baseline and endpoint time-points. The relative abundance of *Enterobacteriaceae* was slightly higher at endpoint in the standard MNP-Old microbiome age group than in the low-iron MNP old-microbiome

groups ( $p = 0.076$ ). Low-iron MNP-old microbiome age group had higher relative abundance of *Clostridiales* at endpoint than in the standard MNP old-microbiome age group ( $p = 0.028$ )

Using RDA, microbiota composition between baseline and endpoint was compared within the old-microbiota-standard MNP group, which showed modest evidence of statistical difference; variation explained 2.63% ( $p = 0.088$ ). The endpoint was associated with *Bifidobacteriaceae*, *Lactobacillaceae* and *Enterobacteriaceae* (Fig. [7](#)).

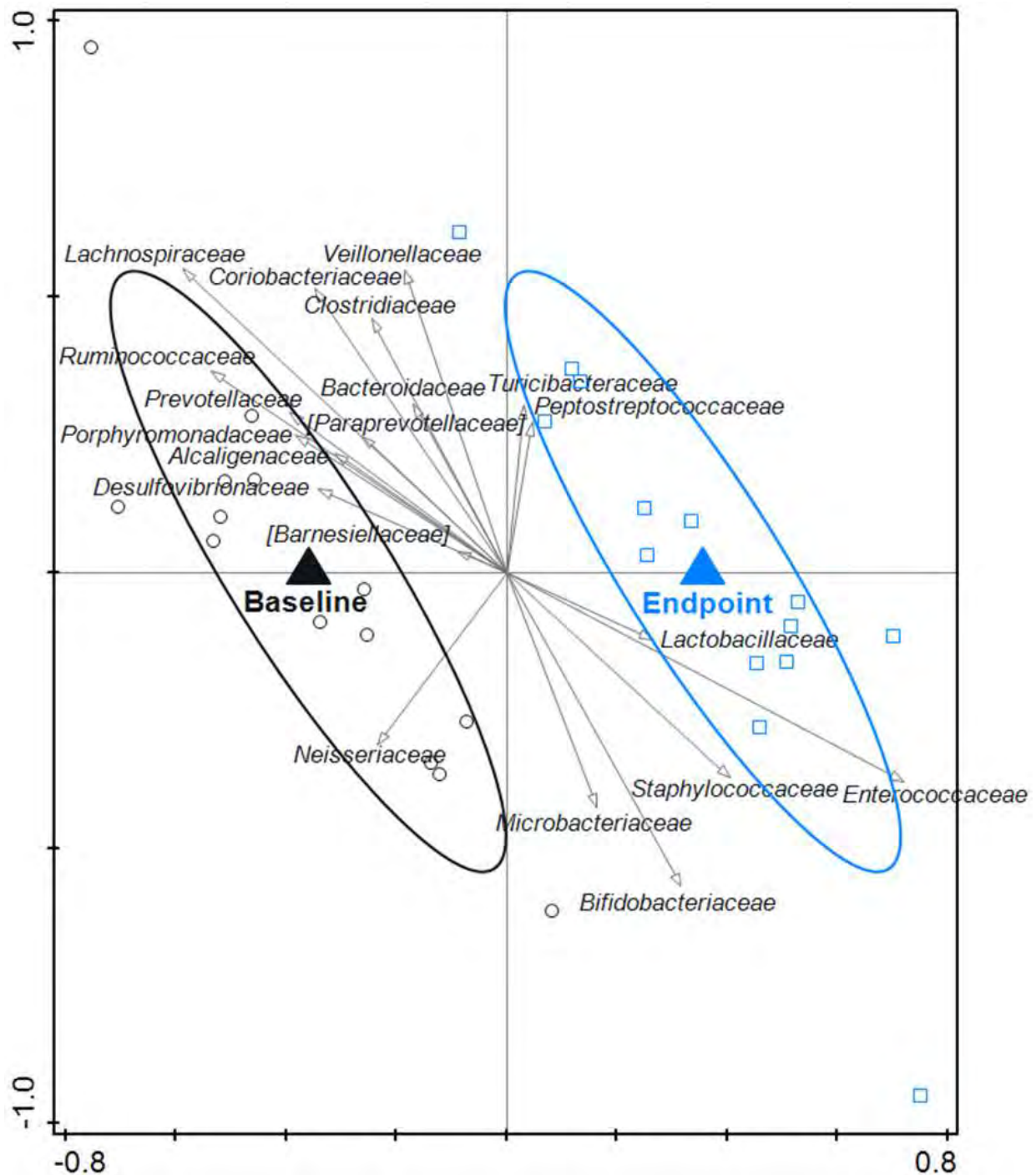


Fig. 7: RDA on the OTU level, assessing the within-group effect of the standard MNP on the gut microbiota composition in the old microbiome-Standard MNP group. OTUs were used as response data and time point was explanatory data, the bacterial families that contributed most were plotted supplementary. The covariance attributable to subject was first fitted by regression and then partialled out (removed) from the ordination. Variation explained by time point was 2.6%,  $p=0.088$ . The end-point was associated with *Bifidobacteriaceae* and *Lactobacillaceae*

There was no association observed between the iron status markers (hemoglobin and ferritin) and the composition of the microbiota at baseline and endpoint, as analyzed by RDA. At baseline, CRP, a biomarker for acute infection/inflammation, was associated with the gut microbiota composition; variation explained 1.88% ( $p = 0.04$ ). CRP was associated with, e.g., *Peptostreptococcaceae* (a family that includes *Clostridium difficile* [29] but not with *Enterobacteriaceae*). Similarly, AGP, a biomarker for chronic infection/inflammation, was associated with the baseline gut microbiota composition with modest statistical evidence; variation explained 1.09% ( $p = 0.07$ ) [Supplementary Fig. 2].

## Discussion

The present study explored the effect of a low-iron MNP compared to the standard MNP on the gut microbiota composition of Bangladeshi children exposed to a high amount of natural iron acquired from drinking groundwater. The iron doses in the low-iron MNP and the standard MNP were 5 mg and 12.5 mg per sachet, respectively, and were consumed by the children at one sachet per day for 2 months. The children of both groups consumed 84–100% of the doses (data not shown). Mean concentration of iron in groundwater was ~13 mg/L, which was several-fold higher than the cutoff (2 mg/L) [30].

In the present study, the baseline relative abundance of *Bifidobacteriaceae* was 15.6%, which was much lower than that reported in the studies of Kenyan infants (63–65%) conducted by Jaeggi et. al. [15] and Paganini et. al. [16]. The main reason for the difference is probably the age of the studied populations. The participants were infants in the African studies [15, 16] while the mean age  $\pm$  SD of the present study participants were  $43.5 \pm 7.7$  months. During infancy, the preponderance of *Bifidobacteriaceae* is linked with breast milk [31], which is the predominant form of food. The observation of a reasonable presence of *Prevotella* (12.2%) in the present study can be explained by the predominantly cereal-based, fiber-rich diet [32] which is the dietary characteristic in this setting. *Prevotella* is known for polysaccharide hydrolysis of the fibrous residue in the intestines. The baseline level of *Enterobacteriaceae* (2.8%) was similar to the results of the African studies (2.40–3.54%) [15, 16].

At baseline, the concentration of iron in groundwater was negatively associated with the relative abundance of the generally beneficial microbiota members *Bifidobacterium* and *Lactobacillus*. Drinking groundwater with a high concentration of iron is expected to increase the amount of iron in the intestines. Therefore, this observation is consistent with earlier studies [14,15,16] as iron tends to suppress the growth of these beneficial bacteria. Following the intervention, the combined intake of iron from MNP and groundwater at endpoint was not associated with the microbiota composition. A possible explanation for this is the fact that, at the endpoint, the intestinal load of iron was increased from consumption of MNPs in both groups. As a result, the association of a high load of iron in the intestine and the lower relative abundance of *Lactobacillus* and *Bifidobacterium*, at baseline, was attenuated at endpoint.

Our study showed: (A) no overall treatment effects on gut microbiota composition; and (B) some treatment effects on the old-microbiome subgroup, i.e., an apparent higher relative abundance of *Bifidobacterium* and *Lactobacillus* in the standard MNP group compared to the

low-iron MNP group was observed. However, the difference was not statistically significant. For the potentially pathogenic *Enterobacteriaceae*, seeming modestly higher relative abundance ( $p = 0.076$ ) was observed in the standard MNP, which is consistent with the Kenyan and the Côte d'Ivoire studies [3,4,5] as the high level of iron flared up the pathogens. The apparent higher relative abundance of *Lactobacillus* and *Bifidobacterium* in the higher iron group was unexpected. The trend of a higher relative abundance of *Enterobacteriaceae* in the old-microbiome-standard MNP group in combination with slightly higher relative abundances of *Bifidobacterium* and *Lactobacillus* is not considered a clear adverse effect of the standard MNP. The observation of seemingly modest treatment effects in the old-microbiome group only is difficult to explain, warranting further research. The apparent effect on *Enterobacteriaceae* in the small old-microbiome group is supported by the significant RDA on the old-microbiome group ( $p = 0.014$ ; 3.5% variation explained), while RDA on the young-microbiome group was not significant ( $p > 0.05$ ; 0.0%).

Overall, we speculate that the older calendar age of the children compared to the Kenyan infants' studies could be one of the reasons for not finding the effects of MNP iron on the microbiota composition of the present study population. In the African studies assessing the effect of iron-fortified food and/or iron-containing MNP supplementation on the gut microbiota in infants, the salient observation was that iron supplements at various doses (2.5–20 mg) resulted in a significantly higher abundance of the (potentially) pathogenic microbiota, e.g., *Escherichia/Shigella*, *Clostridium* and *Enterobacteriaceae*, and pathogenic *E. coli* compared to “no-iron” and/or placebo intervention [14,15,16]. These studies further documented that the supplementation led to a suppression of the beneficial bacteria *Bifidobacterium* and *Lactobacillus*. The present study differed from these African studies in many ways. Such as the effect of two different doses of iron in MNP (12.5 mg vs. 5 mg) on the composition of the gut microbiota was compared in children aged 2–5 years old. Second, there was no placebo group. Third, the subjects were exposed to a high level of iron from groundwater, the natural drinking source; and finally, children taking antibiotic medicines during the intervention were excluded from microbiota assessment. Hence, different outcomes on the microbiota composition in the present study can be acceptable.

Similar to our study, a 38-week South African study providing 200 mg iron per week to 6- to 11-year-old children residing in a relatively malaria-free zone did not find any treatment effect on gut microbiota [33]. However, there were crucial differences in the design of that study to ours. In the South African study, the subjects were iron deficient, and the intervention was compared to a placebo. In a state of iron deficiency and low-infection burden, the supplements perhaps were absorbed well (Ferritin increased by ~threefold after the intervention). Hence, conceivably less iron might have remained unabsorbed in the intestines which could be consistent with the lack of iron-induced adverse effects on the gut microbiome composition. On the other hand, a plausible explanation for the absence of an overall treatment effect in the present study is the comparison of the two MNPs, both containing iron (albeit, in different doses) and the absence of a placebo group, together with chronic exposure to a high level of iron from drinking groundwater. For ethical reasons, the trial did not include a placebo group [20]. Of note, when we assessed the pre-post differences for both the MNP groups separately, no treatment effect on microbiota composition was observed.

As stated elsewhere, excess iron might affect the gut microbiota adversely, resulting in morbidities. Hence, we assessed clinical morbidities, such as the occurrence of loose stools,

in the treatment groups. The mean number of loose stools in the standard MNP and the low-iron MNP groups were  $1.65 \pm 5.78$  and  $1.48 \pm 3.33$ , respectively; the difference was not statistically significant.

Modest evidence of association of the infection biomarkers CRP and AGP with the composition of gut microbiota was observed at baseline, but not at endpoint. *Enterobacteriaceae* were expected to be positively associated with the infection biomarkers, but this was not substantiated by our analysis. The difference in AGP between groups at baseline is not believed to have influenced our findings. In both the groups, only a few subjects ( $n = 2$  in the low-iron MNP and  $n = 3$  in the standard MNP group) showed AGP values slightly above the threshold of 100 mg/dL that indicates the presence of an infection.

In the parent trial, the low-iron MNP was non-inferior on hemoglobin response compared with the standard MNP and resulted in significantly fewer incidence of some key clinical morbidities, e.g., diarrhea, nausea and fever [20]. However, the gut microbiota assessment did not show a significant treatment effect on the overall gut bacterial composition, while some effects were observed in a subset of the population. Taking all these findings into consideration exemplifies that, despite there being fewer clinical morbidities from the low-iron MNP than the standard MNP, no clear comparative adverse effect of the standard MNP on gut microbiota composition was found. It is uncertain whether further lowering of the dose of iron ( $< 5$  mg) would result in a favorable influence on the gut microbiome in this setting, and whether the iron reduction might compromise the efficacy on hemoglobin outcome; this should be a subject of future research. Further research is also needed to document the effect of the 5 mg Fe MNP on the gut microbiome in children residing in predominantly low-groundwater-iron areas.

A limitation of the trial is that the final samples were selected purposively for microbiota assessment. As such, the generalizability of the finding is somewhat compromised. However, strengths of the study are that the purposive sampling enabled high compliance children to be selected, and antibiotic users excluded. The effect of the MNP treatments on the composition of the microbiota was, therefore, not influenced by the effect of antibiotic treatments. Of note, this selection might in part have contributed to the observed lack of effects on microbiota composition, as oral antibiotics have been described to modify the effect of iron-containing MNPs on the gut microbiota composition in infants [34].

In conclusion, in Bangladeshi children naturally iron-replete from drinking groundwater, there was no overall significant treatment effect of the low-iron MNP on gut microbiota composition compared with the standard MNP. However, in a subpopulation with relatively adult-like gut microbiota, a seemingly higher relative abundance of potentially pathogenic *Enterobacteriaceae* was observed in children who received the standard MNP. Although we do not consider this as a clear adverse effect, this finding indicates a need for further research into the response of child gut microbiota types to iron supplementation.

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#### Contributions

Study concept and design: S.R., F.A., P.L., and M.R.K.; acquisition of data: S.R.; analysis and interpretation of data: G.K., J.B., and S.R.; manuscript drafting: S.R.; all the authors were involved in revising the article critically for important intellectual content, and all the authors approved the final version to be published.

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#### Ethics declarations

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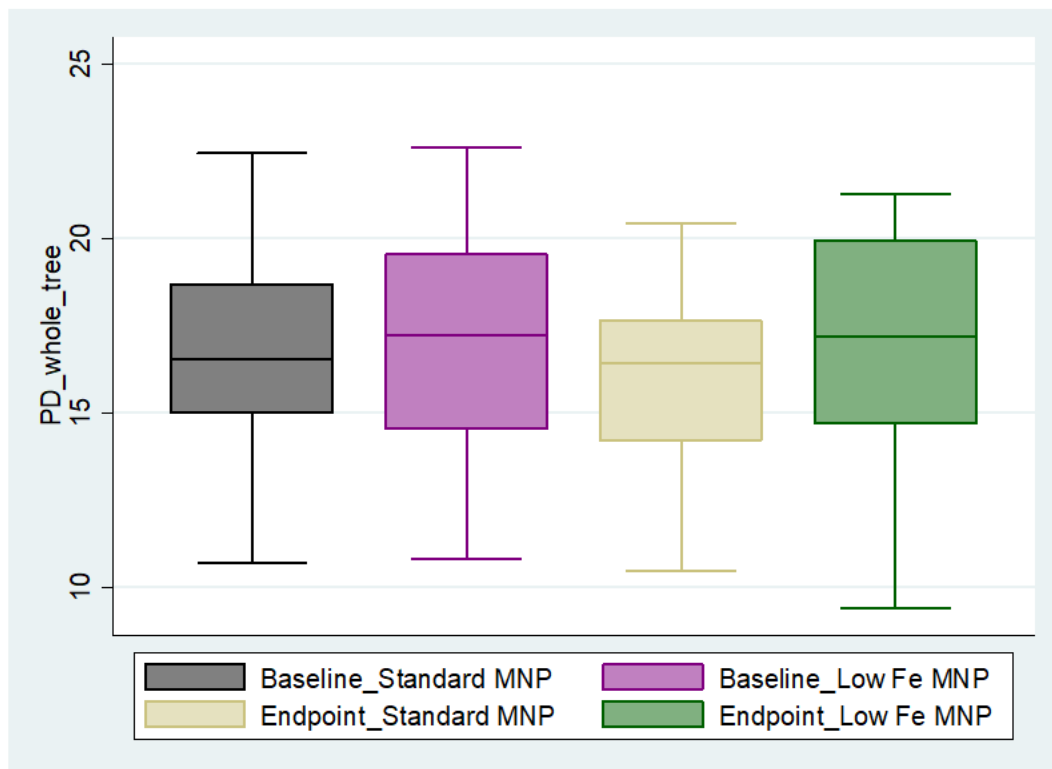
#### Conflict of interest

The authors declare no competing interests.

#### Ethical approval

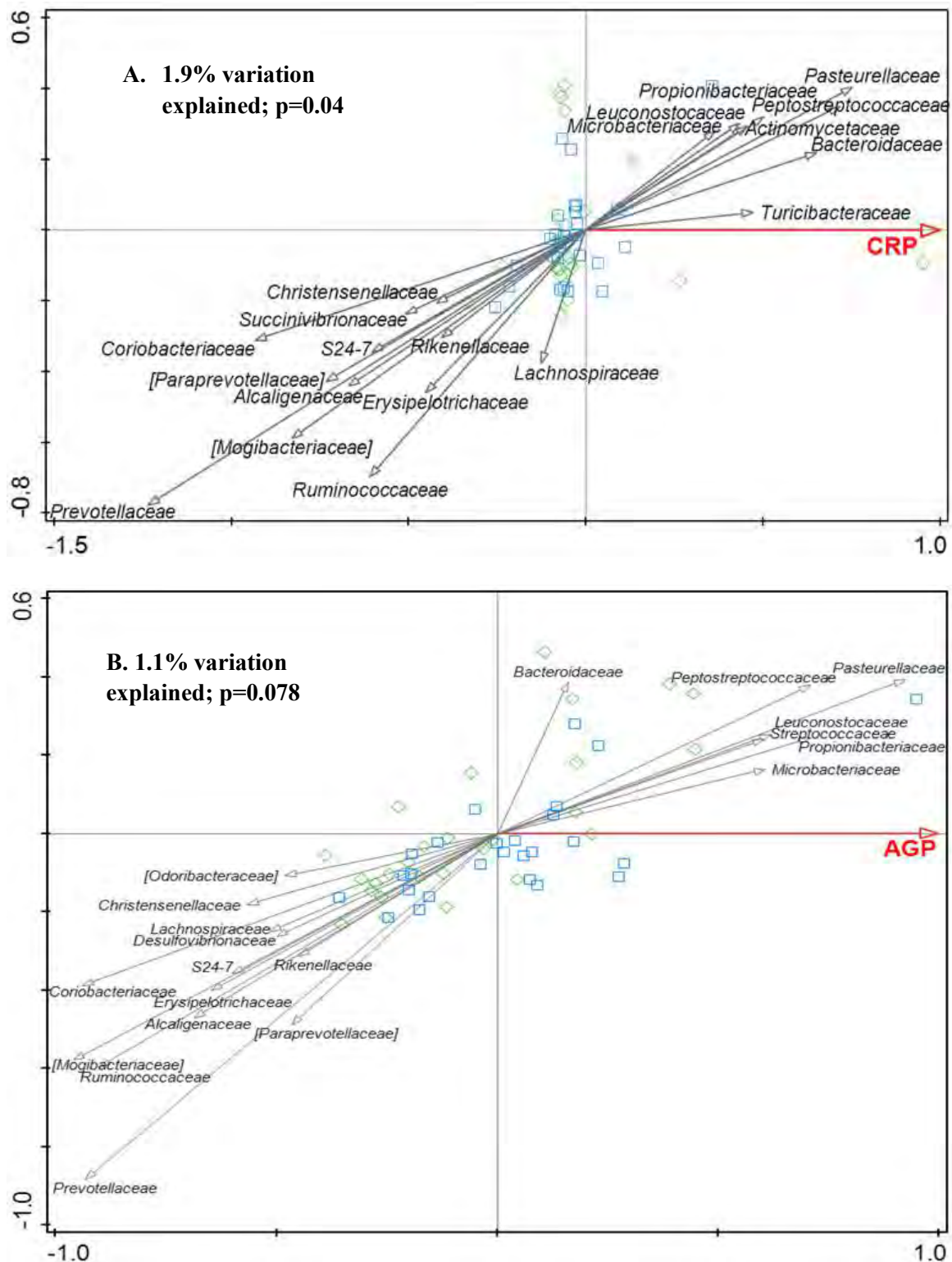
The trial received ethical approvals from the Faculty of Biological Science, the University of Dhaka, Bangladesh (Ref# 46 /Biol. Scs. /2017–2018), and the Griffith University Human Ethics Committee, Australia (Ref# 2017/467). The trial was registered with the International Standard Randomized Controlled Trial Register, number ISRCTN60058115.

## Supplementary materials

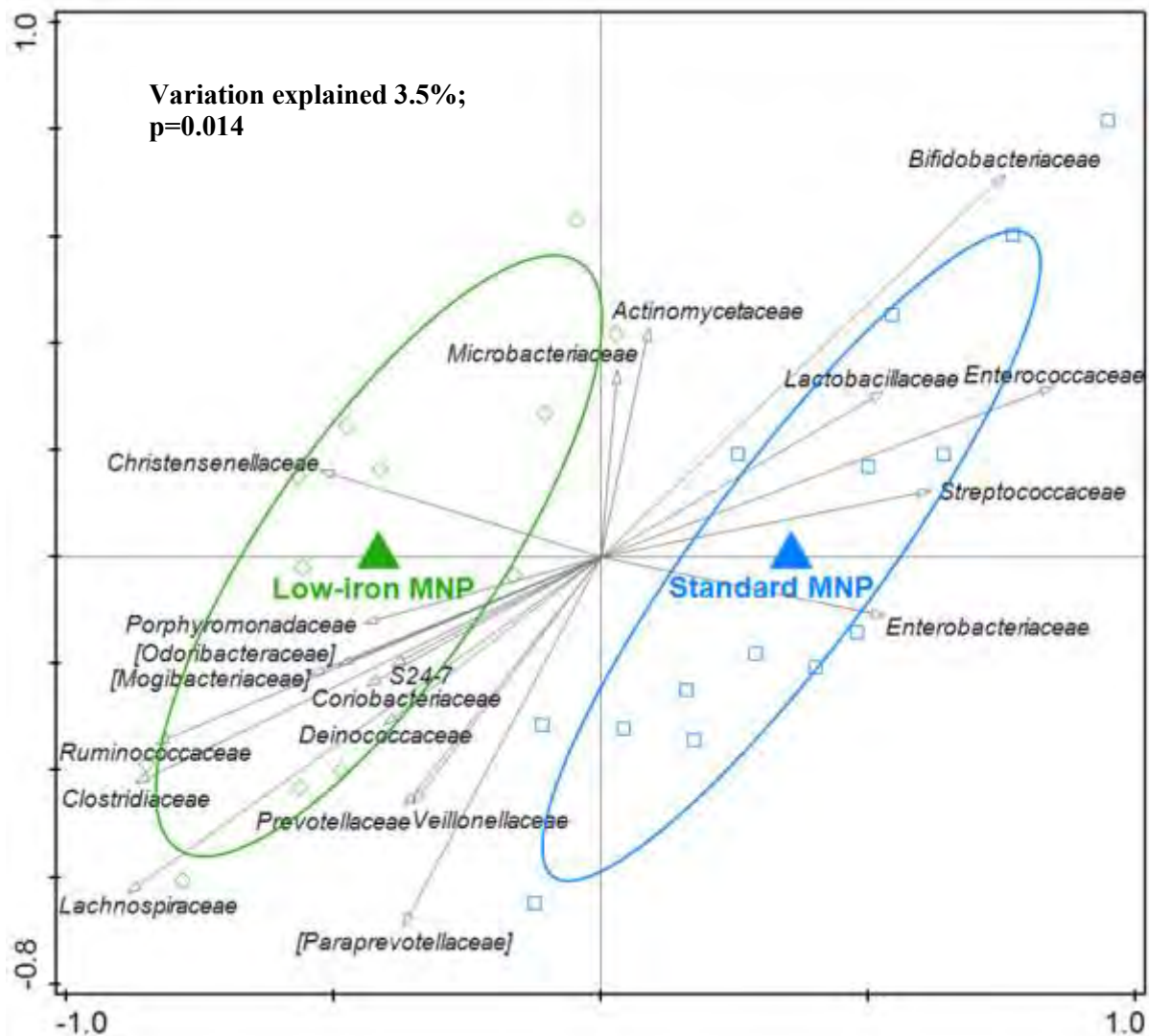


Supplementary Fig. 1: Alpha-diversity or within-sample diversity (PD whole tree index) of the microbiota per treatment group and time point.

Indifferent over standard- and low-iron MNP groups at both the time points.



Supplementary Fig. 2: RDA on the OTU level, assessing the effect of CRP (Panel A) and AGP (Panel B). OTUs were used as response data and CRP or AGP was explanatory data, the bacterial families that contributed most were plotted supplementary. The covariance attributable to age was first fitted by regression and then partialled out (removed) from the ordination. Variation explained by CRP and AGP was 1.9% ( $p=0.04$ ) and 1.1% ( $p=0.078$ ), respectively. Effect of CRP and AGP on the relative abundance of microbiota corrected for age (baseline). Blue squares indicate samples from the standard MNP group and green diamond samples represent children assigned to the low-iron MNP group.



Supplementary Fig. 3: RDA on the OTU level, assessing the effect of treatment on the relative abundance of the gut microbiota composition within the “old- microbiome-age group” groups at endpoint (corrected for real age). OTUs were used as response data and treatment was explanatory data, the bacterial families that contributed most were plotted supplementary. The covariance attributable to calendar age was first fitted by regression and then partialled out (removed) from the ordination. Variation explained by treatment was 3.5% ( $p=0.014$ ). Blue squares indicate samples from the standard MNP group and green diamond samples represent children assigned to the low-iron MNP group.

### **5.2.3 OVERVIEW OF THE SUPPLEMENTARY PAPER 1 [SUPPLEMENTARY STUDY 1]**

Title: Thalassaemia carrier status and groundwater iron: Implication for iron supplementation program for children in Bangladesh

Status: Submitted to International Journal of Hematology-Oncology and Stem Cell Research (Citescore: 2.6);

Outcome: under review

#### **5.2.3.1 INTRODUCTION**

Thalassaemia is a congenital condition characterised by premature destruction of red blood cells resulting in a state of low haemoglobin. The drive for haemosynthesis is high and the potential for absorption of iron from food and supplement sources is generally high. For this, in a setting where diet is a poor source of iron, thalassaemic carriers, besides being anaemic, may suffer from iron deficiency. However, Bangladesh poses a different context. The Bangladeshi population is exposed to a fair- to high-level of iron from drinking groundwater which maintains a good iron status in them. Since the burden of childhood anaemia is high, the national policy advocates for MNP supplementation for prevention of anaemia which contains an iron dose of 12.5 mg. Since a large number of the children are iron-replete, an iron supplement programme with a blanket approach is possibly unnecessary for thalassaemia carriers who are susceptible to iron overload. The additional supplemental iron might be unabsorbed due to its intrinsic metabolism and might induce gastro intestinal side effects. Therefore, the present study was conducted to assess the effect of thalassaemia on haemoglobin and iron status in the trial participants who were taking the MNP supplements and were exposed to a high amount of iron from drinking groundwater. Results showed that haemoglobin concentration of the children with thalassaemia at the end-point remained unchanged relative to the baseline value;  $11.56 \pm 0.59$  (Endpoint) versus  $11.6 \pm 0.54$  (Baseline),  $p=0.83$ ; while in the children without thalassaemia, it tended to increase;  $12.54 \pm 0.72$  (Endpoint) vs.  $12.41 \pm 0.72$  (baseline);  $p=0.06$ . As expected, the baseline reserve

of iron was higher in the carrier children; 594 mg vs. 558 mg;  $p=0.03$ . Intestinal side-effects such as loose stools were apparently higher in the thalassaemia children.

The study indicates that under a blanket iron supplementation programme to control childhood anaemia, in the absence of screening for thalassaemia, a low-iron MNP is possibly a better alternative to the standard MNP. This might minimise the iron loading and the side effects. The findings provide supplementary data for promotion of low-iron MNP for the national anaemia programme for children.

#### **5.2.3.1.1 SUBMITTED PAPER [SUPPLEMENTARY PAPER 1]**

Thalassemia carrier status and groundwater iron: Implication for iron supplementation program for children in Bangladesh

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Running title: Thalassemia, Groundwater Iron and Anemia

## Abstract

**Background:** Thalassemia, a congenital disorder of hemoglobin synthesis is characterised by low hemoglobin and high iron status, and the condition is prevalent in Bangladesh. Iron, acquired through drinking groundwater increases the population iron status in Bangladesh. Hence, the effect of iron containing micronutrient powder (MNP) on the hemoglobin and ferritin status in Bangladeshi thalassemia and non-thalassemia children was examined.

**Methods:** In 327 children aged 2-5 years were recruited for an MNP efficacy trial. Of them a subsample (n=222) were screened for thalassemia. Hemoglobin and ferritin levels were measured in the children with and without thalassemia at baseline and the endpoint. Intake of iron from the key sources- diet, groundwater and MNP was measured. Statistical tests- Mann Whitney, t-test were employed to compare the groups.

**Results:** Hemoglobin concentration of the children with thalassemia at the end-point remained unchanged relative to the baseline value;  $11.56 \pm 0.59$  (Endpoint) vs.  $11.6 \pm 0.54$  (Baseline),  $p=0.83$ . In the children without thalassemia hemoglobin tended to increase;  $12.54 \pm 0.72$  (Endpoint) vs.  $12.41 \pm 0.72$  (baseline),  $p=0.06$ . Baseline reserve of body iron was significantly higher in the thalassemia carriers compared to their non-carrier peers; 594 mg vs. 558 mg;  $p=0.03$ . The increase of the infection adjusted ferritin level from baseline to the endpoint was 7.37% ( $p=0.7$ ) and 10.17% ( $p=0.009$ ) in the carrier and non-carrier groups respectively.

**Conclusion:** In Bangladesh with the coexistence of thalassemia carrier states and the environmental exposure to a high concentration of iron from drinking groundwater; anemia prevention program with a low iron MNP can be potentially beneficial to the thalassemia carriers.

**Key words:** Thalassemia, Groundwater iron, Anemia, Bangladesh

## Introduction

Thalassemia, a group of hereditary disorders resulting from genetic mutations involving hemoglobin synthesis, showing a wide range in severity from fetal death to mild anemia. The hallmark of thalassemia is the hyperplasia of the erythroid marrow and unproductive erythropoiesis affecting the tetramer structure of hemoglobin<sup>1,2</sup>. This condition can lead to the secondary iron overload, defined as the iron overload which is not the result of direct mutations to proteins involved in iron absorption. Instead, it is a result of the inefficient erythropoiesis and a higher demand for and absorption of iron<sup>3</sup>. It has been reported that certain thalassemia carriers can have the iron absorption rates 3 to 4 times more than normal, increased serum ferritin levels, and decreased levels of hepcidin<sup>3, 4</sup>. Hence, a number of guidelines suggest the avoidance of routine supplementation of iron, unless there is a deficiency of iron<sup>5-7</sup>. However, in the developing country settings where dietary iron is sparse and predominantly plant-based, often a coexistence of thalassemia carriers and iron deficiency anemia is reported<sup>8</sup>, which warrants a correction by iron supplementation.

Bangladesh poses a different context. To control water-borne communicable diseases and the related mortalities and morbidities, the country had embarked upon the massive operation of utilization of groundwater in the 1970-80s; and since then, 97% of the rural population rely on groundwater for potable supply<sup>9</sup>. Iron concentration in ground water is high in many parts of the country<sup>9</sup>. In a nationally representative survey, a high level of iron in groundwater was observed to be independently associated with high iron status in children, adolescents, and women<sup>10</sup>. The similar observation was reported in another study<sup>11</sup>. Groundwater iron is fairly bioavailable<sup>12</sup>, which may provide a wholesome supply of iron in the thalassemia carriers; and thus, may augment their iron stores. In this particular context, additional supplemental/fortified iron may not be required for erythropoiesis and could be counterproductive by loading of excess iron and the associated side-effects. Currently, there is no nationally representative data on the magnitude of thalassemia carrier in Bangladesh. However, several small-scale studies suggest that the prevalence of the condition is variable by regions and the reported estimates were 2.9-16%<sup>13</sup>, 17.2% in pregnant women<sup>14</sup>, 28% in non-pregnant women<sup>11</sup>, and 13.1 % in children aged 2-5 years old<sup>15</sup> - suggesting that a fair proportion of the population might be affected with the condition. In the public health approach to manage population level anemia in association with thalassemia and/or the carrier states, there is no specific global guidelines taking into consideration the complex interplay of the conditions. The issue of groundwater iron in Bangladesh might have further compounded the scenario. The recent national guidelines for the control of anemia in Bangladesh<sup>16</sup> did not provide the directives as there is a lack of pertinent data.

Therefore, we conducted an exploratory study to observe the effect of supplementation of micronutrient powders (MNP) containing iron (12.5 mg or 5 mg iron) on hemoglobin and ferritin concentrations; and on iron-related side-effects in Bangladeshi children aged 2-5 years with or without thalassemia, who were exposed to a high concentration of iron from drinking groundwater.

## Materials & Methods

The study was a nested sub study within a randomized controlled trial examining the effect of a low-iron MNP in preventing anemia and iron-related side-effects in Bangladeshi 2-5 years old children exposed to a high level of iron from drinking ground water. The trial received ethical approval from the Faculty of Biological Science, the University of Dhaka, Bangladesh

(Ref# 46 /Biol. Scs. /2017-2018), and the Griffith University Human Ethics Committee, Australia (Ref# 2017/467). The trial was registered with the International Standard Randomized Controlled Trial Register, number ISRCTN60058115. Written informed consent was obtained from the parents allowing the children to take part in the study. Three hundred and twenty-seven rural children who drank groundwater containing high level of iron ( $\geq 2$  mg/L) were randomly allocated to receive the standard MNP (containing 12.5 mg of iron) or the low-iron MNP (containing 5 mg of iron); 1 sachet per day for 60 days. In a subsample of children ( $n=222$ ), hemoglobin, ferritin, infection-biomarkers (CRP and AGP) were measured at baseline and at the end of intervention period. After drawing a venous blood sample, hemoglobin was measured immediately using a hemocue photometer (Hemocue 301, Angleholm, Sweden). Ferritin was measured by an automated immunoassay analyzer (Cobas C311; Roche Diagnostics, Mannheim, Germany). Ferritin values were adjusted for infection for the raised values of CRP ( $> 5$  mg/L) and AGP ( $> 1$  g/L) by applying the Thurnham's principle<sup>17</sup>. Thalassemia carrier state was assessed by capillary zone electrophoresis of Hb at pH 9.4 (Capillary 2 system; Sebia, Evry, France). Concentration of iron in groundwater was measured by a hand-held portable colorimeter (HI-721; Hanna Instruments, USA) at baseline. At baseline and the end-point, the intake of water was assessed by a 24-hour recall method by using 6 time-prompts<sup>11, 15</sup> and the intake of iron from water was estimated by multiplying the concentration of iron and the volume of water drunk over the preceding 24 hours. Dietary intake of iron was assessed by a validated 7-day semi-quantitative food frequency questionnaire. Details of the methodology are provided in the parent trial<sup>15</sup>.

We compared the thalassemia carriers with the non-carriers, irrespective of the intervention groups (standard MNP and low-dose MNP), in regard to- hemoglobin and ferritin concentrations, prevalence of iron deficiency, episodes of iron related side-effects; and the intakes of iron from multiple sources- groundwater, diet and the MNP supplements.

### Sample size

The present study is nested in a larger study (i.e. RCT) examining the effect of a low-dose iron supplement on hemoglobin status in children of rural Bangladesh. Among the children selected for blood sample collection ( $n=222$ ) ~13% ( $n=29$ ) had thalassemia or Hemoglobin E disease carrier states, irrespective of the intervention groups. Daniel et al. reported that for an exploratory/pilot study the minimum requirement of the cases is 20<sup>18</sup>. The cases of the thalassemia carriers of the present study are 29 which are higher than the requirement of an exploratory study. Furthermore, 30 samples would approximate a Gaussian distribution to provide a valid mean with standard deviation as per the central limit theorem<sup>19</sup>. Hence, the number of the thalassemia cases roughly conforms to the standard.

### Statistical analysis

Data were first assessed for normality by histogram and the Shapiro-Wilk test. Since the participant and household characteristics e.g. age of the children, household expenses on food, intake of iron from various sources such as diet, groundwater and MNP were non-normally distributed, non-parametric test (Mann-Whitney U test) was used to compare between the children with thalassemia and children without thalassemia. Distribution of hemoglobin was largely bell-shaped by histogram and non-significant by the Shapiro Wilk test (results not shown). Therefore, the group comparison was done by the Independent Sample t-tests—i.e., a. between the children with thalassemia vs. children without

thalassemia and b. between baseline and endpoint sorted by the presence or absence of the carrier children.

Regarding the unadjusted and infection-adjusted ferritin, the data were non normal (significant results of the Shapiro Wilk test; results not shown). The ferritin values were first log-transformed. This improved the appearance of the normality curve consistent with the “bell shape” on histogram. We did t-test with unequal sample option on the log-transformed ferritin data; and back transformed to report the geometric mean of ferritin sorted by the groups and between baseline and end-point. The statistical significance for the difference in ferritin between the groups and between the study points was tested by the Mann Whitney test. For all analyses, the p-value <0.05 was considered statistical significance.

## Results

Table 1 shows the selected characteristics of the subjects by thalassemia status. The proportion of the children who were thalassemia carriers and non-carriers was 13.1% and 86.9% respectively ( $p<0.001$ ). Age of the subjects was  $41.13\pm9.9$  months and  $39.81\pm8.9$  months in the children with and without thalassemia respectively. Household expenses on the weekly food purchases did not differ between the groups ( $p=0.06$ ). In the children with thalassemia, none of the children had iron deficiency (ID) at baseline, whereas 2.07% had ID among their peers without thalassemia. None of the children had ID in either of the groups at end-point. Intake of iron from groundwater apparently was higher in the non-carriers both at baseline and end-points; albeit with a non-significant statistical difference. Dietary intake of iron was similar between the groups at baseline; however, at the end-point the children with thalassemia consumed apparently lesser than their non-carrier peers, but the difference was not statistically significant ( $p=0.10$ ). Over the intervention period, the total intake of iron from MNPs were  $437.58\pm214.75$  mg and  $440.61\pm228.17$  mg in the respective groups ( $p=0.94$ ).

Table 1: Selected participant and household characteristics by the thalassemia carrier status

Variables	With thalassemia	Without thalassemia	p-value
	N=222		
Thalassemia carrier status (%) n	(13.1) ; n=29	(86.9) ; n=193	<0.001
Age (month)	41.13±9.9	39.81±8.9	0.38*
Household expenses on food (BDT. per week)	1473.20±548.1	1804.27±878.14	0.06*
Prevalence of ID+ (%) n			
Baseline	0.0; n=0	2.07; n=5	-
End-point	0.0; n=0	0.0; n=184	-
Intake of iron from groundwater‡ (mg/d); mean ± SD, n			
Baseline	4.91±6.1,29	6.08±6.2,193	0.17*
End-point	3.45±3.41,28	4.70±4.85,186	0.09*
Dietary intake of iron(mg/d), mean ± SD, n			
Baseline	3.04±1.32,29	3.14±1.45,193	0.96*
End-point	2.60 ±1.02,29	3.27±1.78, 193	0.10*
Intake of iron from MNPs§	437.58±214.75, 29	440.61±228.17,193	0.94*

\*Mann Whitney Test

†ID was defined as the infection-adjusted ferritin<12 ng/ml <sup>20</sup>

‡Intake of iron from groundwater was calculated by multiplying the amount of water taken over the preceding 24 hours and the concentration of iron in groundwater

§Intake of iron was from both the MNPs as per random allocation of the treatment

Figure 1 depicts the effect of iron supplementation (i.e. MNPs) on hemoglobin and ferritin concentrations in the children with thalassemia compared with the children without thalassemia. Concentration of hemoglobin remained largely unchanged in the children with thalassemia over the intervention (11.60±0.58 g/dl; baseline vs. 11.56±0.60 g/dl; end-point). In the children without thalassemia a trend of increase in hemoglobin concentration was observed (12.41± 0.72 g/dl vs. 12.54±0.72 g/dl; p=0.06). The infection-unadjusted ferritin marked a statistically non-significant increasing trend in children with thalassemia showing 4.4% increase at endpoint. This showed smaller increment of 1.76% (statistically non-significant) at endpoint in children without thalassemia. The infection-adjusted concentration of ferritin showed increasing trend (non-significant) in children with thalassemia (7.37%). However, in their non-career peers the increase of ferritin was higher (10.17%) and statistically significant (p=0.009).



Figure 2 shows the episodes of the pertinent iron-related intestinal side-effects over the two-month course of the intervention in the children with and without thalassemia receiving MNP supplements and consuming the comparable amount of iron over the intervention period (Table 1). In children with thalassemia, the mean number of loose stool episodes were 3.5 and 0.6 in the subjects receiving the standard MNP and the low-iron MNP respectively ( $p=0.10$ ). The mean episodes of diarrhea were 0.43 and 0.20 ( $p=0.39$ ), respectively. In children without thalassemia, there were 1.86 and 1.4 episodes of loose stools in the subjects receiving the standard MNP and the low-iron MNP respectively ( $p=0.42$ ).

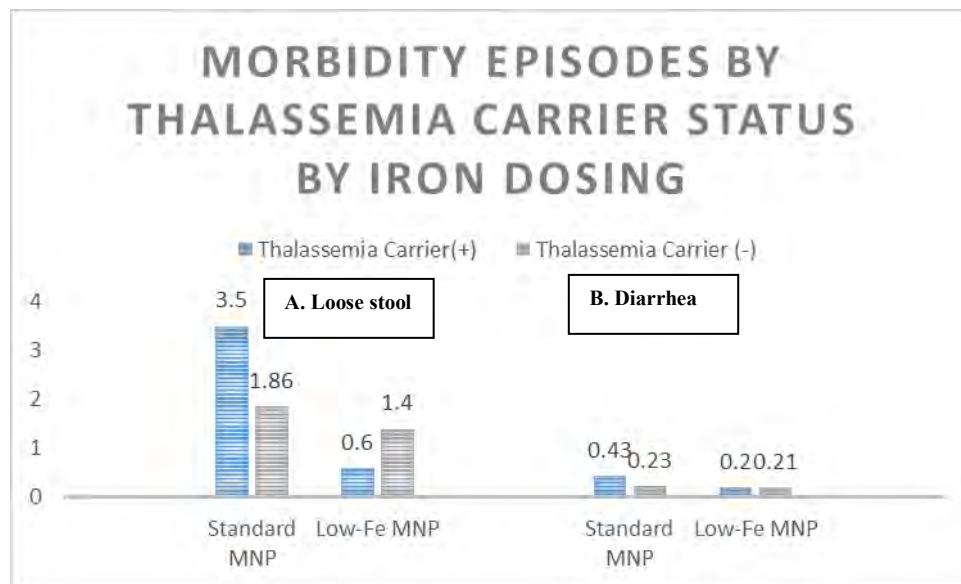


Figure 2: Iron-related side-effects by the thalassemia carrier status by iron dosing

## Discussion

We assessed the changes in hemoglobin and ferritin concentrations (unadjusted and infection-adjusted) in Bangladeshi children with and without thalassemia carrier status after receiving MNPs containing iron (1 sachet per day) for two months, exposed to a high concentration of iron from drinking groundwater.

The hemoglobin concentration in children with thalassemia was statistically significantly lower than in the children without thalassemia both at baseline and end-points. Despite there was no difference in the intake of iron from MNPs between the groups, the hemoglobin concentration of the children with thalassemia at the end-point remained unchanged relative to the baseline value, while that in the children without thalassemia tended to increase. The relative static level of hemoglobin despite a fair degree of supplementation of iron is consistent with the subjects of the thalassemia carrier states who usually maintain a low level of hemoglobin<sup>5</sup>.

The mean concentrations of infection unadjusted ferritin at baseline were not statistically different between the groups; and albeit a bit high relative to a less-diversified, predominantly cereal based rural diet in the country<sup>21</sup> and considering a low level of infectious burden (%  $\uparrow$ CRP<10%; results not shown). This relative high values of the baseline ferritin possibly accounted for a high concentration of iron in groundwater which is the potable supply to the children. Over the two-month long MNP intervention, the ferritin concentration remained nearly static, showing just 1.75% increase in children without thalassemia. On the other hand, this rate of increase was slightly higher (4.4%) in their thalassemia carrier peers, however the increase was statistically non-significant. This observation is consistent with a predisposition of increased absorption of iron in thalassemia carriers when iron supplementation is provided<sup>5, 6</sup>.

For the infection-adjusted ferritin, the children with thalassemia tended to have a slightly higher concentration at baseline than in the children without thalassemia (non-significant). At the end-point, while the increase (7.37%) of ferritin was non-significant in the *carrier* children; the non-carrier peers registered a significant increase (10.17%;  $p=0.009$ ). However, yet they (children without *thalassemia*) tended to have a lower ferritin value than in the children with *thalassemia* (non-significant). The reason for the significant rise in the infection-adjusted ferritin in the children without thalassemia and non-significant increase in the thalassemia group is difficult to explain. It can be speculated that in this setting the thalassemia carriers are in a hyper-ferremic state both due to the medical condition and the groundwater iron. As such they are likely to uptake supplemental iron (e.g. through MNP) less efficiently compared to their non-carrier peers. Complementing this, the total reserve of body iron at baseline was higher in the children with thalassemia compared to the non-carriers (median: 594 mg vs. 558 mg;  $p=0.03$ , results not shown). At the endpoint, the increment of the reserve iron was 3% and 7% respectively in the thalassemia-carrier and the thalassemia non-carrier groups implying a less efficient uptake of the supplemental iron in the thalassemia carrier children. This observation complements the apparent higher episodes of loose stools in the carrier children which might happen when the absorption of supplemental iron is less efficient with a likelihood of the iron remaining unabsorbed in the distal intestines. This might potentially affect the composition of the gut microbiome adversely.

A limitation of the study is the sample size of the thalassemia carrier group, which was small and hence the statistical power was suboptimum to report the significant  $p$  values in relation

to some of the presented estimates. However, the trend and direction of the estimates were expected as per our understanding of the theory and consistent over the assessments. Larger study is required to confirm this preliminary observation. Due to logistical constraints, gut microbiome assessment was not done which could provide additional information on the microbiome composition; and thus complementing its link with the differential iron reserve and absorption between the children with and without thalassemia. Hence, lack of this measurement is a limitation. Strength of the study is that we have measured multiple sources of iron to account for ferritin and hemoglobin status- e.g. groundwater, dietary and MNP supplements.

In summary, the children with thalassemia had significantly lower hemoglobin concentrations than the children without thalassemia at both the assessment points. The infection-unadjusted ferritin showed a slightly higher predisposition for ferritin build up in the thalassemia carriers than in the non-carriers. This finding is consistent with Mehta et al study<sup>22</sup>. The findings complement to some current guidelines, which does not recommend the routine iron supplementation in thalassemia carriers unless the ID coexists in them<sup>5-7</sup>.

There were hardly any cases with ID (infection-adjusted ferritin <12 ng/ml), unlike the observed co-existence of ID and thalassemia traits in other studies<sup>8,22</sup>. The lack of ID in the subjects can be plausibly explained by the iron from groundwater; and not accounted for dietary iron, which was suboptimum relative to the requirement in this age-group<sup>21</sup>.

## Way forward

In Bangladesh, there is a national policy for control of childhood anemia with the provision of the blanket iron supplementation in combination of other micronutrients i.e. micronutrient powder (MNP) formulation containing 12.5 mg iron per dose. However, a fair proportion of the population might be affected with thalassemia, mostly carriers; and as per some guidelines, these subjects are not recommended to receive the routine (i.e. unscreened) iron supplementation, as the risk of excess iron may increase. The risk is heightened in the background context of high level of groundwater iron in many parts of the country which contributes independently to a good body iron status. In this particular scenario, supplemental iron at the current dose (i.e. 12.5 mg) in these subjects would likely to exacerbate the excess iron reserve and may increase the risk of the iron-related side-effects. Morbidity findings of the present study apparently complemented this.

In an ideal scenario, all children are required for screening for thalassemia. Following the screening, the body iron status (e.g. serum iron, serum ferritin) of the thalassemia carriers should be assessed<sup>5, 6</sup> before deciding them to enroll for iron supplementation program. However, this is financially and logistically burdensome for a resource-poor setting like Bangladesh where the population size is enormous. Furthermore, operational challenge for the screening-based iron supplementation is enormous in the country setting. Recently, a low-iron MNP containing a dose of 5 mg iron (instead of 12.5 mg iron of the standard MNP) has been shown to be efficacious in preventing low hemoglobin concentration and has shown a decreased incidence of the iron related side-effects, such as diarrhea, loose stool, nausea and fever; compared to the standard MNP in Bangladeshi children exposed to a high level of iron in groundwater<sup>15</sup>. In case the mass-screening for thalassemia and the assessment of iron status are infeasible, a reasonable option is to consider low iron formulation - e.g. low-iron

MNP (5 mg iron). This is likely to benefit the thalassemia carrier children residing among the overall population in two ways- potentially by decreasing the risk of iron overload if not eliminating the possibility; and secondly by decreasing the risk of the intestinal side effects.

In conclusion, in Bangladesh on the backdrop of the co-occurrence of thalassemia carrier states and the environmental exposure to a high concentration of iron from drinking groundwater; under a blanket iron supplementation policy for prevention of childhood anemia, a low iron MNP can potentially be beneficial to the thalassemia carriers.

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#### Conflicts of statement

The authors declare no competing interests

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#### 5.2.4 OVERVIEW OF THE SUPPLEMENTARY PAPER 2 [SUPPLEMENTARY STUDY 2]

Title: Intake of Iron in a Low-iron Groundwater Setting in Rural Bangladeshi Children: Low-iron Micronutrient Powder (MNP) is a potential intervention for prevention of childhood Anaemia.

Status: Submitted to Anaemia (IF: 1.65)

Outcome: Under review

##### 5.2.4.1 INTRODUCTION

The main study (randomized controlled trial) demonstrated that low-iron MNP is effective in the prevention of anaemia and incurs fewer side effects in children whose source of drinking water contain a high level of iron ( $\geq 2$  mg/L). However, Bangladesh has a substantial number of tube wells which contain a low level of iron ( $< 2$  mg/L). Although the use of two different doses of iron supplement is a theoretical proposition suited better for the customized solution of anaemia, it is logistically and administratively cumbersome to operate two different supplements in the programme setting, especially when the tube wells, even those which are adjacent, might widely differ in iron content in the water. A single dose supplement catering for the needs of the maximum number of people is favourable. Hence, the present study was conducted to assess the scope of the low-iron MNP in curbing anaemia in children who drink from tube wells with a low concentration of iron. The results showed that the combined intake of iron from dietary, groundwater, and low-iron MNP in children was  $5.8 \pm 2.0$  and  $6.9 \pm 2.5$  mg/day comprising 193% and 169% of the Estimated Average Requirement in the 2-3 years old and 4-5 years old subgroups, respectively. The mean concentration of haemoglobin in the respective groups (excluding the intake of MNP) was  $12.17 \pm 0.94$  mg/dl and  $11.91 \pm 0.91$  mg/dl ( $p=0.30$ ). The finding complements the results of the main trial about the utilities of the low-iron MNP. It highlights the potential of low-iron MNP in curbing childhood anaemia in low groundwater iron settings.

#### **5.2.4.1.1 SUBMITTED PAPER [SUPPLEMENTARY PAPER 2]**

Intake of Iron in a Low-iron Groundwater Setting in Rural Bangladeshi Children: Low-iron Micronutrient Powder (MNP) is a potential intervention for prevention of childhood Anaemia.

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## Abstract

**Background:** Iron supplementation is associated with side effects and thus reduces the intake compliance. Because it has fewer side effects, a recent trial recommended low-iron micronutrient powder (MNP) for the prevention of anaemia in Bangladeshi children exposed to a high concentration of iron from drinking groundwater. In the present study, we hypothesize that the low-iron MNP is a potential intervention to prevent childhood anaemia in the low groundwater-iron settings in Bangladesh.

**Methods:** A cross-sectional study was conducted in Bangladesh with children aged 2-5 years who drank groundwater containing a low level of iron ( $0 < 2$  mg/L). The combined intake of iron was calculated from the key sources--diet, groundwater and MNPs. The intakes of iron - were compared against the standard reference intake. The children's haemoglobin was measured using a photometer.

**Results:** Combined intake of iron from dietary, groundwater and low-iron MNP in children was  $5.8 \pm 2.0$  and  $6.9 \pm 2.5$  mg/day comprising 193% and 169% of the Estimated Average Requirement in the 2-3 year-old and 4-5 year-old subgroups, respectively. Combined intake of bioavailable iron from dietary and groundwater sources was  $0.42 \pm 0.023$  and  $0.22 \pm 0.019$  mg/day in children exposed to groundwater concentration  $0.8 < 2.0$  mg/L and  $0.0 < 0.8$  mg/L respectively ( $p < 0.001$ ). The mean concentration of haemoglobin in the respective groups was  $12.17 \pm 0.94$  mg/dl and  $11.91 \pm 0.91$  mg/dl ( $p = 0.30$ ).

**Conclusion:** The combined intake of iron from dietary and groundwater sources was associated with maintenance of haemoglobin concentration at the non-anaemic level in most of the children. The finding highlights the potential of low-iron MNP in controlling childhood anaemia in this setting.

**Key words:** Iron, Groundwater, Low-iron MNP, Anaemia, Bangladesh

## 1. Introduction

In Bangladesh, the magnitude of Anaemia among children younger than five is high, with a range that varies from 33% to 51%, according to two nationally representative surveys [1, 2]. Iron deficiency was thought to be the primary cause of Anaemia in the Bangladesh population and thus, a national policy for its prevention recommended iron supplementation, i.e., micronutrient powder (MNP) containing iron, for children aged 6-23 months [3,4]. Consequently, both national and non-government organizations have been running the MNP (containing 12.5 mg of iron) supplementation program for a decade to prevent Anaemia in children up to 59 months old [5]. Nevertheless, current coverage of the MNP program is suboptimum [5], and compliance is poor because of iron-related side effects such as diarrhea, nausea, and vomiting [6].

However, contrary to popular perception, the National Micronutrient Survey 2011–2012 [7] reported that the prevalence of iron deficiency (ID) in children under-five was 10.7%, and iron deficiency Anaemia (IDA) 7.2%. Recent studies attributed the low prevalence of ID and IDA in the Bangladesh population to drinking iron-containing groundwater from tube wells [2, 8]. Of note, groundwater (extracted from hand-pumped tube-wells) is the principal source of drinking water for the large majority (97%) of the rural population in Bangladesh [9]. Hydrochemistry of Bangladesh groundwater reveals that it contains varying concentrations of dissolved iron, with a predominantly high concentration in many parts of Bangladesh, but a largely low concentration in approximately half of the country [2, 9]. Furthermore, a prominent feature of groundwater is that the iron concentrations vary considerably between tube-wells located near each other. Hence even in predominantly high iron groundwater areas, some wells contain a low concentration of iron in the water.

Acknowledging the presence of a high concentration of iron in groundwater, the national Anaemia consultation of Bangladesh [3] recommended examining the efficacy and side effects of MNP supplementation with a low dose of iron for children residing in areas with predominantly high groundwater iron. A recent trial conducted among Bangladeshi children aged 2-5 years demonstrated that low-iron MNP (containing 5 mg iron) was equally efficacious in preventing Anaemia compared to the standard MNP containing 12.5 mg of iron [10]. In addition, low-iron MNP was associated with significantly fewer incidence of key side effects, such as diarrhea, loose stools, nausea, and fever compared to the standard MNP [10]. The study recommended low-iron MNP for the prevention of Anaemia in children under five residing in high iron groundwater areas.

Given the geologically variable iron-content scenario in groundwater in Bangladesh, it would be difficult to introduce low-iron MNP for children drinking high-iron groundwater and standard MNP for children drinking low-iron groundwater. Administratively it would be much more feasible and cost-effective to have a single composition (e.g., low-iron MNP) for the whole population. Therefore, the present study hypothesizes that the low-iron MNP is a potential intervention in preventing childhood Anaemia in predominantly low-iron groundwater areas or in children whose potable supply is groundwater with a low concentration of iron ( $0 < 2$  mg/L). Ideally, this should have been examined by conducting a clinical trial. However, we have attempted to assess the potential of the low-iron MNP in the prevention of Anaemia from a different perspective. We assessed the combined intake of iron from the key sources (diet, drinking groundwater, and low-iron MNP) and compared it with the dietary reference intakes. The comparison contributed to understanding the extent of potential iron intake, and thus an assessment of the possible protection from iron deficiency/Anaemia.

## Methods

### 2.1 Study subjects and selection process

The study subjects are children aged 2-5 years residing in Belkuchi- a rural sub district of north-west Bangladesh. The children were selected from a household if the groundwater iron concentration of the tube wells they drink from was low, i.e. 0-<2 mg/L. Low concentration (<2 mg/L) is defined as per the cut-off recommended by the joint technical committee of Food and Agricultural Organization and World Health Organization FAO/WHO [11]. The sampling was done over three stages. At the first stage, an inquiry was made with local residents to establish 1) households with a drinking water source (tube well) containing “low” or “no” iron; and 2) if the designated household had a child who met the age criteria. At the second stage, the initial verification of the iron status was done in the designated households by using a novel sensorial tool—taste-rating of a groundwater sample for the level of iron [12]. In case the taste-rating suggested a low level of groundwater iron, at the final stage, a colorimetric device was used to confirm low-level iron in the groundwater (0-<2 mg/L). A child was selected for the study if both conditions (age, level of drinking water iron) were met.

### 2.2 Sample size

There is a paucity of data on the intake of iron from drinking groundwater in children. The only available study that estimated the intake of iron from groundwater reported a standard deviation (SD) of 6.5 [10]. That study was conducted considering tube-wells with a high concentration of groundwater iron, and had a mean concentration of iron ~8 mg/L (max. 43.3 mg/L). So, the wide range of the values of iron intake yielded a relatively large SD. The present study considered children who drank from groundwater with a concentration of iron 0-<2 mg/L. So, assuming a reasonably lower SD for the mean intake of iron from water in

this population, a SD of 3.25, which is equivalent to 50% of the SD of the above study, was considered reasonable in the present study. Allowing for a margin of error of 0.7 mg [10], with a variance ( $SD^2$ ) of 10.2 and at 95% confidence level, the required sample size was 80, as per online sample size estimation software [13].

To estimate the mean hemoglobin, considering the variance ( $SD^2$ ) of 0.608 [10], and an error margin of 0.15 mg/dl at 95% confidence level, the required sample size was 104 [13].

## 2.3 Data Collection

### 2.3.1 *Assessment of dietary iron intake*

The mother of the recruited child was asked about her child's intake of food over the 24-hours preceding the interview. The intake was captured over six time prompts— breakfast, mid-morning, lunch, afternoon, dinner and bedtime. The amount of the reported food intake was assessed by the usage of food albums and utensils—plates, bowls, spoons, and packets/brand of the food items (i.e., processed foods). An updated food composition table on Bangladeshi foods [14] was used to calculate the intake of iron from the food items.

### 2.3.2 *Assessment of iron intake from groundwater (drinking water)*

The concentration of iron in the drinking water sample was measured by a colorimetric test kit device (Hanna 3831, Hanna Instruments, USA) by using the manufacturer's provided manual. To calculate the intake of iron from drinking groundwater, the total amount of water intake was first assessed over the 24-hours preceding the interview using six time prompts, following the methods described by Merrill et al [8]. Intake of iron was calculated by multiplying the concentration of iron in the groundwater sample and the volume of intake of water over the 24-hours preceding the survey [8,10].

### 2.3.3 *Possible intake of iron from MNP supplements*

A hypothetical intake of the low-iron MNP was calculated. Since the compliance of MNP supplement consumption is generally suboptimum in the programmatic context of Bangladesh, the study considered the hypothetical intake of MNP representing two different compliance levels. Compliance of 85% and 50% was considered satisfactory [15] and sub-optimum consumption, respectively. Intake of iron from the low-iron MNP was calculated by multiplying the dose (5 mg/day) by 0.85 (at 85% intake compliance) and 0.50 (at 50% intake compliance).

#### *2.3.4 Assessing the total iron intake from different sources*

The children's intake of iron from drinking groundwater (i.e., tube-wells) and their dietary intake of iron were estimated. Further, intake of iron from the low-iron MNPs was assessed hypothetically. The combined intake of iron from the sources was calculated and compared with the dietary reference intakes (i.e., Recommended Dietary Allowance and Estimated Average Requirements) for the stipulated age group of the children. The amount of bio-available iron was calculated and compared with the reference values.

#### *2.3.5 Reference standard for comparison*

Recommended Dietary Allowance and the Estimated Average Requirement were used as references to assess the extent of iron intakes from all sources. For children aged 2-3 years and 4-5 years, the RDAs of iron were 7 mg/day and 10 mg/day [16], while the EARs were 3 mg/day and 4.1 mg/day respectively [17]. The intake of bio-available iron was compared to the median and the 95<sup>th</sup> percentile of the joint FAO and WHO reference [18]. The medians were 0.46 mg/day and 0.50 mg/day in the 2-3 year-old and 4-5 year-old groups respectively. The 95<sup>th</sup> percentiles were 0.58 mg/day and 0.63 mg/day in the respective age subgroups.

#### *2.3.6 Assessing the total intake of bioavailable iron*

The children's age varied between 2 to 5 years and therefore they had been exposed to iron from drinking water for different periods of time. Additionally, the groundwater iron concentration of the tube-wells could have been any value of the 0-2 mg/L range. Hence, taking these factors into account, it was assumed that some children might have had a depleted status of body-iron, particularly the children who drank from the wells with zero or close to zero concentration of iron. On the other hand, some children, principally those who drank from the tube-wells with water iron concentration in the higher side of the 0-2 mg/L range, might have had replete body-iron reserves. Therefore, to calculate the bioavailable iron from groundwater and presenting both the scenarios, we used 40% efficiency of absorption of iron from the water considering iron-deplete subjects and 10% considering iron-replete subjects as suggested by Worwood et al [19]. Average absorption efficiency (23%) [19] was used in determining the amount of the bioavailable iron from the groundwater during the sub-group assessment (i.e. subgroups  $0 < 0.8$  mg/L &  $\geq 8-2.0$  mg/L) (Table 3). This is because the range of the iron concentration at the sub-group level would not have had a similar scale of water iron concentration for the overall (all samples) range (0-2 mg/L). Assuming 40% absorption in the iron-depleted children (at a near-zero concentration of iron) and 10% absorption in the iron-replete children (near the 2 mg/L iron concentration) is not feasible. Hence, the average absorption potential of 23% was considered at the sub-group level.

Regarding absorption of iron from the MNP that contains ferrous fumarate as the iron-complex and ascorbic acid as the absorption enhancer, for the subjects who were iron-replete, an absorption rate of 4.65% was considered [20]. For subjects with iron-depleted status, the absorption rate of 4.48% was considered [20]. The combined intake of bioavailable iron from all-sources (dietary + groundwater + MNPs) was calculated considering the hypothetical usage of the low-iron MNP at the satisfactory (85%) [15] and suboptimum (50%) level of

compliance. No children were consuming any iron supplements (including MNP) at the time of the study and in the preceding 6 months, and therefore such supplements were not considered for calculating the combined iron intakes. Regarding absorption of dietary iron, taking into consideration the predominantly cereal-based traditional diet, a 5% absorption was considered [21].

Since the focus of the study was the assessment of the potential of the low-iron MNP on hemoglobin status in a low-iron groundwater setting, an appraisal of the intake of iron from the key sources in conjunction with the intake of low-iron MNP is provided in the results section. However, a similar assessment of the standard MNP, the existing MNP formulation in the country, is presented as supplementary data (Supplementary Table 1, 2).

#### *2.3.7 Measurement of hemoglobin*

The hemoglobin concentration of 105 children was measured on venous blood samples by a photometer (Hemocue 301, Hemocue AB, Angleholm Sweden). The blood was drawn from the median cubital vein with the subjects seated on their mothers' laps. Following proper asepsis of the puncture site with an alcohol pad, 0.5 ml of blood was drawn using a 3 ml disposable syringe. The blood sample was gently placed on a cover slip and sucked into a microcuvette of the photometer device. The measurement was performed observing the manufacturer's supplied manual.

#### *2.3.8 Statistical analysis*

General characteristics of the children were estimated as mean  $\pm$ SD and median (interquartile range) for the quantitative variables, and proportion (%) for the categorical variables. The histogram visualization appeared nearly consistent with normality; however, the Shapiro-Wilk U coefficient, which is a stricter metric of normal distribution, was significant (results not shown). Hence the aggregated intake of iron from all foods consumed over the preceding 24 hours of the interview was computed both as mean  $\pm$ SD and median with interquartile

range (IQR). Iron from drinking groundwater was similarly presented. Intake of iron from standard (12.5 mg iron) and low-iron (5 mg iron) MNPs was hypothetically deducted as the function of 85% (satisfactory compliance) [15] and 50% consumption (suboptimum compliance). Combined intake of iron from all sources – diet, water and MNPs – was estimated as mean  $\pm$ SD and median (IQR). Similarly, bioavailable iron from all sources combined sorted by different compliances (50% and 85%) of MNP intakes was presented. Children were sub-grouped by the median concentration (0.8 mg/L) of iron in their drinking water. Mann-Whitney tests were performed for comparison of intakes of iron between the groups with p-values < 0.05 considered significant. However, since the hemoglobin concentrations were normally distributed (non-significant Shapiro-Wilk U coefficient; results not shown), a student's t-test was performed to compare the groups. Prevalence of Anaemia between the groups was compared by chi-square test. Spearman rank correlation coefficient (rho) was computed to assess the association of hemoglobin concentration and a) the groundwater iron concentration, b) intake of water iron, and c) dietary iron. Furthermore, linear regressions were performed to assess the association of groundwater iron concentration and intake of water iron with children's hemoglobin concentration after adjusting for dietary iron.

## 2.4 Ethical considerations

The study was nested in a community-based trial examining the efficacy of a novel micronutrient powder formulation in children residing in areas with a high level of iron in groundwater. The trial received approval from the Research Ethical Committee of the Faculty of Biological Science, Dhaka University, Bangladesh (Ref# 46 /Biol. Scs. /2017-2018) and Griffith University Human Research Ethics Committee, Australia (Ref# 2017/467). Informed consent was obtained from each subject's guardian before the interview and blood collection.

Data are kept in a secure place with the lead investigators. The subjects will remain anonymous during the presentation of the aggregated results.

### 3.1 Results

Table 1 depicts the general characteristics of the children in the study. Mean  $\pm$ SD age of the children was  $43 \pm 10.6$  months. The proportion of female children was 44.3%. The common occupations of the household head were: unskilled labor (32.8%), skilled labor (14.8%), business (12.3%) and farmer (11.5%). The mean  $\pm$ SD duration of institutional education of the mothers was  $6.3 \pm 4.6$  years. The mean  $\pm$ SD weekly expense on purchasing basic food items was BDT.  $1801.3 \pm 676.1$  [USD  $21.3 \pm 8.0$ ]. The mean  $\pm$ SD amount of cultivable land possessed by household was  $23.6 \pm 43.5$  decimals. The mean  $\pm$ SD intake of water by the children over the preceding 24 hours was  $885.5 \pm 428.4$  ml.

Table 2 shows the mean  $\pm$ SD intake of dietary iron, groundwater iron, and the combined intake from these sources by age groups of the children. Intake of dietary iron, groundwater iron and the combined dietary and groundwater iron in children aged 2-3 years was  $2.62 \pm 1.84$  mg/day,  $0.64 \pm 0.51$  mg/day and  $3.3 \pm 2.0$  mg/day, respectively. The respective intakes in children aged 4-5 years were  $3.51 \pm 2.34$  mg/day,  $0.85 \pm 0.73$  mg/day and  $4.4 \pm 2.5$  mg/day. Combined intakes of dietary iron and water iron comprised 47.1% and 44% of the RDA in the respective age groups. The aggregated intakes exceeded the EAR for both the subgroups at 110% and 107.3% respectively.

Table 3 depicts the intakes of actual and bioavailable iron from dietary and groundwater sources and the mean concentration of hemoglobin and the prevalence of Anaemia in children, stratified by the median concentration of iron in groundwater (0.8 mg/L). The children's mean (SE) intake of iron from drinking groundwater was  $1.11 \pm 0.07$  mg/day and  $0.24 \pm 0.04$  mg/day in the sub-groups, defined as groundwater iron concentration ( $\geq 0.8$ -<2.0)

mg/L and (0.0-<0.8) mg/L respectively; the difference was statistically significant,  $p<0.001$ . There was no statistical difference in the mean  $\pm$ SE intake of the dietary iron between the sub-groups;  $3.32\pm0.38$  mg/day vs.  $3.26\pm0.23$  mg/day,  $p=0.79$ . Combined mean  $\pm$ SE intake of bioavailable iron from dietary and groundwater sources was  $0.42\pm0.023$  mg/day and  $0.22\pm0.019$  mg/day in the above defined sub-groups ( $\geq 0.8$ -<2.0 mg/L) and (0.0-<0.8 mg/L) respectively;  $p<0.001$ . Mean $\pm$  SE concentration of hemoglobin in children did not differ statistically;  $11.91\pm0.91$  mg/dl (0.0-<0.8 mg/L subgroup) vs.  $12.17\pm0.94$  mg/dl ( $\geq 0.8$ -<2.0 mg/L subgroup),  $p=0.30$ . Prevalence of Anaemia was 6.25% and 12.2% in the ( $\geq 0.8$ -<2.0 mg/L) and (0.0-<0.8 mg/L) subgroups respectively ( $p=0.29$ ).

Figure 1 shows the correlations of hemoglobin concentration in children with a) groundwater iron concentration, b) intake of iron from groundwater and c) dietary intake of iron. The iron concentration of groundwater and intake of iron from water were positively correlated with children's hemoglobin concentration;  $\rho=0.22$ ;  $p=0.0248$  (Fig 1A) and  $\rho=0.21$ ,  $p=0.0257$  (Fig 1B) respectively. Dietary intake of iron was positively correlated with hemoglobin;  $\rho=0.19$ ,  $p=0.0495$  (Fig 1C).

The linear regression (Table 4) showed that the concentration of groundwater iron is positively associated with hemoglobin concentration after adjusting for dietary iron (Beta=0.19;  $p=0.049$ ). Intake of iron from groundwater had an apparent positive association with hemoglobin; but the relationship marginally missed the statistical significance.

Table 5 presents the total intake of iron from all sources—dietary, groundwater and the low-iron MNP by children using the different levels of compliance - satisfactory (85%) and suboptimum (50%). The all-sources mean  $\pm$ SD intakes of iron in children aged 2-3 years were  $7.5\pm2.0$  mg/day [median (IQR): 7.35(6.1-8.5) mg/day] and  $5.8\pm2.0$  mg/day [median (IQR): 5.6(4.3-6.7) mg/day] at the 85% and 50% compliance level of MNP intakes,

respectively. In the 4-5 year-old group, the respective intakes were  $8.6 \pm 2.5$  mg/day [median (IQR): 7.92(6.6-10.1) mg/day] and  $6.9 \pm 2.5$  mg/day [median (IQR): 6.2(4.9- 8.4) mg/day]. At the satisfactory compliance of MNP intake (85%), all sources intakes were 250% and 210% of the average reference intake (EAR) in the 2–3 year-old and 4-5 year-old sub-groups, respectively. At the suboptimum compliance of intake (50%) of the MNP, all sources' aggregated intakes were 193% and 169% of the EARs, respectively.

In the case of the low-iron MNP intake at suboptimum compliance, the intake of iron from all sources was 83% and 69% of the RDAs in the respective age groups. In the case of satisfactory compliance, the fulfillment of RDAs met was 107% and 86% respectively (Table 5).

Table 6 presents the mean  $\pm$ SD intake of bioavailable iron from all sources (dietary + groundwater+ low-iron MNP) by differential absorption potential of water iron and differential MNP intake compliances. In case the children were considered iron-depleted (consistent with 40% absorption of water-iron) and consumed the low-iron MNP at the satisfactory compliance level (85%), the intakes of combined sources of bioavailable iron was  $0.58 \pm 0.24$  mg/day and  $0.71 \pm 0.32$  mg/day in the 2-3 year-old and 4-5 year-old children respectively. In the event of the children being iron-replete (consistent with 10% absorption of water-iron), the intakes of the bioavailable iron in the 2-3 year-old and 4-5 year-old children would be  $0.39 \pm 0.11$  mg/day and  $0.46 \pm 0.14$  mg/day respectively.

When the low-iron MNP is used at the suboptimum compliance level (50%), the intakes of combined sources of bioavailable iron would be  $0.50 \pm 0.24$  mg/day and  $0.63 \pm 0.32$  mg/day in iron-deplete 2-3 year-old and 4-5 year-old children respectively. The intake of the same MNP with the same compliance would render the intake of bioavailable iron in iron-replete 2-3

year-old and 4-5 year- old children, as  $0.31 \pm 0.11$  mg/day and  $0.38 \pm 0.14$  mg/day respectively (Table 6).

Table 6 further depicts that the combined all-sources intake of bioavailable iron exceeded the median of the reference intake [17] regardless of the intake compliance in the iron-depleted children. It exceeded the 95<sup>th</sup> percentile of the reference in the 4-5 year-old children who were iron depleted while in the 2-3 year-old group, the intake was equaled (i.e., to the 95<sup>th</sup> percentile). In the iron-replete children, the all-sources intakes of bio-available iron were lower than the median reference intake regardless of the intake compliance (Table 6).

### 3.2 An account of the iron intake in relation to the intake of the standard MNP

In the case of the standard MNP, the estimated all sources intake of iron exceeded the EAR considerably in both the age groups; 365.8-463.3% (at the satisfactory compliance) and 258.5-316.6% (at the suboptimum compliance; Supplementary Table 1). All-sources intake of bio available iron exceeded both the median and the 95<sup>th</sup> percentile of the reference [17] if the intake compliance was considered satisfactory irrespective of the iron status of the children (i.e., iron-depleted or iron-replete). In the case of the suboptimum compliance, the all-sources intake of bio-available iron exceeded the median regardless of the iron status of the children, and exceeded the 95<sup>th</sup> percentile in the iron- depleted children (Supplementary Table 2)

### Discussion

The present study examined the intake of iron in 2-5 year-old rural Bangladeshi children from all the key sources, including the drinking of groundwater with a low concentration of iron ( $0 < 2$  mg/L), diet, and the hypothetically-consumed low-iron MNP (or the standard MNP) at different compliances of consumption. Hemoglobin concentration of the children was

measured to appraise the potential scope of low-iron MNP to prevent Anaemia in children who drink groundwater with a low concentration of the mineral iron.

Our results revealed that the combined intake of iron from diet and groundwater marginally (107-110%) exceeded the EAR in both age subgroups. However, when low-iron MNP was hypothetically added to that of the diet and groundwater, the higher amount relative to the EARs was further increased- (210-250%) for satisfactory compliance and (168-193%) for suboptimum compliance of MNP intake. To complement this finding, the prevalence of anaemia (in absence of the hypothetical MNP intervention) was low (8.6%). Therefore, the key revelation of the study is that in this low groundwater iron setting in Bangladesh, the low-iron MNP has the potential to prevent childhood anaemia, favoring the hypothesis of the study.

Furthermore, the comparative intakes of water iron, dietary iron and hemoglobin concentrations in children sub-grouped by the median groundwater Fe concentration ( $0 < 0.8$  mg/L vs.  $\geq 0.8 < 2$  mg/L) were examined. There was a small difference in the concentration of hemoglobin between the groups, but it was not statistically significant ( $p=0.20$ ). Dietary intakes of iron in both groups were predominantly cereal-based and were grossly suboptimum relative to the recommended intake, implying that the absorption efficiency of iron was low. The statistical parity in hemoglobin concentrations between the sub-groups was observed along with a) the statistical parity in the amount of the dietary iron and b) about 4.5 times lower intake of groundwater iron in the subgroup with groundwater Fe concentration below the median ( $0-0.8$  mg/L). This clearly demonstrated that even a very modest amount of daily consumption of iron from drinking water was plausibly associated with a fair concentration of hemoglobin at the non-anemic level.

We observed a significant positive correlation of hemoglobin concentration with a) iron concentration in groundwater, b) intake of iron from groundwater and c) intake of dietary iron. The total sample estimate of the amount of iron consumed from drinking groundwater was roughly 25% of the dietary iron consumption (0.80 mg/d vs. 3.28 mg/d). Despite this, the correlation coefficient of the intake of water iron and hemoglobin was slightly larger than that between dietary iron and hemoglobin, and the coefficient of iron concentration in water and hemoglobin was the largest. The findings suggest that water iron is slightly more efficient than dietary iron in favorably influencing hemoglobin concentration in children. We also observed (results not shown) that the effect of water iron on the concentration of hemoglobin is further complemented when cases with zero value of iron concentration in the drinking water are omitted. This shows that the concentration of groundwater iron (non-zero) at the lower sub-group (iron concentration (0-<0.8 mg group) and the children's hemoglobin concentration had a larger correlation coefficient;  $\rho=0.41$ ,  $p=0.06$ .

Further, we performed linear regressions to study the association of the concentrations of iron in groundwater and hemoglobin. After adjusting for the intake of dietary iron, concentration of the water iron was positively associated with hemoglobin concentration. However, a significant association was marginally missing between the intake of water iron and hemoglobin concentration after adjusting for dietary iron. We assume that the low intake of drinking water in this age group of children, with subsequent low intake of water iron, might have resulted in a statistical non-significance despite a positive trend of association. Nonetheless, overall, the findings of the correlational and regression analysis were complementary.

The amount of the combined bioavailable iron from dietary and drinking water sources (0.22 mg/day) in children drinking from the wells in the bottom sub-group (0-<0.8 mg/L) was lower than the FAO/WHO recommended median value of the daily requirement of the

bioavailable iron (0.46 mg/d) [17]. Despite the low amount of bioavailable iron, the mean concentration of hemoglobin in this subgroup was 11.93 mg/dl (median 12 mg/dl), which is well-above the cut-off for defining Anaemia (<11 mg/dl), and most of the children (87.8%) were non-anemic (results not shown). This is difficult to explain on the back of low absorption (5%) of dietary iron due to their predominantly cereal-based food intake. But we assume that despite being in small amount, a constant daily dose of highly bioavailable (23% assumed in the present study) iron from drinking water over the years might have replenished and developed the body iron reserve in the children to support hemoglobin synthesis. Furthermore, this outcome is complemented by the fact that the combined intake of iron from dietary and groundwater sources exceeds the EAR for iron.

Since a small proportion of the children in this subgroup were anemic, a supplement with a low dose of iron (low-iron MNP) may potentially be useful to prevent Anaemia in them. This proposition can be assessed to some extent when the bioavailable iron from all the sources combined is appraised, sorted by MNP types and different MNP intake compliances. In this setting (i.e., children drinking from the low-Fe groundwater), if low-iron MNP is consumed at the suboptimum compliance (i.e., 50%), the estimated amount of bioavailable iron in the iron deficit children would be  $0.50 \pm 0.24$  mg/d and  $0.63 \pm 0.32$  mg/d in the 2-3 year-old and 4-5 year-old subgroups respectively, which are above the median level of the requirement of bioavailable iron in the groups. Higher compliance of the low-iron MNP or usage of the standard MNP at either level of compliance (satisfactory or suboptimum) would result in an even higher amount of bioavailable iron with a potentially higher likelihood of side effects. These observations suggest that low-iron MNP in low groundwater iron settings has the potential to prevent Anaemia in children.

The strength of this study is that we used venous blood which has a higher accuracy for measurement of hemoglobin [22,23,24]; hence the hemoglobin measured in the study better reflects the actual status. The study has a number of limitations. Firstly, we did not consider the real-life consumption of MNP to account for its actual contribution to the intake of iron; instead, we modeled to show hypothetical intake levels of MNPs for determination of the combined intakes of iron from the multiple sources. Secondly, consumed iron from multiple channels might have inter-source interactions in the gastro-intestinal tract which might influence the ultimate absorption in the intestines. Studying such interactions was beyond the scope of the study. In addition, an iron status biomarker, e.g., ferritin, was not included in the study. This could have been used to explain explicitly the satisfactory level of hemoglobin in children, despite their being exposed to a low amount of iron from groundwater.

In conclusion, the study suggests that in low-iron groundwater settings in Bangladesh, the combined intake of iron from dietary and groundwater sources was associated with maintenance of hemoglobin concentration at the non-anemic level in the majority of the 2-5 year-old children. The small proportion of the anemic children exposed to a very low level of iron from drinking groundwater might plausibly benefit from a low-dose iron supplement. However, a randomized controlled trial is required to confirm the findings.

#### Author's contributions

Sabuktagin Rahman: Conceptualization, Methodology, Data curation, Data analysis, Original draft preparation; Patricia Lee: Conceptualization, Data analysis, Writing-Reviewing and Editing; Faruk Ahmed: Conceptualization, Methodology, Data analysis, Writing- Reviewing and Editing.

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Table 1: Selected Child and Household Characteristics

Child and Household Characteristics	Estimates (n=122)
Age (months)	43.0±10.6
Sex (Female), n (%)	54(44.3)
Occupation of household head	
Business, n (%)	15(12.3)
Skilled labor, n (%)	18(14.8)
Unskilled labor, n (%)	40(32.8)
Farmer, n (%)	14(11.5)
Mother's education (years)	6.3±4.6
Weekly expenses on principal food items* (BDT.)**	1801.3±676.1
Amount of cultivable land (decimal unit)	23.6±43.5
Intake of water (ml)	885.5±428.4

Variables are reported as mean ±SD unless stated otherwise

\*Rice, flour, oil, fish, meat, sugar, salt

\*\*Bangladeshi Taka

Table 2: Intake of dietary iron, groundwater iron and combined intakes (mg/day) sorted by the age subgroups

Age subgroup	RDA	EAR	Intake of Fe (dietary)*		Intake of Fe (water) †		Intake of Fe (dietary +water) ‡		Combined intake % of reference intakes	
P	A	R	Mean ±SD	Median (IQR)	Mean ±SD	Median (IQR)	Mean ±SD	Median (IQR)	% of RDA	% of EAR
2-3 year-old	7	3	2.62±1.84	2.14 (1.48-3.22)	0.64±0.51	0.55 (0.26-1)	3.3±2.0	3.10 (1.84-4.26)	47.1	110
4-5 year-old	10	4.1	3.51±2.34	2.87 (1.77-4.6)	0.85±0.73	0.71 (0.31-1.26)	4.4±2.5	3.67 (2.37-5.92)	44.0	107.3

RDA, Recommended Dietary Allowance; EAR, Estimated Average Intake; SD, Standard Deviation; IQR, Inter-Quartile Range

\*Dietary iron was measured by a 24-hour recall

†Intake of water iron was measured by a 24-hour recall

‡Combined intake was calculated by summation of intake of dietary iron and groundwater iron.

Table 3: Comparative intake of actual and bioavailable iron from groundwater and diet, and hemoglobin and Anaemia status in children in the subgroups derived by the median concentration (0.8 mg/L) of iron in groundwater

Subgroups	Intake of water iron Mean $\pm$ SE ,n	p-values
Groundwater iron concentration <0.8mg/L	0.24 $\pm$ 0.04*,44	<0.001
Groundwater iron concentration $\geq$ 0.8 mg/L	1.11 $\pm$ 0.07,78	
Combined	0.80 $\pm$ 0.06,122	
	Intake of dietary iron Mean $\pm$ SE ,n	
Groundwater iron concentration <0.8mg/L	3.32 $\pm$ 0.38*,44	0.79
Groundwater iron concentration $\geq$ 0.8 mg/L	3.26 $\pm$ 0.23,78	
Combined	3.28 $\pm$ 0.20,122	
	Intake of bioavailable water Fe§ Mean $\pm$ SE ,n	
Groundwater iron concentration <0.8mg/L	0.056 $\pm$ 0.009*,44	<0.001
Groundwater iron concentration $\geq$ 0.8 mg/L	0.26 $\pm$ 0.017,78	
Combined	0.18 $\pm$ 0.014,122	
	Bioavailable dietary Fe Mean $\pm$ SE,n	
Groundwater iron concentration <0.8mg/L	0.16 $\pm$ 0.02*,44	0.79
Groundwater iron concentration $\geq$ 0.8 mg/L	0.16 $\pm$ 0.01,78	
Combined	0.16 $\pm$ 0.11,122	
	Bioavailable Fe-- groundwater and dietary combined Mean $\pm$ SE ,n	
Groundwater iron concentration <0.8mg/L	0.22 $\pm$ 0.019*,44	<0.001
Groundwater iron concentration $\geq$ 0.8 mg/L	0.42 $\pm$ 0.023,78	
Combined	0.35 $\pm$ 0.018,122	
	Hemoglobin concentration Mean $\pm$ SE ,n	
Groundwater iron concentration <0.8mg/L	11.91 $\pm$ 0.91 †,41	0.30
Groundwater iron concentration $\geq$ 0.8 mg/L	12.17 $\pm$ 0.94 ,64	
Combined	12.07 $\pm$ 0.93,105	
	Anaemia, n (%)	
Groundwater iron concentration <0.8mg/L	41(12.2) ‡	0.29
Groundwater iron concentration $\geq$ 0.8 mg/L	64(6.25)	
Combined	105(8.6)	

\*Mann-Whitney U test; <sup>†</sup>Student's t-test; <sup>‡</sup>Chi-Square test; §Groundwater iron absorption 23%  
 ||Subgroups based on the median concentration of groundwater iron

Table 4: Linear regression showing association of the water iron estimates (I. iron concentration of groundwater and II. iron intake from groundwater) and hemoglobin after controlling for the intake of dietary iron

Independent variables	Covariate	Coefficient	p-value	Beta
I. Concentration of groundwater iron		0.317	0.049	0.19
II. Intake of iron from groundwater	Dietary iron	0.184	0.11	0.14

Table 5: Estimates of the combined intake of iron from the key sources—dietary, groundwater and the low-iron MNP and the contribution to the Dietary Reference Intakes at different intake compliances of the low-iron MNP										
Age subgr oup (years ,n	RD A mg/d	EA R mg/ d	Intake of iron (Dietary +Groundwater + MNP)				% of RDA* at 85% compliance	% of EAR* at 85% compliance	% of RDA* at 50% complian ce	% of EAR* at 50% complia nce
2- 3,31	7	3	Low-Fe MNP @ 85%		Low-Fe MNP @ 50%		107	250	82.8	193
			Mean ±SD	Median (IQR)	Mean ±SD	Median (IQR)				
			7.5±2.0	7.35 (6.1-8.5)	5.8±2.0	5.6 (4.3-6.7)				
4- 5,91	10	4.1	8.6±2.5	7.92 (6.6-10.1)	6.9±2.5	6.2 (4.9-8.4)	86	210	69	169

RDA, Recommended Dietary Allowance; EAR, Estimated Average Intake; SD, Standard Deviation; IQR, Inter-Quartile Range;

MNP, Micronutrient Powder;

\*Proportions of RDA and EAR were calculated as the mean intake as proportion of the RDA/EAR values

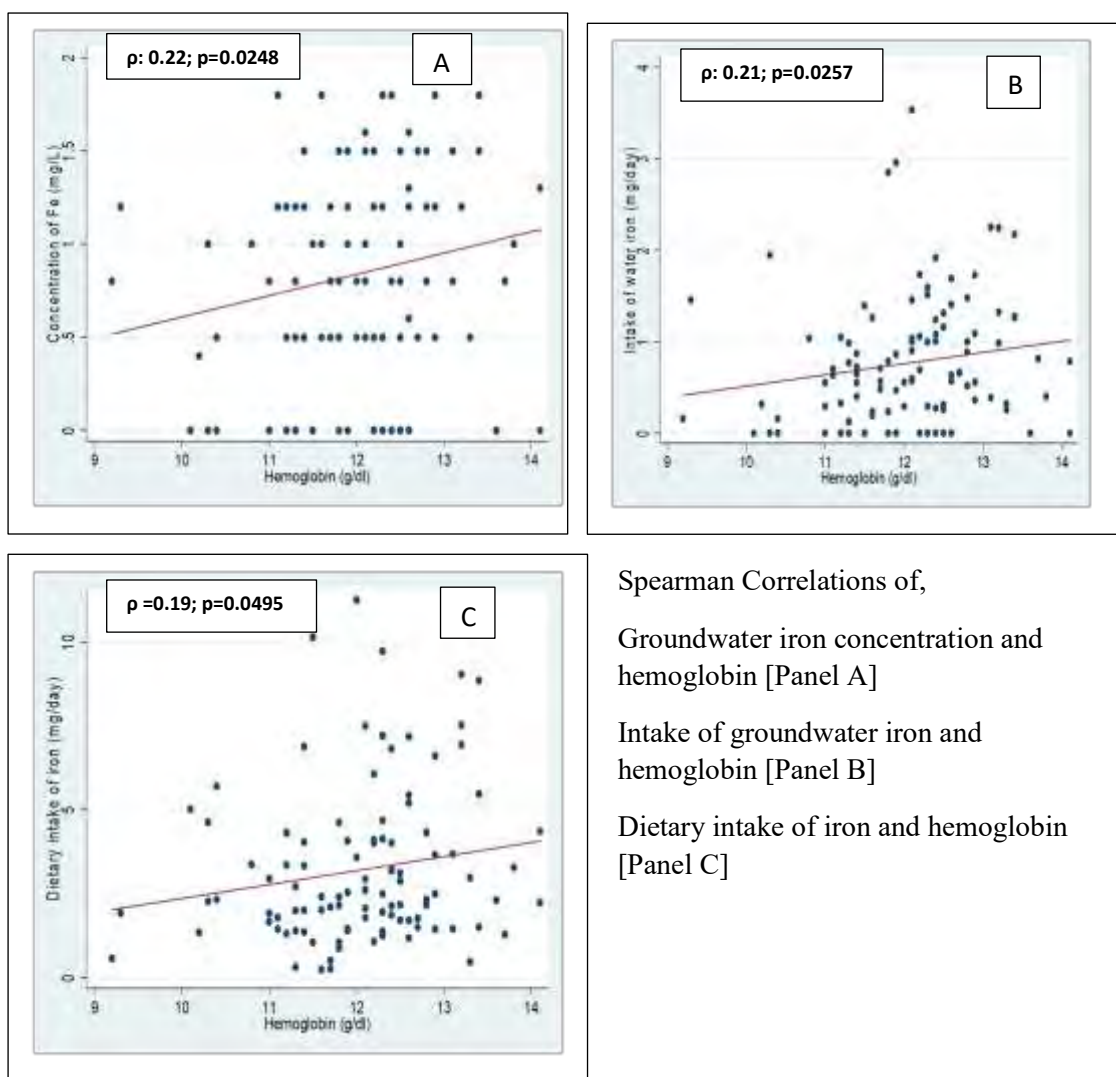
Table 6: Combined Intake of Bioavailable Iron from the Key Sources when the Low-iron MNP is consumed at different intake compliances in iron-depleted and iron-replete statuses

Age subgroup (years)	Absolute requirement of bioavailable Fe (mg/day)		Amount of all-sources bioavailable Fe from the key sources (Dietary +Groundwater +MNP) as per differential absorption potential of water iron sorted by differential MNP intake profiles and body- iron status			
			Differential absorption of groundwater iron			
			In case the subjects are iron-depleted *		In case the subjects are iron-replete†	
			At 85% intake of low-iron MNP			
	Median	95 <sup>th</sup> percentile	Mean ±SD ,n mg/day	Median (IQR),n mg/day	Mean ±SD, n mg/day	Median (IQR),n mg/day
2-3	0.46	0.58	0.58±0.24,31	0.49(0.40-0.78)	0.39±0.11, 31	0.37(0.31-0.45)
4-5	0.50	0.63	0.71±0.32,91	0.61(0.47-0.91)	0.46±0.14, 91	0.42(0.35-0.52)
2-5			0.67±0.31,122	0.58(0.44-0.86)	0.44±0.14, 122	0.40(0.33-0.50)
			At 50% intake of low-iron MNP			
			Mean ±SD ,n mg/day	Median (IQR),n mg/day	Mean (SD),n mg/day	Median (IQR),n mg/day
2-3	0.46	0.58	0.50±0.24, 31	0.41(0.32-0.70)	0.31±0.11,31	0.29(0.23-0.37)
4-5	0.50	0.63	0.63±0.32, 91	0.53(0.39-0.84)	0.38±0.14, 91	0.34(0.27-0.44)
2-5			0.60±0.31,122	0.50(0.36-0.79)	0.36±0.14, 122	0.32(0.25-0.42)

SD, Standard Deviation; IQR, Inter-Quartile Range; MNP, Micronutrient Powder

\*Absorption of iron 40%

†Absorption of iron 10%



Spearman Correlations of,  
 Groundwater iron concentration and hemoglobin [Panel A]  
 Intake of groundwater iron and hemoglobin [Panel B]  
 Dietary intake of iron and hemoglobin [Panel C]

**Figure 1: Spearman Correlation of hemoglobin concentration and A. Groundwater iron concentration, B. Intake of groundwater iron and C. Dietary iron.**

Supplementary Table 1: Distribution of the combined intake of iron from the key sources—dietary, groundwater and the standard MNP sorted by different intake compliances of the MNP

Age subgroup	RDA	EAR	Intake of iron (Dietary +Groundwater + MNP)			
			Standard MNP @85%		Standard MNP @ 50%	
			Mean $\pm$ SD, n	Median (IQR)	Mean $\pm$ SD, n	Median (IQR)
2-3 years	7	3	13.9 $\pm$ 2.0, 31	13.7 (12.5-14.9)	9.5 $\pm$ 2.0, 31	9.35 (8.1-10.5)
4-5 years	10	4.1	15.0 $\pm$ 2.5, 91	14.3 (13-16.5)	10.6 $\pm$ 2.5,91	9.92 (8.6-12.1)

RDA, Recommended Dietary Allowance; EAR, Estimated Average Intake; SD, Standard Deviation; IQR, Inter-Quartile Range

In children 2-3 years old, the combined intake i.e. mean  $\pm$ SD of iron from all sources when standard MNP is considered is 13.9 $\pm$ 2.0 mg/day [median (IQR): 13.7 (12.5-14.9) mg/day] and 9.5 $\pm$ 2.0 mg/day [median(IQR): 9.35(8.1-10.5) mg/day] at 85% and 50% compliances respectively. In children 4-5 years- old the combined intake of iron from all sources when standard MNP is considered is 15.0 $\pm$ 2.5 mg/day [median (IQR): 14.3(13-16.5) mg/day] and 10.6 $\pm$ 2.5 mg/day [median (IQR): 9.92(8.6-12.1) mg/day] at the respective compliances of MNP intakes. All-source intake of iron exceeded the RDA levels in both the age groups irrespective of the MNP intake compliances. The intake is 198.5% and 150% in the respective age groups (at the satisfactory compliance) and 135.7% and 106% (at the suboptimum compliance). If the EAR is used as the dietary reference, the exceeds were higher—463.3% and 365.8% (at the satisfactory compliance) as well as 316.6% and 258.5% (at the suboptimum compliance).

Supplementary Table 2: Combined Intake of Bioavailable Iron from the Key Sources when the Standard MNP is consumed at different intake compliances in iron-depleted and iron-replete statuses

Age subgroup	Absolute req. of bioavailable Fe (Median)/95 <sup>th</sup> Percentile		Amount of all-source bioavailable Fe from the key sources (Dietary +Groundwater +MNP) as per differential absorption potential of water iron sorted by differential intake profiles of the standard MNP and body- iron status			
	Median	95 <sup>th</sup> p	Differential absorption of groundwater iron			
			In case the subjects are iron-depleted *		In case the subjects are iron-replete†	
			@85% intake of standard MNP			
			Mean ± SD	Median(IQR)	Mean ± SD	Median(IQR)
2-3 years	0.46	0.58	0.86±0.24	0.78(0.69-1.06)	0.69±0.11	0.66(0.61-0.75)
4-5 years	0.50	0.63	0.99±0.32	0.89(0.75-1.2)	0.75±0.14	0.72(0.64-0.82)
			@50% intake of standard MNP			
			Mean±SD	Median(IQR)	Mean ± SD	Median(IQR)
2-3 years	0.46	0.58	0.66±0.24	0.58(0.49-0.87)	0.49±0.11	0.46(0.41-0.54)
4-5 years	0.50	0.63	0.79±0.32	0.70(0.56-1.0)	0.55±0.14	0.51(0.44-0.62)

SD, Standard Deviation; IQR, Inter-Quartile Range

\*Absorption of iron 40%

†Absorption of iron 10%

In case the children are considered iron-depleted (consistent with 40% absorption of water-iron)--if standard MNP is used at the satisfactory level of compliance (85%), the intake of combined sources of bioavailable iron would be 0.86±0.24 mg/day and 0.99±0.32 mg/day in 2-3 years and 4-5 years old children respectively. In the event of the children being iron-replete (consistent with 10% absorption of water-iron), the intake of the bioavailable iron in 2-3 years and 4-5 years old children would be 0.69±0.11 mg/day and 0.75±0.14 mg/day respectively.

In case the standard MNP being used at the suboptimum compliance (50%), the intake of combined sources of bioavailable iron would be 0.66±0.24 mg/day and 0.79±0.32 mg/day in iron-deplete 2-3 years and 4-5 years old children respectively. Using the same MNP with the same compliance, in iron-replete 2-3 years and 4-5 years old children the intake of the all-sources bioavailable iron would be 0.49±0.11 mg/day and 0.55±0.14 mg/day respectively.

## Chapter 6: General Discussion and Conclusion

## Introduction

Sections 5.2.1.3, 5.2.2.3, 5.2.3.1.1, 5.2.4.1.1 and annexes 1, 2 and 3 have included separate discussions –that explained the individual research findings and recommendations. Those sections also provided the strengths and limitations of each study. The chapter 6 summarises the key findings from the main trial and two supplementary studies and provides a general discussion of the study findings. It also discusses the strengths and limitations, as well as the policy implications and recommendations for future research.

The prevalence of childhood anaemia in children aged 6-59 months old is high in Bangladesh, and has been reported by various national surveys. However, contrary to the widely held assumption, iron deficiency in children, the most common cause of anaemia, is surprisingly low (NMS 2011-12, NMS 2021, preliminary report). Bangladesh poses a unique context; on the one hand, the burden of anaemia is high (NMS, 2011-12, BDHS, 2011); but on the other hand, the iron status in the population is satisfactory. The satisfactory iron status at a population level is associated with a fair-to-high level of iron in drinking groundwater (Merrill et al., 2011, NMS, 2011-12, Ahmed et al., 2018). Nonetheless, due to the high burden of anaemia, the national policy supports blanket supplementation of micronutrient powder (which includes 12.5 mg of iron per dose) for the prevention of childhood anaemia (National Anaemia Consultation, 2016). These programmes are run under government and NGO activities. However, the MNP programmes often suffer from low coverage (Sarma et al., 2020) and side effects such as nausea, diarrhoea, vomiting, and constipation are reported as the reasons for the discontinuation and poor coverage (Mitra et al, 2015).

It is important to note that iron is a pro-oxidant and its entry into the body is tightly regulated by the status of the iron reserve and the presence of infection and/or inflammation. In case the body is endowed with sufficient reserves of iron, its absorption is inhibited at the

gastro-intestinal tract (Saito et al., 2014). Thus, the unabsorbed iron adversely influences the composition of the gut microbiota in the intestines. This disturbance in the microbiota composition is consistent with the biological mechanism of the iron-induced clinical side effects.

To address this problem, the present research project was planned to assess the effect of the MNP supplement with a low dose of iron (5 mg) in preventing childhood anaemia in children whose potable water contains a high amount of iron ( $\geq 2$  mg/L). A randomized controlled trial (RCT) was conducted to assess the treatment effect of the low-iron MNP against the standard MNP (12.5 mg iron). The study also compared clinical side effects between the treatment groups. Since the iron-induced side effects are rooted in the adverse changes of the composition of the intestinal microbiota, on a subsample, the treatment groups were compared for the effects on the composition of the microbiota. It is important to note that there is a paucity of studies involving low-iron MNP on efficacy of iron status and anaemia outcome and the iron-induced side effects in a context where populations are largely iron-replete from the iron-rich drinking groundwater source. To my knowledge, the trial is the first of this kind globally.

Before the main study (i.e., the RCT), three substudies, necessary for facilitating the RCT with high quality data, were conducted. The first preparatory study (annex 1) was conducted for developing a non-device-based taste-rating tool for the initial screening of the tube wells with a high level of iron in groundwater. The intent was to limit the use of expensive, imported iron-measuring chemical reagents. Taste-ratings were standardized as “no-iron”, “some iron”, and “heavy iron”, based on the perceived taste sensation of the water sample experienced, such as *sweet*, *bitter*, *burning*, or *sticking*, for example. The iron concentration of the well-water was measured using a portable colorimetric test kit device to compare and standardise the test ratings. Results revealed that there was a significant positive

association of taste-rating for the presence of iron in water, and actual iron concentration. Thus, the tool was used during the site selection for the RCT to be conducted, and for the initial screening of the households with a high level of iron in tube well water, for possible inclusion in the trial.

The second preparatory study was the validation of a 7-d semiquantitative food frequency questionnaire (SQ-FFQ) for the dietary assessment of Bangladeshi children; the details of this study are presented in the annex 2. This SQ-FFQ has been in use in Bangladesh in national surveys and dietary assessment studies, but had not been validated. Because the present trial took into consideration the study children's dietary intake data, especially the micronutrients of haemopoietic potentials such as iron, vitamin A, zinc, folic acid, and vitamin B12, the validation of the tool was essential. For the study, the 7-d SQFFQ was administered to different children (not the trial children) aged 2-5 years old. The intake measured by the SQFFQ was compared to the intakes measured by two 24-hour dietary recalls recorded in the same children and held on non-consecutive days. Results revealed that after adjusting for the energy intake and de-attenuation for within-subject variation, the tool demonstrated a *good* correlation for the key micronutrients: i.e., iron, zinc, calcium, and vitamin A. Thus, this validated 7-d SQ-FFQ tool was used for assessing the dietary intake of the children enrolled in the RCT.

The third preparatory study, described in the annex 3, was the assessment of the temporal concentration of iron in groundwater over 6 hours. Iron in groundwater might rapidly oxidise upon contact with air and precipitate, which might affect the availability of iron from water. Hence, this study was conducted to get an understanding of the extent of decline of the iron concentration in water when it is stored. The implication was for possible adjustment of the intake of water iron in the children participating in the trial. The extracted

groundwater samples were measured at six time points- 0.0 hr, 0.5 hr, 1.0 hr, 2.0 hr, 3.0 hr and 6.0 hr. The concentration of iron over the time was measured and compared.

Furthermore, I have conducted two supplementary studies to answer some important questions with the possibility of replacing the standard MNP programme with a low-dose iron MNP for alleviating the problem of anaemia in Bangladeshi children.

The first supplementary study (section 5.2.3.1.1) was a subanalysis of the main trial conducted by stratifying the study samples with and without thalassaemia. The basis of this study was that the thalassaemia carriers are prone to have a hyperferremic state; thus, the comparative effects of the MNP supplements on the iron and haemoglobin level of the children in conjunction with the exposure to high level of groundwater iron might indicate which MNP is more suitable for the thalassaemic carriers. This was essential because Bangladesh does not have a mass screening programme for thalassaemia. Among the trial participants, iron and haemoglobin status were compared at baseline and the endpoint between the children with and without thalassaemia.

The second supplementary study (section 5.2.4.1.1) examined the scope of the low-iron MNP in alleviating anaemia in children drinking groundwater containing a low level of iron.

The main trial proved that the low-iron MNP was effective in the prevention of anaemia in children drinking groundwater with a high concentration of iron. Nonetheless, there are areas in the country where the groundwater contains a low concentration of iron. However, operating programmes with multiple MNP formulations would be logistically and administratively challenging to manage. Therefore, an observational study was conducted among the users of groundwater with a low concentration of iron, to assess the potential utility of the low-iron MNP in the prevention of childhood anaemia. The study measured the

combined intake of iron from the key sources: dietary, groundwater, and the low-iron MNP (hypothetical). The intakes were triangulated with the measured haemoglobin concentration and anaemia prevalence.

I appraised the findings of the trial and the two supplementary studies to assess the potential of using low iron MNP in the programme setting to curb childhood anaemia irrespective of the level of iron in groundwater and the presence of the thalassaemia carriers.

#### Summary of the Key Findings

The main findings of this research (section 5.2.1.3) revealed that the low-iron MNP was non-inferior to the standard MNP in the prevention of anaemia in children aged 2-5 years old who drink groundwater with a high concentration of iron. Furthermore, the intake of the low-iron MNP resulted in fewer incidence of iron-induced side effects: e.g., diarrhoea (IRR: 0.29,  $p=0.01$ ), nausea (IRR: 0.24,  $p=0.002$ ), and fever (IRR: 0.26,  $p<0.001$ ) in the recipient children compared to the children who consumed the standard MNP. The other component of the trial was the assessment of the effect of the MNP supplements on the composition of gut microbiota (section 5.2.2.3). Overall, the low iron MNP did not show significantly different effects on the composition of gut microbiota relative to the standard MNP.

In addition, two supplementary studies were conducted to justify the possible use of low-dose (5.0 mg) iron MNP for alleviating the problem of anaemia in Bangladeshi children and thereby replacing the standard MNP programme.

The first supplementary study (section 5.2.3.1.1) examined the effect of thalassaemia on the comparative status of haemoglobin and iron in the trial children, exposed concurrently to MNP supplementation and high iron content in drinking groundwater source. The findings revealed that there was no change in haemoglobin concentration between the baseline and the endpoint in the thalassaemia carriers. The reserve of body iron at baseline was significantly

higher in the thalassaemia carriers than in non-carriers. Iron-induced side-effects, such as episodes of loose stools over the intervention period were apparently higher in the children with thalassaemia.

The second supplementary study (section 5.2.4.1.1) was conducted to examine the potential of the low-dose iron MNP in preventing anaemia among the children drinking the groundwater with a low level of iron ( $0 < 2$  mg/L). The findings revealed that the median iron concentration of the water was 0.8 mg/L. Based on this median estimate of the groundwater concentration, two subgroups were compared for the level of haemoglobin. The findings further revealed that the children who drank water containing a very low level of iron ( $0 < 0.8$  mg/L) had the mean and median haemoglobin concentrations at the non-anaemic level. Haemoglobin concentration and the prevalence of anaemia of the children of the ( $0 < 0.8$  mg) sub group were statistically not different from those of the children belonging to the ( $0.8 < 2$  mg/L) sub group.

## **Discussion of the Findings**

### **Effect of Low Iron MNP on Preventing Low Haemoglobin Level**

The concentration of haemoglobin in the standard MNP group was 12.23 g/dl (baseline) and 12.46 g/dl (endpoint). In the low-iron MNP group, the respective concentrations of haemoglobin were 12.37 g/dl and 12.40 g /dl. The low-iron MNP resulted in a 0.14 g/dL lower effect on the haemoglobin concentration compared with the standard MNP ( $\beta = -0.14$ , 95% CI:  $-0.30, 0.013$ ;  $p = 0.07$ ). The lower bound ( $-0.30$  g/dL) of the 95% CI for the difference in the effect was higher than the priori non-inferior margin ( $-0.50$  g/dL). The findings indicate that the low-dose iron (5 mg) MNP was non-inferior to the standard dose iron MNP (12.5 mg) when comparing the effect on haemoglobin level, thus supporting the hypothesis of the research.

The children in both treatment groups had high body iron reserves at baseline. The standard MNP group consumed about 2.5 times supplemental iron over the intervention period. Interestingly, the efficiency of absorption of iron was superior in the low-iron MNP group compared to the standard MNP group. This is supported by the finding that there was a significant association between the total iron intake from different sources (diet + groundwater + MNPs) and the body iron reserve in the low-iron MNP group, while no such association was observed in the standard MNP group.

Though there is a paucity of literature showing the effect of the low iron MNP or iron supplements in prevention of childhood anaemia compared with the standard dose of iron supplement (e.g. 12.5 mg), the findings of the present trial complement a study on the same topic which compared the low iron treatment with a placebo. In South African primary school children in a malaria-free setting, 2.5 mg iron as NaFeEDTA and 2.5 mg zinc over the 23 weeks intervention significantly increased serum ferritin and decreased the prevalence of ID. The intervention decreased anaemia from 6.3% to 5.3% in the intervention group, while in the placebo group anaemia increased from 8.2% to 12.4% (Troesch et al., 2011). In the present trial setting, non-endemic for malaria and subjects being exposed to a high amount of iron from drinking groundwater, the low iron MNP (5 mg iron as ferrous fumarate) reduced the prevalence of anaemia from 5.8% to 2.5%. It proved non-inferior to the standard MNP (12.5 mg iron) which decreased anaemia from 5.4% to 1%. Hence, despite the difference in the design and the setting, the findings of the trial complement the trial conducted by Troesch et al (2011).

Another study conducted by Teshome et al.(2017) in Kenyan children aged 12-36 months old tested the efficacy of MNPs containing iron-EDTA (3 mg daily) versus the MNP with ferrous fumarate (12.5 mg daily), given over a short, 30-day period. They concluded that both the interventions did not improve iron status or haemoglobin concentrations, as

compared to a placebo, and questioned the effectiveness of MNPs to reduce iron deficiency and anaemia prevalence in their setting. However, as described by the authors, the study was primarily designed to compare the two different iron formulations. Furthermore, the duration of the intervention was short. It is worth noting that the placebo of the Teshome et al. trial essentially was not a true placebo, but contained several haemopoietic micronutrients except iron. The other contextual issue of the Teshome et al. trial was that they conducted the study in a malaria-endemic setting. A malaria setting is unfavourable for haemoglobin synthesis due to hemolysis of RBCs and the hepcidin-induced hindrance to intestinal iron absorption (Spottiswoode et al., 2014). But as the study subjects were administered with anti-malaria medication before the trial, the inhibitory effect of malaria for haemoglobin synthesis was somewhat ameliorated. Although there was a lack of iron, it did not preclude the placebo from exerting some haemopoietic effects and aiding haemoglobin synthesis. Therefore, no net significant effect was detected between the two doses of iron –12.5 mg ferrous fumarate, 3 mg NaFeEDTA and the placebo with haemopoietic nutrients minus iron.

In comparison, the present trial compared two different iron doses –the MNPs containing 12.5 mg versus 5 mg iron (ferrous fumarate); there was no placebo. Although the setting is malaria-free, children of both groups were exposed to a high concentration of groundwater iron which is the potable source in the setting. Groundwater iron is highly absorbable (Worwood et al., 1996) and consequently, the infection-adjusted baseline level of serum ferritin was high. The serum transferrin receptor (sTfR) level was low: below the cut-off indicating iron sufficiency. The reserve of body iron was high in both groups and statistically non-significant differences existed between them. Hence, the baseline prevalence of anaemia was low in both groups. The groundwater iron induced high-iron status hindered an efficient absorption of supplemental iron. Therefore, the reduction of the prevalence of anaemia after the intervention was small and occurred in a slightly higher magnitude in the

standard MNP group. Nonetheless, the low-iron MNP group was statistically non-inferior on the haemoglobin outcome. Hence, despite there being major differences in the design and context between the Teshome et al. study and my trial, no treatment effect of the low-iron MNP with statistical significance was detected.

### **Effect on Iron-Induced Side-Effects**

The findings of the present trial are complementary to the previous evidence on the adverse effects of iron supplementation. The present trial within an environmental context of high serum iron status acquired from drinking iron-rich groundwater, found that the incidence of diarrhoea, nausea, and fever were significantly fewer among the users of the low-iron MNP than in the users of the standard iron MNP. The number of mean episodes of loose stool and diarrhoea was also lower in the low-iron MNP group over the intervention period. Of note, a large cluster-randomized trial in a non-malarious region of Pakistan showed a significant rise in the proportion of days with diarrhoea ( $P=0.001$ ), bloody diarrhoea ( $P=0.003$ ) and in-drawing of the chest (indicative of lower respiratory tract infection) ( $P=0.03$ ) in predominantly anaemic children who received an MNP containing encapsulated iron (12.5 mg/d) compared with a placebo over a 1-year intervention (Soofi et al., 2013). In the absence of malaria and a suboptimum infection burden, such incidence of diarrhoea and bloody diarrhoea in the Pakistan setting seems somewhat unusual, as iron absorption could have been efficient, leaving a minimal load of unabsorbed iron in the intestines. However, the British Geological Survey 2001 reported that Pakistan groundwater is not free from iron, and it ranges between 0 -3.5 mg/L in parts of the country. At the time of the Soofi et al. (2013) study, the issue of groundwater iron was not apparent in the conceptual framework of determinants of iron status and/or anaemia. Overall, there is a complexity to triangulate the involvement of groundwater iron with the iron-induced side effects in the Pakistan trial

setting. First, the lower Sindh region of Pakistan where the trial (Soofi et al) was conducted (Nawabshah-Mirpurkas area), the concentration of groundwater iron is mostly low (0.5 mg/L) with sporadic high levels up to 3.5 mg/L (British Geological Survey 2001).

On the other extreme, the context of that trial was non-endemic for malaria and the trial also reported a low infection burden in the participants. Moreover, the participants were predominantly anemic. These factors favored a net inward gradient for absorption of iron which is associated with low stress on the intestinal microbiome. Therefore, on this premise, the incidence of the iron induced side effects plausibly points out to other factor, such as the groundwater iron.

However, acknowledging the predominant low concentration of iron (0.5 mg/L), this association of groundwater iron and the supplemental iron induced diarrhoea may not be strongly claimed in Soofi et al trial. At the same time, its association may not be completely ruled out. There are two reasons for this---it is difficult to ascertain the exact study areas of the Soofi et al trial. The Nawabshah-Mirpurkas area is a stretch of 135 kilometers, and geologically the sporadic high iron areas usually occur at random. Second, with our present project, it was observed that even at low concentration of groundwater iron ( $0 < 0.8$  mg/L), the haemoglobin concentration in children was 11.91 g/dl (Table 3, pg 175) which is well above the cut-off defining anaemia. This exemplifies that even at a low concentration of groundwater iron, the iron status in children is possibly fair. The low groundwater iron concentration might have a modest effect for the iron-related side effects.

In the present study, the results of three-step hierarchical regression models revealed a large adjusted effect size of the protection from morbidities (i.e. diarrhoea) compared to that in the unadjusted model. In the series of Poisson regression analysis, various household, maternal and child's characteristics including the intake of colostrum were used as covariates in the model.

While AGP at baseline was found slightly un-balanced between the two treatment groups, it was not included in the analysis. The reason for this, in the present study, there was no association observed between the incidence of diarrhoea and AGP. Of note, AGP is a marker of long-term, chronic infection/inflammation (WHO/CDC, 2007, Gannon et al., 2019) and it is generally associated with chronic intestinal inflammation (Kamng'ona et al., 2019, Iqbal et al., 2018) and less likely to be associated with acute incidence of diarrhoea.

In the unadjusted model the independent variable (i.e. treatment group) and the diarrhoeal incidence (dependent variable) was included. The results revealed that the incidence rate ratio (IRR) was 0.614,  $p=0.062$ . In the subsequent modeling procedure, first, the household and maternal characteristics, such as the drinking groundwater iron concentration; mother's hand washing practices before child feeding and after toilet were included as these were associated with diarrhoea. Evidence of association with diarrhoea justified the inclusion of these covariates-- socio economic status, household expenses (Kundu et al, 2021, Khan et al, 2018, Connel et al, 2017), household food insecurity (Ullah et al, 2019), and education of mother (Kundu et al, 2021, Alebel et al, 2018, Santika et al, 2020). The results revealed the IRR: 0.58,  $p=0.042$ .

Finally, the model was further adjusted by addition of some child characteristics on the basis of previous literature suggesting association of these variables with diarrhoea. These covariates are-- age (Aziz et al, 2022) and sex of the child (Kundu et al, 2021, Mosisa et al, 2021), nutritional status of the child (height-for-age z score) (Kundu et al, 2021), intake of iron from groundwater and diet, and diarrhoea over preceding 2 weeks (Patel et al, 2011). The intake history of colostrum was included in the model for its documented association with diarrhoea (Delelegn et al, 2020.) as well as for the theoretical consideration, since

colostrum is an innate immune booster (Cacho et al., 2017, Ballard et al., 2013, Goldman et al., 1982). The adjusted result of the full model showed that the IRR became even stronger and level of significance for the association also increased, IRR: 0.29,  $p=0.01$ . The modelling procedures with gradual adjustments for different sets of covariates (household, maternal and child characteristics) clearly demonstrated a tendency of greater effect sizes and levels of significance, thus indicating a strong protective effect of the low iron MNP from the incidence of diarrhoea.

In the present trial the effect size of the protective effect of the low iron MNP relative to the standard MNP was large when compared with other relevant trial (Soofi et al, 2014). As for example, the low iron MNP incurred ~70% fewer incidence of diarrhoea compared to the standard MNP (IRR: 0.29, 95%CI: 0.11-0.77,  $p=0.01$ ). On the other hand a relevant study in the Subcontinent, the Soofi (2014) trial showed a smaller protective effect i.e. ~25% fewer incidence of diarrhoea in placebo group compared to the MNP groups. These two trials differ in several aspects—

- 1) Age of the children; 6-18 months (Soofi et al) vs. 23-59 months (present study),
- 2) Context; urban and rural (Soofi et al) vs. rural (present study),
- 3) Composition of MNP; (iron, vitamin A, vitamin D, folic acid, vitamin C, and zinc) in Soofi et al trial vs. (iron, vitamin A, vitamin C, folic acid, zinc) in the present trial
- 4) Length of the intervention; 12 months (Soofi et al) vs. 2 months (present trial),

All these contextual and design related differences possibly has resulted in different magnitude of the protective effects of low iron supplements. In this connection, in a Kenyan trial the incidence of diarrhoea in the infants receiving the MNP with no iron was 70% fewer than their peers receiving the standard MNP (12.5 mg iron).

The other possible reason for somewhat lower protection from side effects in Soofi et al study is that the children were mostly anaemic. This signifies a smaller reserve of body

iron (ferritin 14-17 ng/ml) and due to this, the inward gradient for iron perhaps was satisfactory, leaving the intestinal microbiome somewhat less affected by the 12.5 mg iron. Unlike the Pakistan setting, our study children were largely non-anaemic and overtly iron replete (baseline ferritin ~65 ng/ml) which perhaps resulting in diminished inward drive for iron through the intestinal mucosa. This might have led much higher stress on the intestinal microbiota, especially due to 12.5 mg iron. Therefore, a low dose of iron (5 mg) ensued in relatively a high magnitude of protection compared to the standard MNP (12.5 mg iron).

Another possible reason for low degree of the protective effect in the Pakistan trial relative to the present study is the duration of the intervention. A much longer duration of intervention (12 months) in the Soofi et al trial might have possibly induced a moderation of stress on the intestinal mucosa. A much shorter duration of the present trial (2 months) suggests that the high iron dose of the standard MNP (12.5 mg) in the background of the iron-replete status in children could have induced high degree of stress on the mucosa resulting in incidence of diarrhoea more frequently, so much so that the low-iron MNP group appeared to have conferred relatively a large degree of protection.

Another randomized controlled trial conducted by Stoffel et al. (2020) has shown that the total incidence of the gastrointestinal side effects that were assessed (epigastric pain/ nausea/ diarrhoea/ vomiting) was 40% lower with 100 mg dosing than with 200 mg dosing of iron. However, this difference was not statistically significant ( $P=0.105$ ) (Stoffel et al., 2020). Among Kenyan infants, during a trial on examining the effect of low iron MNP (2.5 mg) versus standard MNP (12.5 mg), 27.3% of infants in the standard MNP group required treatment for diarrhoea versus 8.3% in the placebo group ( $p=0.092$ ) (Jaeggi et al., 2015). Salam et al. (2013) in a systematic review considering the studies of developing countries, using a fixed-effect model with no significant heterogeneity, reported that the use of 5-15

components MNP including iron over duration of 2-12 months was associated with a significant increase in diarrhoeal episodes.

The recent scientific evidence suggests that supplementation of iron with or without MNP induces adverse changes in the composition of gut microbiota compared to a placebo, i.e., induces the proliferation of pathogenic species such as pathogenic *E. coli*, *Salmonella*, and *Shigella*, while decreasing the population of the health promoting protective bacteria, e.g., *Lactobacillus*, *Bifidobacterium* (Zimmermann et al., 2010, Jaeggi et al., 2015, Tang et al., 2017).

The present study has attempted to investigate if the clinical side effects demonstrated in the trial are consistent with the composition of the gut microbiota which is considered as the biological mechanism of the iron-induced side effects. Overall, there was no significant effect of the low iron MNP on the composition of gut microbiota, unlike the anticipation that the lower dose of iron might have decreased the pathogenic, disease-causing microbiota and increased the beneficial bacteria. However, on a subgroup (older adult-like microbiome), the higher dose iron (12.5 mg) of the standard MNP showed a trend of positive association ( $p=0.07$ ) with pathogenic *E. coli*, a common microorganism known for causing diarrhoea. There are two possible reasons for not observing the overall treatment effects of the low-iron MNP. First is the lack of a placebo group (i.e., 0 mg iron group) in the trial. The other studies in African settings conducted on a similar topic used a placebo group besides different doses of iron groups, and was able to detect the significant treatment effect of the iron groups (Jaeggi et al., 2015, Tang et al., 2017). In the present trial, the comparison of the 5 mg versus 12.5 mg iron doses might have nullified each other's effect to some extent, and that failed to capture the difference in the composition of the microbiota with statistical significance. Second, users of antibiotics before and/or during the intervention time were excluded. This exclusion might in part have contributed to the observed lack of effects on microbiota

composition, as oral antibiotics have been described as modifying the effect of iron-containing MNPs on the gut microbiota composition in infants (Paganini et al., 2019). Interestingly, Tang et al. (2017) in a trial on assessing the effect of MNP on microbiota composition in Kenyan infants aged 6-9 months old also excluded users of antibiotics from analysis. However, Tang et al. reported the increased pathogenicity in the composition of the microbiota in relation to the MNP group containing 12.5 mg iron (as ferrous fumarate). Possible reason for this could be the consideration of the placebo group (0.0 mg iron) for comparison in the Tang et al trial.

### **Findings of Supplementary Studies**

As explained elsewhere, thalassaemia is a congenital condition characterised by high body iron status. In Bangladesh, thalassaemia carriers who live in a hyper-ferric medical condition are exposed to a fair-to-high level of natural iron coming from drinking groundwater. This has a benefit in that often, thalassaemia carriers suffer from coexisting ID due to premature breakdown of red blood cells. Hence, they might suffer from ID especially in low-income country settings with poor dietary quality (National Micronutrient Survey 2011-12). Occasionally, thalassaemia carriers need exogenous iron supplements. The abundance of wholesome iron from drinking groundwater in Bangladesh might have protected the thalassaemia carriers from ID. However, tube wells (even adjacent wells) differ considerably in respect to the iron content in water. So, some thalassaemia carriers might need supplements as they are not protected from natural water. Furthermore, it is logistically not feasible to screen the entire population of the country to detect thalassaemia, along with the measurement of iron from carriers' drinking sources. Hence, a blanket supplementation is the policy arrangement to control anaemia for the population at large. Presently, under the blanket supplementation, a standard dose of iron is provided, such as the standard MNP with

12.5 mg iron for children. This amount might be excessive for thalassaemia carriers and may exacerbate iron loadings, thus resulting in side effects. To complement this, a subanalysis of the main trial (Supplementary Study 1, Section 5.2.3.1.1) has shown that at baseline, the carriers of thalassaemia had a significantly higher amount of body iron reserve than the children without thalassaemia ( $p=0.03$ ). Therefore, in Bangladesh with a geological presence of iron in the drinking water and absence of screenings for thalassaemia, the low-iron MNP under the blanket programming would be beneficial in children with thalassaemia by minimising the iron loading and probable decrease of iron-induced side effects.

Furthermore, Supplementary Study 2 (Section 5.2.4.1.1) demonstrated that low-iron MNP potentially can be used in children whose source of drinking water contains a very low level of iron ( $0 < 0.8$  mg/L); and are likely to be protected from anaemia. As per the DPHE/BGS 2001, roughly two-thirds of the country's tube wells contain iron with concentrations above 0.8 mg/L. Hence, potentially low -iron MNP can be useful for the large majority of children aged 2-5 years old to prevent anaemia.

Therefore, to summarise, the low-iron MNP demonstrated its utility in several aspects. First, it is effective in the prevention of childhood anaemia with fewer iron-induced side effects in children residing in high-iron groundwater settings. Second, potentially it could be effective in the prevention of anaemia in children whose potable water supply contains a very low level of iron. As such, overall, the low-iron MNP could be useful for the large majority of Bangladeshi children under 5 years old for the prevention of anaemia. With the demonstrated fewer incidence of side effects in the trial children, it brightens the prospect of improved compliance and coverage of the national anaemia control programmes. Third, the low-iron MNP is promising that it might be beneficial to thalassaemia-carrier children in Bangladesh in terms of limiting their iron overload and minimising side effects.

## Strengths

1. The major strength of this research is that it has provided a comprehensive picture for potential utility of the low-iron MNP for control of childhood anaemia. For instance, the RCT has shown the efficacy of low-dose iron MNP compared to standard MNP supplementation in a high-iron groundwater setting. Additionally, a scoping substudy highlighted the potential utility of low-iron MNP even in low iron groundwater settings. Further, another substudy suggested the potential usefulness of the low-iron MNP in thalassaemia carriers exposed to high-iron groundwater by minimising iron overload and the possible reduction of gastrointestinal side effects. Put together, the findings of the trial and the supplementary studies make a substantial case for low-iron MNP for control of childhood anaemia in Bangladesh.
  
2. The study has employed a plethora of quantitative measurements, such as:
  - (a) iron status markers (haemoglobin, ferritin, serum transferrin receptor, and measurement of the body's iron reserve);
  - (b) measurement of infection biomarkers (C-reactive proteins, and 1-alpha acetylated glycoprotein);
  - (c) screening for thalassaemia and haemoglobin E diseases;
  - (d) assessment of helminth status in stool samples;
  - (e) assessment of gut microbiota to examine the effects of the supplementation;
  - (f) quantitative assessment of dietary micronutrient intakes using a validated assessment tool;
  - (g) measurement of groundwater iron intake;

(h) quantitative measurement of the concentration of iron in groundwater and its covariates, e.g., pH, temperature, oxidation-reduction potentials (ORPs)

These measurements offer a robust battery of quantitative data which have sufficiently demonstrated the expected scientific relationship of iron supplements and related biochemical and environmental factors. This strength has aided the substantiality of the findings.

3. We considered covariate adjustment in the RCT. It offered potential beneficial effects such as---First, to correct for probable imbalances in baseline prognostic covariates despite randomisation; second, to increase power by modelling the variability in outcome explained by relationships with highly prognostic covariates; third, to obtain treatment effect estimates that would be more closely relevant to individual patients than to an average population (Assmann et al., 2000, Altman et al., 1985, Hauck et al., 1998). In addition, the CONSORT statement on RCT recommended for adjustment of the baseline covariates. Thus, adjusting the baseline covariates was strength of the study.

## Limitations

1. A limitation of the trial was that double-blindness could not be implemented in its entirety. One of the main investigators could not be blinded to the treatment group coding. This might have introduced some risk of bias. Unfortunately, this could not be avoided as the MNP preparations were imported from India and the customs clearance required the declaration of the composition of the different MNP preparations. However, all field personnel involved in the distribution of MNPs and collection of the compliance of MNP consumption and morbidity data, together with parents of the children, remained blind to the treatment group coding.

2. Because MNP intervention is recommended by the national anaemia control policy (National Anaemia Consultation, 2016), a placebo arm (0 mg iron) was not considered in the trial on ethical grounds. One of the possible reasons for the absence of the significant effect of the low iron MNP on the composition of gut microbiota is the absence of the placebo arm. This constitutes a limitation.
3. The study compared the effect of thalassaemia carrier status on haemoglobin and iron status in children who are exposed to a high amount of iron from drinking groundwater. The findings were consistent with our understanding of scientific knowledge. However, the statistical results fell short of reaching a significant level due to the suboptimum number of the cases of thalassaemia carriers. A larger study is likely to confirm these findings with higher statistical power. The lack of statistical power in the substudy constitutes an additional limitation.
4. In the literature review, the reported intake of iron was assessed by comparing with the EAR of the Institute of Medicine (IOM), which considers a higher bioavailability of iron compared to Bangladeshi cereal based diet. In absence of an indigenous Bangladeshi EAR, customarily the IOM reference is used in Bangladeshi studies. Nonetheless, using the IOM reference is admitted as a limitation.
5. We did not consider the inclusion of the drinking water into the list of the food items for the FFQ used in the trial. The reason was that the intake of iron from the drinking water was recorded separately by using a 24 hour recall (Merrill et al., 2011) which served the purpose of the study. The other reason for not considering the water intake in this FFQ is the fact that the FFQ keeps record of the actual amount of the intake over a period of 7 days. This will be extremely cumbersome for the respondents to recall and report, since recalling the amount of water intake is harder

than other foods. Nevertheless, non-inclusion of the intake of water in the FFQ constituted a limitation.

6. Hepcidin has emerged as the master regulator of iron metabolism (Saito et al., 2014). This protein is critical to understanding the access to or inhibition of *free* iron from entering into the body and into the reticulo-endothelial system to prevent oxidative cellular damage. It maintains appropriate correlations with iron/ferritin (i.e., negative) and infection indication biomarkers (i.e., positive). It depicts a clearer picture of the overall dynamics of iron metabolism. Logistical constraints handicapped the measurement of hepcidin in the trial and this constitutes a limitation. Nonetheless, this limitation is somewhat addressed by calculating the total body iron reserve and has shown an anticipated three-component axis of association in a cascading manner.

These are:

- a. the intrinsic body iron reserve (baseline and endpoint status accrued through groundwater iron, diet, and low iron MNPs) which was effective in the prevention of anaemia;
- b. the superior efficiency of iron absorption in the low-iron MNP (accounted for ongoing combined sources of dietary, groundwater, and MNP iron supplements); and
- c. a reduction of the intestinal side effects in the low iron MNP.

This has aptly addressed the metabolic paradigms for the low-iron MNP supplementation in this particular setting which demonstrated both the effect on prevention of anaemia and incurred fewer side effects.

### **Implication for National Policy and the Process**

As mentioned previously, Bangladesh poses a unique context of a high level of childhood anaemia and a low magnitude of iron deficiency. Furthermore, it was stated that a fair-to-high amount of iron in the potable source groundwater is associated with low ID. Low ID, i.e., good iron status, indicates that the blanket supplementation of iron/MNP might be unnecessary for the large majority of the population and further, it might result in exacerbated iron-induced side effects. The Government of Bangladesh recommended investigating the efficacy and side effects of an MNP with a lower dose of iron (National Anaemia Consultation, 2016). Hence, the present trial was conducted, and demonstrated that low-iron MNP was effective in the prevention of anaemia in Bangladesh children who drink groundwater containing a high amount of iron. The low-dose iron MNP is also found to be associated with fewer incidence of side effects, such as diarrhoea, nausea, and fever. This is the first research to examine the efficacy and side effects of a low-iron MNP (5 mg iron) taking into consideration a setting where the population is largely iron-replete from natural drinking source groundwater (NMS 2011-12). On the other hand, the uses of the standard MNP containing 12.5 mg iron per dose (supported by national anaemia control policies) has reported poor compliance and coverage of the programmes (Mitra et al., 2015). Furthermore, the findings of the supplementary observational studies under this research project indicated two additional insights:

- Low-iron MNP potentially can prevent anaemia in children whose drinking groundwater contains a low level of iron;
- Low-iron MNP is potentially less hazardous to children with thalassaemia.

Therefore, the findings of the present trial and its associated substudies have clear implications for the national childhood anaemia control policy. If the low-iron MNP containing 5 mg iron is introduced in the national policy instead of the standard MNP, it

would benefit the large majority of children under-five years old. The compliance and coverage of the supplementation programmes is therefore likely to be improved.

Further, the issue of groundwater iron is a geological phenomenon. Positive association of groundwater iron and iron status in a population has been reported in other countries (Karacochuk et al., 2015). Therefore, the findings of the present study may also help developing strategies to combat childhood anaemia in other countries and settings similar to Bangladesh, where the potable source is groundwater.

### **The Next Steps in the Research**

The present RCT has proved the efficacy of the low-iron MNP (containing 5 mg iron) in prevention of anaemia along with reducing the side effects in children aged 2-5 years old who drink groundwater with a high concentration of iron ( $\geq 2$  mg/L). The findings of a supplementary observational study indicated that the low-iron MNP may have the potential to curb anaemia in children who are drinking groundwater with a low concentration of iron ( $0 \leq 2$  mg/L). However, to confirm the finding of the latter study, there is need for a randomized control trial examining the efficacy of low iron MNP in children drinking groundwater with low iron content.

As MNP supplementation is considered a short term measure, this need to be phased out by an appropriate long-term intervention. Therefore, future studies should also focus on developing food-based approaches including awareness programmes for controlling anaemia and iron status in Bangladeshi children.

### **Conclusion**

The findings of this research revealed that low-iron MNP containing 5 mg iron per dose is non-inferior to standard MNP in preventing anaemia in Bangladeshi children aged 2-5 years old who drink groundwater with a high concentration of iron. In addition, the low iron

MNP resulted in fewer incidence of iron-induced side effects, such as diarrhoea, nausea, and fever compared to the standard MNP with a higher dose of iron (12.5 mg). Furthermore, the findings of a supplementary observational study suggested that the low-iron MNP can potentially be effective in controlling anaemia in children who drink groundwater containing a low concentration of iron. Additional results of this research project also suggest that the use of the low-iron MNP would be potentially favourable to thalassaemia carriers by minimising excess deposits of iron in the body. This supplement might be most appropriate in the context of Bangladesh where screening of thalassaemia and iron status markers are not feasible due to limited resources.

To aggregate the key results, low-iron MNP appears to be a superior intervention for Bangladesh to prevent childhood anaemia, and thus the findings merit the recommendation to replace the standard MNP. Similar research needs to be undertaken in the settings where groundwater is the common source of potable water, and groundwater iron and thalassaemia are prevalent.

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## 8. Annexure

## 8.1 ANNEX 1

### 8.1.1 OVERVIEW OF PREPARATORY PAPER 1

Title: Development and standardization of taste-rating of the water sample as a semi-quantitative assessment of iron content in groundwater

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### 8.1.2 INTRODUCTION

The present research project *Efficacy of micronutrient powder (MNP) with low-dose of iron supplementation in Bangladeshi children living in areas of high level of iron in groundwater* required a fundamental consideration, tube wells (groundwater) containing iron at and above a certain concentration ( $\geq 2$  mg/L). This requirement was pertinent both for the site selection for the trial and more importantly, to select the tube wells for inclusion in the trial. One way of accomplishing this was to test the random geographical areas and the tube wells by using the reagent-based measurement of iron. However, the reagents were not readily available and imported; therefore, their unrestricted use was logistically infeasible.

Hence, in this preparatory study we developed a non-device-based, non-reagent-based tool for a sensorial assessment of approximated level of iron content in the groundwater samples. The method is the *taste-rating* of the groundwater sample, which was based on anecdotal experience that the *harsher/bitter* taste of the water sample was associated with a higher level of iron.

In this observational study, the method of the taste-rating of the groundwater sample was developed and standardised as the following categories: “no iron”, “some iron”, and

~~heavy iron~~", based on the perceived taste sensation of the water sample experienced, such as ~~sweet~~", ~~bitter~~", ~~burning~~", or ~~sticking~~". The iron concentration of the well-water was measured using a portable colorimetric test-kit device to compare and standardise the test ratings. The results showed that the higher (i.e. numerical values) the taste-ratings, the higher the concentration of iron in the water ( $p < 0.001$ ). Taste-ratings of the water samples have the potential to be used as a semi-quantitative tool for assessment of iron content in groundwater.

The application of the taste-rating tool aided in: (a) the selection of the trial site, and (b) the initial screening of the tube wells with a high level of groundwater iron. At first, the taste-rating tool was used on the water sample of the tube-well. If the taste-rating suggested a potentially high concentration of iron, the reagent-based colorimetric device was used for confirmation, and thus a decision was made about the provisional inclusion of the well in the trial.

### 8.1.3 PUBLISHED PAPER

#### Development and standardization of taste-rating of the water sample as a semi-quantitative assessment of iron content in groundwater

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#### Abstract

The present study attempted to develop and standardize a simple tool of taste-rating of groundwater sample for the level of iron as a semi-quantitative assessment of iron content in groundwater. An observational study was conducted involving randomly selected 666 households with the drinking source of groundwater (e.g. tube-wells) in a high-iron and in a low-iron groundwater area of rural Bangladesh. The respondents of the households were asked to provide their taste-rating of the respective tube-well water. Two external assessors also taste-rated the water samples. Taste-ratings were standardized as “no-iron”, “some iron” and “heavy iron”, based on perceived taste sensation of the water sample experienced, such as “sweet”, “bitter”, “burning”, “sticking” etc. The iron concentration of the well-water was measured using a portable colorimetric test-kit device to compare and standardize the test ratings. In the high-groundwater-iron area (n=400), the mean iron concentrations were 3.69 mg/L, 3.93 mg/L and 7.71 mg/L in the “no”, “some” and “heavy” categories respectively (p=0.001). In the low-groundwater-iron area (n=266), the respective concentrations of iron in the well-water for the taste-categories were 0.08 mg/L, 0.68 mg/L and 3.26 mg/L (p<0.001). There was a small agreement between the assessors and the respondents (Kendall’s  $\tau_b$ =0.14, p=0.004; Lin’s concordance=0.13, p=0.004), however the inter-assessor agreement was high (Kendall’s  $\tau_b$ =0.79, p<0.001; Lin’s concordance=0.77, p<0.001). Taste-ratings of the water

samples have the potential to use as a semi-quantitative tool for measuring iron content in groundwater. External assessors may provide a reliable assessment.

Keywords: Taste-rating; Groundwater iron; Semi-quantitative

## 2. Introduction

Usage of groundwater for drinking is ubiquitous in Bangladesh as 97% of the rural people of the country rely on it for potable supplies (British Geological Survey 2001). Hydrochemistry of Bangladesh groundwater reveals that iron concentration in groundwater is variable, and a high concentration is observed in many parts of the country (British Geological Survey 2001, Bangladesh National Drinking Water Quality Survey 2009). Population-based studies have shown that iron in groundwater is positively associated with iron and hemoglobin status in Bangladeshi populations (Merrill et al 2011, Rahman et al 2016). The national prevalence of iron deficiency (serum ferritin concentration below a specified level according to age) in children and women is low (<10%); and this low prevalence was most likely attributable to the presence of iron in groundwater (Rahman et al 2016). However, iron is a key determinant of hemoglobin synthesis (Stoltzfus et al 2003). The government of Bangladesh maintains the program of iron supplements, as a measure to prevent Anaemia (hemoglobin concentration below a specified level according to age and sex). High prevalence of Anaemia in children (33.1%) and women (26%) still poses a public health concern (Rahman et al 2016). While iron is essential for many physiological functions, an excess amount of iron may lead to various side effects, e.g. diarrhea, nausea, vomiting, and respiratory tract infection (Soofi et al 2013). Further, iron is a pro-oxidant, which in the free-state may lead to the potential damaging effects (Scholl 2005); with possible adversaries in key organs- heart, liver, pancreas (Bowman et al 2006). Hence, on the backdrop of a fair amount of iron naturally

acquired from drinking groundwater, an optimization of the body-iron level is pertinent in the context of a blanket supplementation of iron/iron-containing micronutrients for prevention of population-level Anaemia. This optimization is required to decrease the iron-related side-effects and to increase compliance and coverage of the supplementation programs. Rahman et al. recommended for assessment of groundwater iron prior to the design of Anaemia prevention/iron supplementation programs (Rahman et al., 2016).

Presence of groundwater iron can be assumed by clogging/rusty discoloration of household water supplies, household appliances, and staining of clothes and body parts, such as teeth and nails (Rahman et al 2018). However, this iron is usually quantified by various devices. In the low-income countries, the devices and reagents for measuring the concentration of iron in groundwater are imported, expensive and not readily available. Hence, we attempted to develop and standardize a simple and non-device based method of taste-rating of groundwater sample for the level of iron for a semi-quantitative assessment of groundwater iron. The basis of the taste-rating is the anecdotal experience that the metallic/bitterly taste of the water sample is associated with iron level in groundwater. Recently, we conducted a small pilot study (n=13) which observed a positive association of taste-rating of groundwater sample and its concentration of iron (Rahman et al 2018). The present study with a much larger sample attempted to develop and standardize the method.

## 1. Methods

### 2.1 Study area and sample size

The study was conducted in two sub-districts- Belkuchi and Pirganj, situated in the north-west and northern part of Bangladesh respectively (Figure 1).



Fig. 1. Study sites: Map of Bangladesh showing the location of the study sub districts (upazila)- Belkuchi (24.2917°N 89.7000°E) and Pirganj (25°51.3'N 88°22'E)

According to the report of the Department of Public Health Engineering of Bangladesh and the British Geological Survey (BGS) (British Geological Survey 2001), Belkuchi and Pirganj represented a predominantly high- and a predominantly low-groundwater-iron areas respectively. We considered the high level of iron in groundwater based on the Joint FAO/WHO Expert Committee on Food Additives (World Health Organization 2004) defined upper-limit ( $\geq 2$  mg/L) of iron in the water.

There is a paucity of data on the number of tube-wells at the sub-district level of Bangladesh. As per the British Geological Survey 2001, the total number of tube wells in Bangladesh is up to 11 million and the total population of the country is ~166 million (Worldometers 2017).

This translates in one tube well for roughly 15 people. We triangulated the usage rate of the tube-wells and the population of the sub-districts (Banglapedia 2012) (Supplementary Table 1) and estimated the number of tube wells in Belkuchi and Pirganj as 20,178 and 14,383 respectively.

The sample size was estimated based on the proportion (p) of tube wells which the assessor taste-rated to have “some” level of iron in the water sample observed in our pilot study. Considering  $p=76\%$  (Rahman et al 2018), 5% error margin, 95% confidence level and an estimated total number of 20,178 tube wells, the required sample size in Belkuchi was 277. However, as the study was nested in a larger trial on the efficacy of a novel micronutrient formulation, we assessed 400 tube wells for taste-rating.

Similarly, in Pirganj with an estimated total number of tube wells of 14,383, and considering the above stated statistical parameters, the required sample size was 270. We could assess 266 wells. Hence, a total of 666 households with a tube-well were assessed for taste-rating of the water samples in the two areas.

## 2.2 Procedure

The tube wells were selected at random from 8 and 3 villages of Belkuchi and Pirganj respectively. After obtaining the consent of the household head, two external assessors undertook the assessment of the well-water samples separately and recorded their taste-ratings to one of the following categories- (a) no iron, (b) some iron, and (c) heavy iron. Then the assessors asked the respondent of the households, how they perceive the taste for iron of the water of the tube-wells used by them for drinking. The respondents rated their taste-experiences to one of the above categories. This sequence of the taste- assessment was followed, so that the external assessors could avoid the potential information bias for their ratings from the respondents. The proportional magnitude of different taste-ratings and

concentration of iron in groundwater by the taste-ratings was studied in both study areas. The association of the taste-ratings of the water sample and its concentration of iron; and the agreement of the taste-ratings between the various raters were studied in the high groundwater iron area (Belkuchi). The weighted average of concentration of iron in groundwater of the study sub districts was calculated by summation of the product of the proportions of different taste-categories and the corresponding reference iron concentrations (Supplementary text 1).

### 2.3 Development and standardization of taste-rating of the water sample

At first, the selected tube well was pumped for 5 minutes to get away with any buildup of oxidized residual iron at the inner surface of the tube-well wall which might have come in contact with the upcoming water and contaminate the sample. Further, pumping water for some time would help reach the deeper aquifer which represents the actual milieu of groundwater (Merrill 2011). The sample water was taken in a 50-ml beaker. Before tasting the water sample, the assessors gurgled and washed their mouth with distilled water to neutralize the surface of the tongue and to remove any food particles/dirt which might otherwise interfere with the taste sensation. The assessor took a sip of the sample water in the mouth, held it for up to 10 seconds to assess the taste and sensation experienced of the sample. The assessment needed careful observation to determine the category of the taste and sensation. At the time of the assessment, the taste of the water sample might change. For example, the taste might appear “sweet” when the sample is put in the mouth first time, however, after a few seconds, it might be felt “bitter”. It might appear sharply “bitter” immediately after putting the water in the mouth; however, tasting of the subsequent sips might appear just mildly “bitter”. Hence, the decision was made after several attempts when the assessor felt confident of the type of taste and the presence of any sensation.

To define the taste categories for the study, we did a brief piloting exercise to disentangle the categories based on iron concentrations of tube- well waters. Before data collection, the assessors were provided with an orientation and practical understanding of the different kind of taste and sensations of the well water samples; and how to pick a rating following the operational definitions and the process of tasting described in the method. Of all the assessors the best two who rated consistently correct ratings (as per the operational characteristics) and had good uniformities between them were selected for the study.

For the study, we devised operational characteristics of the taste and sensations of the iron-rich well water sample as shown in Table 1.

Table 1: Operational characteristics of taste and sensations of groundwater sample

Taste and sensation experiences	Level of iron
<del>–mild bitter</del> ” or <del>–slightly sweet</del> ” or <del>–sweet</del> ”	<del>–some level of iron</del> ”
<del>–intense bitter</del> ” or <del>–salty</del> ” and/or presence of any kind of sensation, e.g. <del>–sticking in the mouth</del> ” or <del>–water feeling heavy in the mouth</del> ” or <del>–burning in the mouth</del> ”	<del>–heavy level of iron</del> ”

Absence of the referred taste and sensation was categorized as ~~–no iron~~’.

#### 2.4 Assessment of iron concentration of the tube well water

The iron concentration of the well water was estimated by the iron test kit devices (HI 3834, Hanna Instruments USA). The iron test-kit device was used, because, it has been validated against the gold standard (Atomic Absorption Spectrophotometry) and was observed to have good correlation (Spearman rank: 0.98) and agreement (Merrill et al 2009). At first, the tube-well was pumped for 5 minutes. The test water sample was taken up to the 10-ml mark in the manufacturer supplied test-beaker. The supplied reagent (phenanthroline) was poured onto the sample water and thoroughly mixed. The reagent-mixed test water was poured into the test chamber of the device. After 4 minutes, the resultant color of the test-water was matched with a reference color given with the device. The matching indicated the concentration of iron in the test-water sample. The test-kit device provides the measurement of iron concentration up to the concentration of 5 mg/L. If the color of the test-sample appeared to have exceeded the reference color equivalent to 5 mg/L, the test was repeated with a two, five or ten-fold dilutions of the test-water sample as needed. The dilution was done by mixing distilled water with the sample water, at an appropriate ratio according to the extent of dilution required. The final reading of iron concentration was adjusted by multiplying by the corresponding units of dilution.

## 2.5 Statistical analysis

We calculated the proportions of the taste-ratings; and correlations and agreements of the taste-ratings given by various assessors. The distribution of concentration of iron in water by various taste-raters and the taste-rating categories was presented, and the non-parametric test was used to determine the statistical difference in the mean estimates of iron concentration in the water. The spearman rank correlation coefficient was used to assess the relationship of the perceived taste-ratings for iron and iron concentration in the water sample. The Kendall's  $\tau_b$  coefficients were estimated to examine the correlation of the taste-ratings between the respondents vs. the assessors and between the assessors since there were many tied values over the corresponding ranks (statisticssolution.com).

To assess the precision and the accuracy of relationship of the taste ratings of the different raters, Lin's concordance correlation coefficient was estimated (Lin 1989, Lin 2000). Lin's coefficient evaluates the extent of deviation of observed data from the line of perfect concordance (i.e. the line at 45-degree angle in the scatter plot). Lin's coefficient increases in value (towards +1 or -1) as a function of the closeness of the data's reduced major axis to the line of perfect concordance (the accuracy of the data) and of the tightness of the data around its reduced major axis (the precision of the data). The concordance correlation coefficient,  $\rho_c$ , is expressed as a product of precision and accuracy (Stata journal 2016).

Data analysis was done with the statistical software, STATA 14 (STATA Inc. College Station, Texas, USA).

## 2.6 Ethics approval

The study was nested in a community-based trial examining the efficacy of a novel micronutrient powder formulation in children residing in the areas with a high level of iron in groundwater. The trial received approval from the Research Ethical Committee of Faculty of

Biological Science, Dhaka University, Bangladesh and Griffith University Human Research Ethics Committee, Australia.

## 1. Results

Assessment of taste-rating for iron and measurement of the iron concentration of the groundwater samples were done of 400 tube-wells in Belkuchi, and 266 tube-wells in Pirganj. Table 2 shows the proportions of taste-ratings for iron in groundwater and the distribution of concentration of iron by taste-ratings and the taste-raters in the predominantly high-iron area.

Table 2: Distribution of concentration of iron in tube well water in the high groundwater iron area by the taste-raters and taste-ratings

Taste-raters	Taste-ratings(N=400)											
	No iron				Some iron				Heavy iron			
	Iron (mg/l)				Iron (mg/l)				Iron (mg/l)			
	n	(%)	Mean(SD)	Median	n	(%)	Mean (SD)	Median	n	(%)	Mean (SD)	Median
Respondents taste rating*	23	5.75	3.23(0.82)	3.0	304	76.0	4.29(1.57)	4.0	73	18.25	5.71(2.98)	4.8
Assessor 1 taste rating†	0	0	-	-	346	86.5	3.96(0.90)	4.0	54	13.5	7.86(3.29)	9.0
Assessor 2 taste rating*	12	3.0	3.69(0.65)	3.5	328	82.0	3.93(0.83)	4.0	60	15.0	7.71(3.19)	8.0

\*Independent Samples Kruskal Wallis test;  $p=0.001$  for mean differences in iron concentration of tube well water across the taste-rating categories

†Two-sample Wilcoxon rank-sum (Mann-Whitney) test;  $p<0.001$  for mean differences in iron concentration of tube well water across the taste-rating categories

The proportion of the respondents who taste-rated the water of their tube-well as “no-iron”, “some iron” and “heavy iron” were 5.75%, 76.0% and 18.25% respectively. The taste-ratings of the assessor 1 for iron in the water samples as “no-iron”, “some iron” and “heavy iron” were 0.0%, 86.5% and 13.5% respectively. The taste-ratings of the assessor 2 were 3.0%, 82.0% and 15.0% respectively. The mean concentration of iron progressively increased over the higher taste-rating categories. The mean differences in the iron concentration were significantly different between the taste-categories rated by the assessor 2 and the respondents ( $p=0.001$  in both instances) and by the assessor 1 ( $p<0.001$ ) (Table 2).

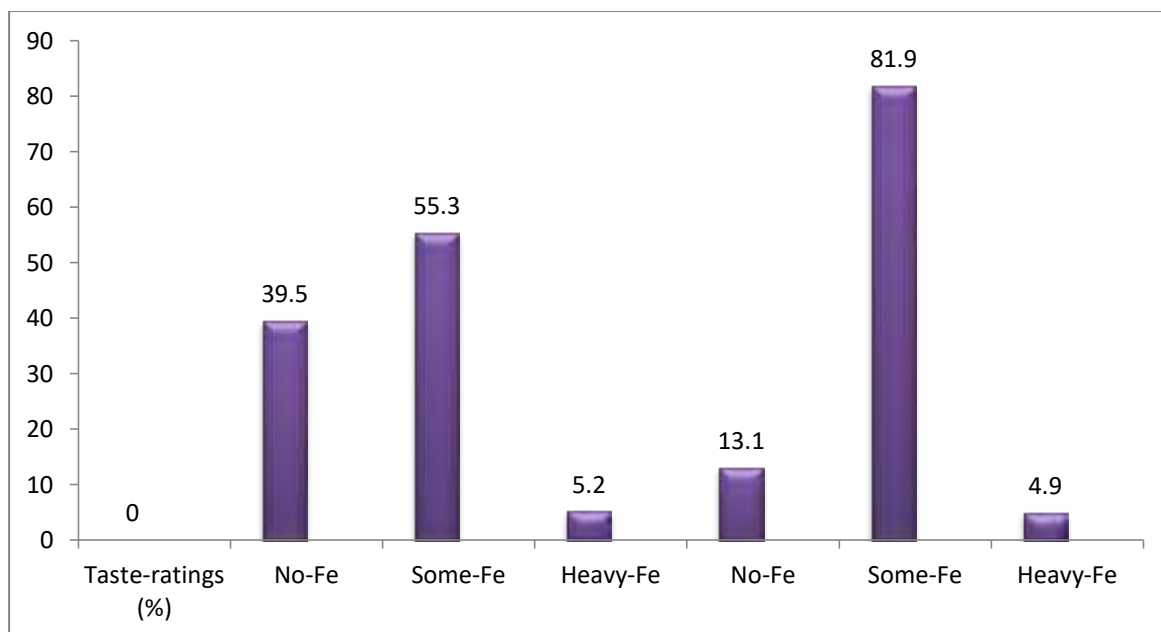


Fig. 2: The profile of taste-ratings of the groundwater samples of the low-groundwater-iron-area (N=266)

<sup>†</sup> For the respondents; n=105 (no-iron), n=147(some -iron), and n=14 (heavy- iron)

<sup>‡</sup> For the assessors; n=35 (no-Fe), n=218 (some- Fe), and n=13 (heavy- Fe)

Figure 2 showed the proportion of taste-ratings in the low-groundwater-iron area, rated by the respondents and assessors. Among the respondents, the proportion of taste-rating of “some iron”, “heavy iron” and “no-iron” was 55.3%, 5.2% and 39.5% respectively. Among the assessors, the respective proportion was 81.9%, 4.9% and 13.1%.

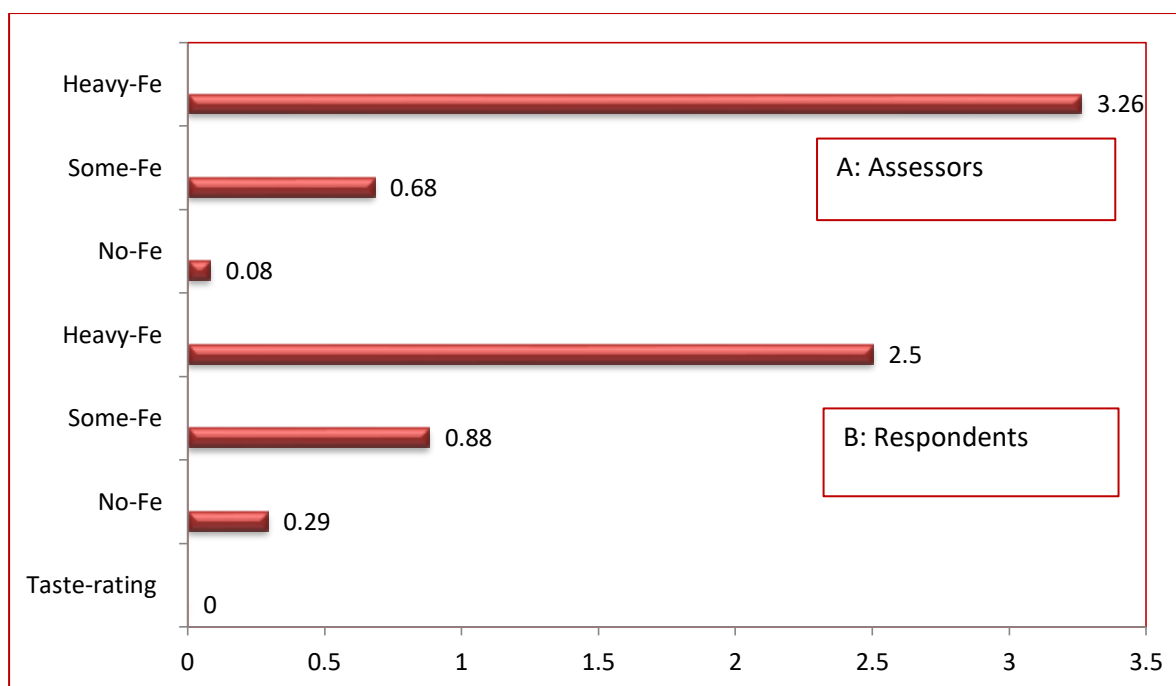


Fig. 3: Concentration of groundwater iron (mg/L)\* by taste-ratings in the low-groundwater-iron area (N=266); by the assessors<sup>†</sup> and by the respondents<sup>‡</sup>

\*Difference in the iron concentration between the taste-rating categories reported by the assessors and the respondents were significant at  $p < 0.001$

<sup>†</sup> For the assessors; n=35 (no-Fe), n=218 (some- Fe), and n=13 (heavy- Fe)

<sup>‡</sup> For the respondents; n=105 (no-iron), n=147 (some -iron), and n=14 (heavy- iron)

Figure 3 depicted the concentration of iron in groundwater in the low-iron-area by the assessor's and respondents' taste-ratings. For the assessor's ratings, the mean concentration of iron was 0.08 mg/L, 3.26 mg/L and 0.68 mg/L in the "no-iron", "heavy iron" and "some iron" categories respectively (Figure 3A). As for the respondent's taste-ratings, the respective concentration of iron was 0.29 mg/L, 2.5 mg/L and 0.87 mg/L (Figure 3B).

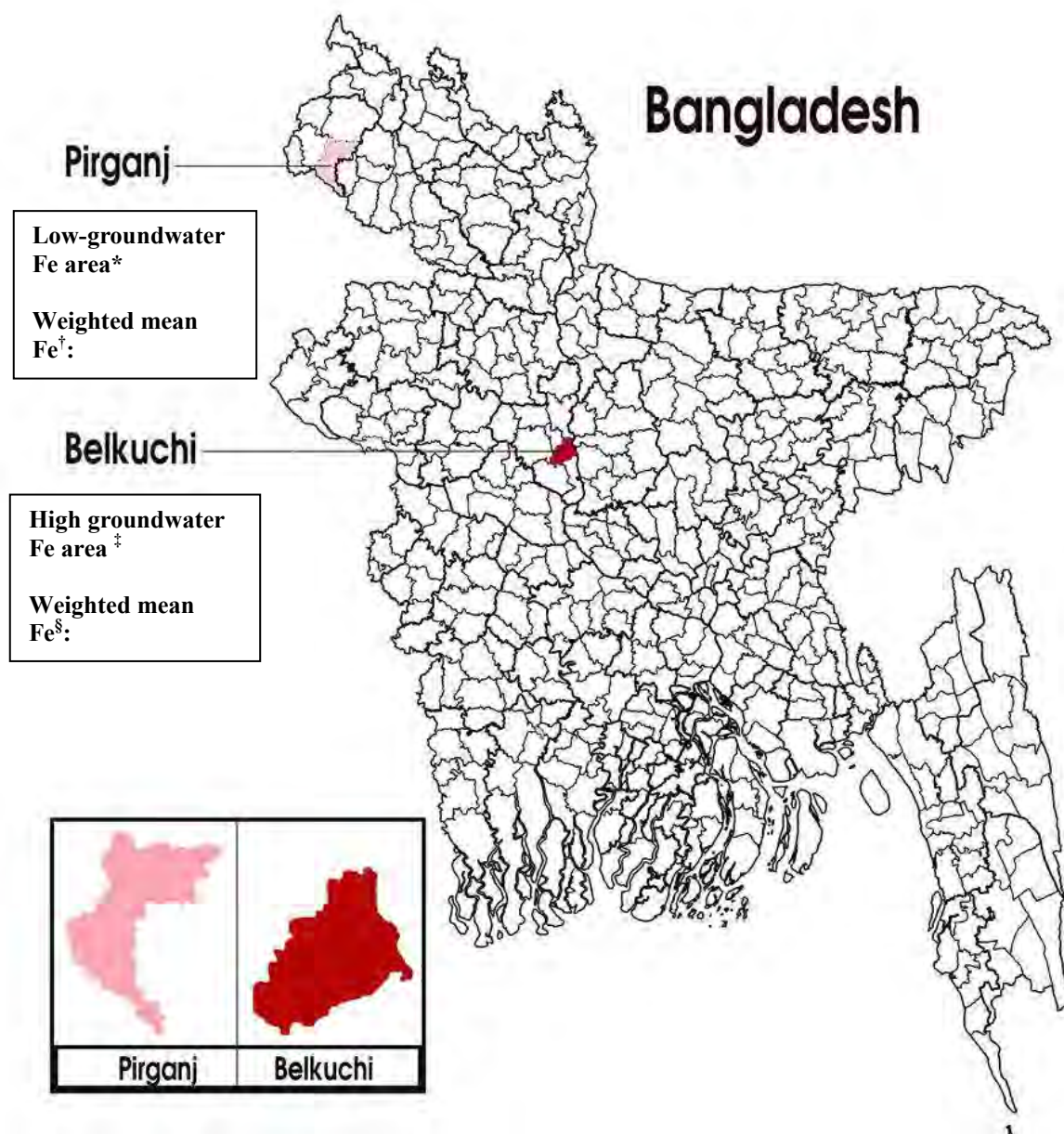


Fig. 4: A spatial presentation of Belkuchi and Pirganj sub districts (upazila) with the estimated weighted-average concentration of groundwater iron measured through the taste-rating profiles of the external assessor

\*Iron concentration in groundwater < 2 mg/L (WHO, 2004)

†Derived by summation of the multiplication of the proportion of the taste-rating categories and the corresponding reference iron concentrations of the low-iron groundwater area (Supplementary Text 1)

‡Iron concentration in groundwater ≥ 2 mg/L (WHO, 2004)

§Derived by summation of the multiplication of the proportion of the taste-rating categories and the corresponding reference iron concentrations of the high-iron groundwater area (Supplementary Text 1)

Figure 4 depicted a spatial presentation of Belkuchi and Pirganj sub districts with the estimated weighted-average concentration of groundwater iron measured through the taste-rating profile of the external assessor.

Table 3 showed the association between taste-ratings of various assessors and concentration of iron in the tube well water in the high iron groundwater area. Respondent's taste-ratings were positively correlated with the iron concentration of their well water ( $\rho=0.3192$ ,  $p<0.001$ ).

Table 3: Association of taste-ratings of groundwater sample for presence of iron and actual concentration of iron in the water sample (in the high groundwater iron area)

Taste assessors	Taste-rating vs. Iron concentration in tube well water
	Correlation coefficient ( $\rho$ ) <sup>*</sup>
Respondents	0.3192 <sup>†</sup>
Assessor 1	0.4571 <sup>†</sup>
Assessor 2	0.4708 <sup>†</sup>

<sup>\*</sup>Spearman Rank Correlation Coefficient

<sup>†</sup> $p<0.001$

Similarly, the external assessor's taste-ratings were positively associated with the iron concentration of the well waters;  $\rho=0.4571$  ( $p<0.001$ ) and  $\rho=0.4708$  ( $p<0.001$ ) for the assessor 1 and assessor 2 respectively.

Table 4 showed the association between taste-ratings of different assessors for the level of iron present in the tube well water in the high groundwater iron area. The taste-ratings of the assessor 1 and the assessor 2 were positively correlated with that of the respondents; Kendall's  $\tau_b = 0.14$  ( $p=0.004$ ) and  $0.15$  ( $p=0.001$ ), respectively. The inter-assessor taste-ratings were highly correlated; Kendall's  $\tau_b = 0.79$  ( $p<0.001$ ).

Table 4: Association<sup>\*</sup> of taste-ratings of groundwater sample for presence of iron between various taste-raters (in the high groundwater iron area)

Taste assessor	Respondent	Assessor 1	Assessor 2
Respondent	1	0.14 <sup>‡</sup>	0.15 <sup>§</sup>
Assessor 1	0.14 <sup>‡</sup>	1	0.79 <sup>†</sup>
Assessor 2	0.15 <sup>§</sup>	0.79 <sup>†</sup>	1

<sup>\*</sup>Kendall's  $\tau_b$ ; <sup>†</sup> $p<0.001$ , <sup>‡</sup> $p=0.004$ , <sup>§</sup> $p=0.001$

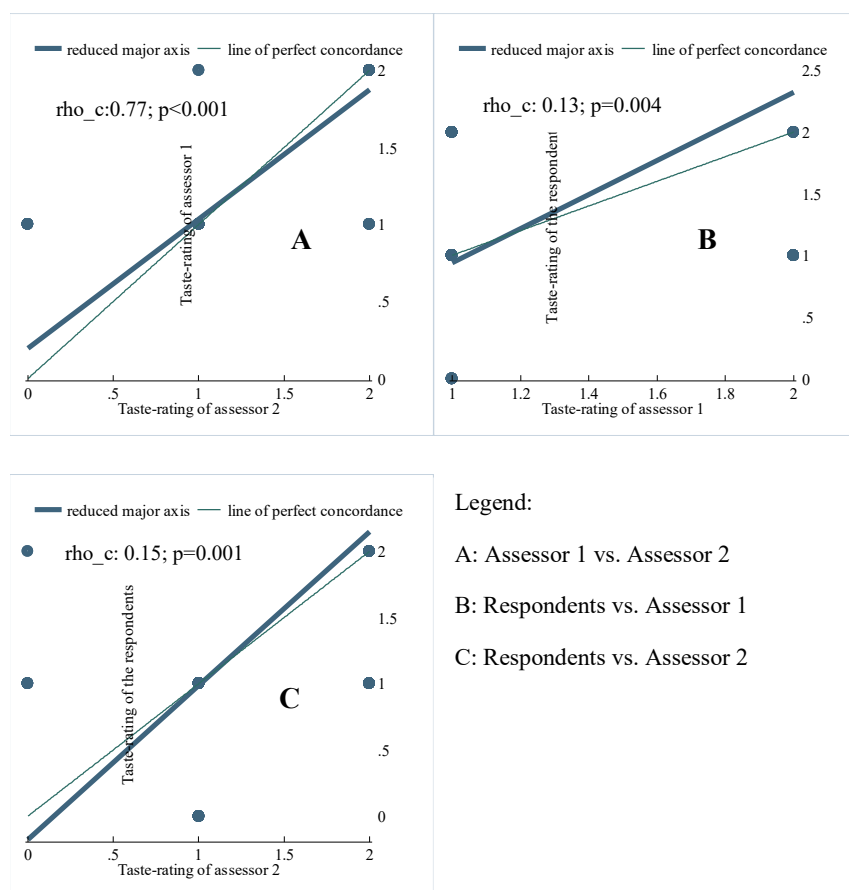


Fig. 5: Concordance correlation coefficients showing the agreement of the taste-ratings given by different raters (in the high groundwater iron area)

Figure 5 depicted the extent of agreement of the taste-ratings given by various raters as shown by Lin's concordance correlation coefficients ( $\rho_c$ ). The coefficient of the inter-assessor agreement was 0.77,  $p < 0.001$  (Figure 5A). The coefficient of agreement between the assessor1 vs. the respondents was 0.13,  $p = 0.004$  (Figure 5B), while that of between the assessor 2 and the respondents was 0.15,  $p = 0.001$  (Figure 5C).

### 3. Discussion

This study reports the development and standardization of the taste-rating of groundwater sample for the level of iron to establish a tool for a semi-quantitative assessment of groundwater iron content. The concentration of iron in groundwater was measured by different taste-rating categories in the predominantly high- and low-groundwater-iron areas in Bangladesh.

The results showed that the taste-rating categories by different raters were significantly different in groundwater iron concentration, and higher concentrations of iron were observed with the higher ratings. This substantiated the utility of the taste-rating tool. The results further showed a significant positive correlation of the taste-ratings by the assessors and the respondents; and the concentration of iron of the well water. The coefficient appeared larger for external assessors than that for the respondents. One way of explaining this is the assumption that the local respondents who are accustomed to using the water might have developed a tolerance to the iron level, and thus tended to under-report. The taste-ratings showed the positive correlations between various raters; however, the coefficient for the inter-assessor ratings was much larger compared to that for between the assessor 1 vs. respondents and the assessor 2 vs. respondents. The findings further indicate that the higher level of mismatch of ratings between the respondents and the external assessors which might have resulted from the respondent's under-reporting of iron. These findings indicate that the trained external assessors may provide the unbiased and objective taste-ratings of the groundwater sample for the levels of iron.

There was a marked difference between the high- and low-iron areas regarding the proportion of the perceived taste category “no-iron”. In the high-iron-area, the reporting of “no-iron” was negligible ( $\leq 5\%$ ), while in the low-iron-area, the proportion of the rating was higher (up to  $\sim 40\%$  among the respondents). This owes to the level of iron in groundwater which was much lower in the low-iron-area and this resulted in higher number of responses from the respondents and the assessors reporting “no iron”. The mean concentration of iron differed over the high- and low-iron-areas within the same taste-rating categories. One way of explaining this is the fact that, we observed a wide range of distribution of iron concentrations within a particular taste rating category (results not shown). This is an inherent limitation of precision of human taste perception, relative to a range of iron concentrations. Hence, the taste-ratings were the same category; despite the mean iron concentrations were considerably different between high- and low-groundwater iron areas. Because of this, the study was conducted both in the high- and low- groundwater iron areas, as the reference iron level by the taste-rating categories from a high-iron-area would not represent for a low-iron-area.

The finding of the positive correlation of the taste-ratings of the water sample and the concentration of iron in groundwater is consistent with our pilot study ( $n=13$ ) (Rahman et al 2018), which is the only similar study available on the topic. Merrill et al studied a slightly

different aspect of the users rating of the their well water for the level of iron based on their overall perception; and the study observed the positive association of the perceived rating for iron in groundwater and its concentration of iron (Merrill et al 2010).

The findings of the present study demonstrate a simple, low-cost (Supplementary Text 2), fast and non-device based way for a semi-quantitative assessment of the iron level of groundwater which has the potential to apply for groundwater iron assessment in the defined geographic areas. The groundwater iron is an emerging issue concerning public health (i.e. iron deficiency Anaemia). Before designing and implementing the Anaemia and micronutrient deficiency prevention programs, it is important to know the background level of iron in groundwater in the areas where population heavily relies on groundwater for potable supplies. This can inform the program designers by identifying and prioritizing the geographical areas for programming and/or modifying the program /interventions of iron supplements (e.g. decreasing the iron doses).

The present study used a large number of water samples from the areas with a predominantly high- and low-level of iron in groundwater. Therefore, the estimates of the mean concentration of iron by the taste categories were likely to be reliable. For the subsequent application of the tool in a defined geographic area, the requirement will be the taste-ratings of the tube well water samples by some trained external assessors. The sampling of the tube wells can be done by applying a random or systematic epidemiological sampling procedure. The proportion of different taste-ratings of the tube well water thus obtained could be used in conjunction with the mean iron concentration by the taste-categories derived from the present study (i.e. reference iron concentration). A weighted average of iron concentration can be estimated to account for differential proportions of taste-ratings (Supplementary Text 1). The reference iron concentrations obtained by the taste categories for the high- and low-groundwater iron areas of the present study can be used for prospective assessment of groundwater iron in a predominantly high- and low- groundwater iron areas respectively (Figure 4). It is important to decide on the appropriate reference iron value, which should be used to calculate the average concentration of groundwater iron of an area. This can be informed by preexisting reference data of groundwater iron- e.g. reports of the department of public health engineering, geological surveys, and geological studies. These data might inform the magnitude of groundwater iron level in the area. Based on the iron levels- high or low, the appropriate reference values of concentration of iron by the taste-categories (from the present study) can be used. If the area to be assessed is geographically large, and both

high- and low-levels of groundwater iron are apparent, both the reference values can be used as per the requirement. If there is no background data of groundwater iron available, a small pilot study to measure iron can be done to have an idea of the iron level in groundwater, before assessing the iron concentration by the taste-rating tool. However, a validation study of the tool in assessing the classification of areas into high- and low- groundwater iron areas against the same assessed through the measurement by the standard laboratory and/or standard devices will greatly endorse its real-life application.

Nearly 100% of the tube wells of the high-iron-area had the iron concentration of  $\geq 2$  mg/l (cut-off defining “high” iron status), and ~91% of the wells of the low-iron-area had the iron concentration  $< 2$  mg/l (results not shown). Hence, the study areas were fairly representative of the high- and low-iron groundwater areas of Bangladesh respectively. Besides, the study used a large number of tube wells to derive the estimates of the iron concentration of groundwater. Therefore, the reference estimates of iron concentration by the taste categories are potentially generalizable in Bangladesh and in similar settings. Although the colorimetric test kit device was validated against the atomic absorption spectrophotometer, and reported to have a good agreement (Merrill et al 2009), the device is not a gold standard. The usage of the device to measure the iron concentration of the water sample is a limitation of the study.

Iron and manganese can give roughly a similar metallic perception of groundwater (WHO 2011). However, iron is found in much higher amount in the crust of the earth and in groundwater (Sandatlas 2020). Although there might be shared presence, the metallic taste of groundwater is predominantly imparted to iron. This is substantiated by the finding of increasingly higher concentrations of iron over the higher taste-rating categories. Nonetheless, the fact that we did not measure manganese was an additional limitation.

#### 4. Conclusion

Taste-rating of groundwater sample for iron may offer a simple, low-cost, fast and a device-free way of semi-quantitative assessment of the level of groundwater iron. External assessors may provide reliable ratings. Subject to validation, the tool can potentially be used in settings where groundwater iron is a source of iron through potable supplies for a rapid appraisal of iron content in groundwater. Such an assessment is important for designing and customization of an appropriate iron-supplementation program for prevention and control of Anaemia in the populations.

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#### Footnotes

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Author's contribution: SR: Conceived and designed the study, collected data, analyzed data and wrote the first draft of the manuscript; FA: Contributed to the design and provided critical review of the manuscript to finalize; PL & KMR: Contributed to the design

#### Conflicts of Interest

The authors declare no conflicts of interest.

Funding source: The study was supported by the Nestle Foundation

Supplementary Table 1: Population of Bangladesh, number of tube-wells, and the estimated tube-wells in Belkuchi and Pirganj sub-districts

	Population	# of tube wells
Bangladesh	166,368,149 (Worldometer 2017)	110,00000 (BGS 2001)
Belkuchi	3,02678 (Banglapedia 2012)	
Pirganj	2,15754 (Banglapedia 2012)	

## Supplementary Text 1

The weighted average of iron concentration of groundwater in an area can be calculated by using the following formula,

Weighted average of groundwater iron concentration of the area= Concentration of groundwater iron in the taste-rating category –no iron” × % of the category –no-iron” rating

+ Concentration of groundwater iron in the taste-rating category –some iron” × % of the category –some-iron” rating

+ Concentration of groundwater iron in the taste-rating category –heavy iron” × % of the category –heavy-iron” rating

The followings are the examples of weighted average concentration of groundwater iron derived from the actual (present study) and some hypothetical taste-rating profiles using the reference iron concentration of the present study,

A. Using reference iron concentration of the high groundwater iron area sorted by the assessor’s taste-rating (Table 2)

[Supposed rating profile, no-iron: 5%; some-iron: 25%; heavy-iron: 70%]

$$3.69 \times 0.05 + 3.93 \times 0.25 + 7.71 \times 0.7 = 0.184 + 0.98 + 5.39 = 6.56 \text{ mg/L}$$

[Supposed rating profile, no-iron: 5%; some-iron: 45%; heavy-iron: 50%]

$$3.69 \times 0.05 + 3.93 \times 0.45 + 7.71 \times 0.5 = 0.18 + 1.76 + 3.85 = 5.79 \text{ mg/L}$$

[Supposed rating profile, no-iron: 20%; some-iron: 60%; heavy-iron: 20%]

$$3.69 \times 0.2 + 3.93 \times 0.6 + 7.71 \times 0.2 = 0.738 + 2.358 + 1.54 = 4.63 \text{ mg/L}$$

[Actual rating profile of the study, no-iron: 3%; some-iron: 82%; heavy-iron: 15%]

$$3.69 \times 0.03 + 3.93 \times 0.82 + 7.71 \times 0.15 = 0.11 + 3.22 + 1.15 = 4.48 \text{ mg/L (Actual estimate of the present study)}$$

[Supposed rating profile, no-iron: 50%; some-iron: 40%; heavy-iron: 10%]

$$3.69 \times 0.5 + 3.93 \times 0.4 + 7.71 \times 0.1 = 1.845 + 1.572 + 0.77 = 4.18 \text{ mg/L}$$

[Supposed rating profile, no-iron: 50%; some-iron: 45%; heavy-iron: 5%]

$$3.69 \times 0.5 + 3.93 \times 0.45 + 7.71 \times 0.05 = 1.8 + 1.76 + 0.385 = 3.98 \text{ mg/L}$$

[Supposed rating profile, no-iron: 65%; some-iron: 30%; heavy-iron: 5%]

$$3.69 \times 0.65 + 3.93 \times 0.30 + 7.71 \times 0.05 = 2.39 + 1.179 + 0.38 = 3.94 \text{ mg/L}$$

B. Using reference iron concentration of the low groundwater iron area sorted by the assessor’s taste-rating (Fig. 3)

[Actual rating profile of the study, no-iron: 13%; some-iron: 81.9%; heavy-iron: 4.9%]

$$0.08 \times 0.13 + 0.68 \times 0.819 + 3.26 \times 0.049 = 0.01 + 0.556 + 0.159 = 0.726 \text{ mg/L (Actual estimate of the present study)}$$

[Supposed rating profile, no-iron: 25%; some-iron: 50%; heavy-iron: 25%]

$$0.08 \times 0.25 + 0.68 \times 0.50 + 3.26 \times 0.25 = 0.02 + 0.34 + 0.815 = 1.175 \text{ mg/L}$$

[Supposed rating profile, no-iron: 5%; some-iron: 60%; heavy-iron: 35%]

$$0.08 \times 0.05 + 0.68 \times 0.60 + 3.26 \times 0.35 = 0.004 + 0.408 + 1.14 = 1.55 \text{ mg/L}$$

## Supplementary Text 2: Financial cost of the study

The tool is low-cost to implement because any future assessment of iron level in groundwater in an area would only require the undertaking of taste-ratings of groundwater samples by the trained assessors. The weighted average of the iron concentration of groundwater of the area can be deducted by summation of the proportions of various taste-ratings multiplied by the corresponding reference iron concentrations derived from the present study. Hence, no device, and reagents for iron measurement will be required which are mostly imported and can be expensive. The cost of the study would be the personnel cost and a few nominal logistics, e.g. beaker (50 ml), distilled water etc.

## 8.2 ANNEX 2

### 8.2.1 OVERVIEW OF PREPARATORY PAPER 2

Title: Validation of an interviewer-administered seven-day Semi-Quantitative Food Frequency Questionnaire for dietary assessment of preschool children in rural Bangladesh

Status: Published

Journal: Journal of Nutritional Science [Impact Factor: 6.96]

Bibliographic details

Rahman, S., Lee, P., Ireen, S., Khan, M. U., & Ahmed, F. (2021). Validation of an interviewer-administered seven-day semi-quantitative food frequency questionnaire for the dietary assessment of preschool children in rural Bangladesh. *Journal of nutritional science*, 10, e26. <https://doi.org/10.1017/jns.2021.19>

### 8.2.2 INTRODUCTION

The trial required the measurement of the children's intake of dietary nutrients. It has not been feasible to use a traditional self-administered FFQ in Bangladeshi rural communities where literacy and numeracy skills are suboptimum. Hence, a new variant of FFQ which is interviewer-administered and suitable for use in the Bangladeshi context has been used extensively in national surveys and other dietary assessment studies in the country. However, it had not been validated.

Therefore, the present study validated the tool prior to its usage to measure the dietary nutrients intakes in the trial children. In this validation study the dietary intake of the studied children (not the trial children) measured by the FFQ was compared against two 24-hour recalls held in the same children on non consecutive days. . Validity was assessed by standard statistical tests. After adjusting for the energy intake and de-attenuation for within-subject variation, the macronutrients (carbohydrate, protein, and fats) had *good* correlations ( $\rho$ : 0.50-0.75;  $p < 0.001$ ); the key micronutrients (iron, zinc, calcium, and vitamin A) also

demonstrated *good* correlations (rho: 0.46-0.85;  $p < 0.001$ ). The FFQ demonstrated adequate validity to assess the dietary intake for most nutrients.

Its usage enabled valid measurement of the nutrient intakes of the children in the trial, especially the micronutrients with haemopoietic potential: iron, zinc, vitamin A, vitamin B12, and folic acid.

### 8.2.3 PUBLISHED PAPER

Validation of an interviewer-administered seven-day Semi-Quantitative Food Frequency Questionnaire for dietary assessment of preschool children in rural Bangladesh

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Short title: Validation of a food frequency questionnaire

Key words: Food Frequency Questionnaire; Dietary Assessment; Preschool Children; Bangladesh

## Abstract

A validation study of an interviewer-administered, seven-day semi-quantitative food frequency questionnaire (SQFFQ) was conducted in Bangladeshi rural pre-school age children. Using a cross-sectional study design 105 children from 103 households were randomly selected. For the SQFFQ, a list of commonly consumed foods was adapted from the Bangladesh national micronutrient survey 2011-12. The data on the actual number of times and the amount of the children's consumption of the foods in the preceding one week were collected by interviewing the mothers. The intake was compared with two nonconsecutive days 24-hour dietary recalls conducted within two weeks after the SQFFQ. Validity was assessed by the standard statistical tests. After adjusting for the energy intake and de-attenuation for within-subject variation, the food groups (cereals, animal source foods, milk and the processed foods) had ~~–good~~ "good" correlations between the methods (rho: 0.65-0.93;  $p < 0.001$ ). Similarly, the macronutrients (carbohydrate, protein and fats) had ~~–good~~ "good" correlations (rho: 0.50-0.75;  $p < 0.001$ ) and the key micronutrients (iron, zinc, calcium, vitamin A) demonstrated ~~–good~~ "good" correlations (rho: 0.46-0.85;  $p < 0.001$ ). The variation in classifying the two extreme quintiles by the SQFFQ and the 24-hour recalls was  $< 10\%$ . The results from Lin's concordance coefficients showed a ~~–moderate~~ "moderate" to ~~–excellent~~ "excellent" absolute agreement between the two methods for food groups, and nutrients (0.21-0.90;  $p < 0.001$ ). This interviewer-administered 7-day SQFFQ with an open-ended intake frequency demonstrated adequate validity to assess the dietary intake for most nutrients, and suitable for dietary assessments of young children in Bangladesh.

## Introduction

Childhood is an important period in the life cycle because it is a phase of intense growth and development. The nutritional needs during childhood are increased significantly, and thus, adequate nutrition and dietary intake are essential.<sup>1</sup> The dietary assessment tool has been used to establish the relationship of a population's eating habits with the presence of morbidity and mortality, allowing early detection of nutritional deficiencies in vulnerable groups, such as children.<sup>2</sup> FFQ is one of the most commonly used dietary assessment tools in nutritional epidemiological studies and surveys.<sup>4</sup> A food frequency questionnaire (FFQ) consists of a predetermined list of foods and beverages with response categories to indicate the usual frequency of consumption over a specified period.<sup>3</sup>

Advantages of FFQ are—easier to administer, usually less time consuming to implement, captures individual-level dietary patterns, and better at estimating 'usual diet' due to longer recall.<sup>5</sup> Traditionally, FFQ is respondent-administered and designed with a close-ended frequency option for the consumption of various food items. However, self-reported FFQ can lead to some measurement error due to within-subject variability, lack of ability to report food consumption, and difficulties in recalling which and how much food was consumed.<sup>6</sup> In rural Bangladesh, the majority of the respondents are functionally illiterate, and thus, the respondent-administered FFQ may introduce bias while assessing the food and nutrient intakes. To ameliorate this respondent issues, recent epidemiological dietary assessments in Bangladesh used an interviewer-administered, open-ended intake-response seven-day semi-quantitative FFQ.<sup>7,8</sup> To date, the FFQ has not been validated. Further, close-ended frequency options of consumption in the traditional FFQ has an inherent limitation, as the respondent's actual consumption often does not match to the specified consumption categories in the questionnaire.<sup>9</sup> Traditional FFQs inquire about the frequency of consumption over a long time (up to a year) and provides information on the habitual frequency of dietary intake of individuals, instead of an actual number of times of consumption over the reference period. Hence, some inaccuracies are expected regarding the amount of consumption.

There is a paucity of FFQ validation in Bangladesh. Lin et al (2017)<sup>10</sup> validated a dish-based FFQ with two 3-day food-records in a mixed population group consisting of children and adults (median age 30 years). Though the dish-based FFQ is contextually relevant in Bangladesh, the lack of specificities of food items is likely to impart a difference in the actual

nutrient intake as the possible different foods within a dish might vary considerably in nutrient content. Additionally, consistent with any FFQ, the study included wide frequency-categories of intake which might put a respondent confused as his/her intake might not belong to any of the categories.

Chen Y et al (2004) <sup>11</sup> validated the other prominent FFQ with Bangladeshi traditional diet in adult male and female subjects. This consisted of commonly consumed foods (39 items) in rural setting of Bangladesh and compared the FFQ with two 7-day Food Diary. Consistent with the proposed SQFFQ, Chen et al employed the interviewer administered FFQ and kept an open ended frequency option. However, the major limitation was that the Food Composition Tables used were either the USDA database<sup>12</sup> or the Food Composition Table (FCT) of the neighbouring India.<sup>13</sup> Usage of the extraneous FCTs unlikely to reflect the most accurate nutrient values of locally produced food, as food composition varies from country to country depending on the species of plants and animals, agricultural technology, climatic condition, processing, and storage circumstances.<sup>14</sup>

Taking into consideration of the above issues, we conducted this study to assess the validity of an interviewer-administered seven-day semi-quantitative food frequency questionnaire (7-day SQFFQ) designed to measure food and nutrient intake, with a particular interest on micronutrient intakes, and to be used in a community-based trial examining the efficacy of a low-iron micronutrient powder (MNP) in preschool children in rural Bangladesh. The distinguishing features of this SQFFQ are a short reference time (1 week) and the open-ended frequency of intake option, i.e. actual number of times of consumption.

## Participants

This study was conducted on 105 children, aged 24-59 months, recruited from 103 households in Belkuchi, a rural sub-district in a north-central district of Bangladesh. The participants were recruited using simple random sampling. The field staff identified the households with children of the stipulated age by a door-to-door visit. The purpose and exact nature of the study were explained to all eligible mothers or the primary caretakers of the children, and those who agreed to participate either signed or put a thumb impression on the consent form. The study was nested in a trial examining the efficacy of a low-iron micronutrient formulation in children of rural Bangladesh. The trial was approved by the

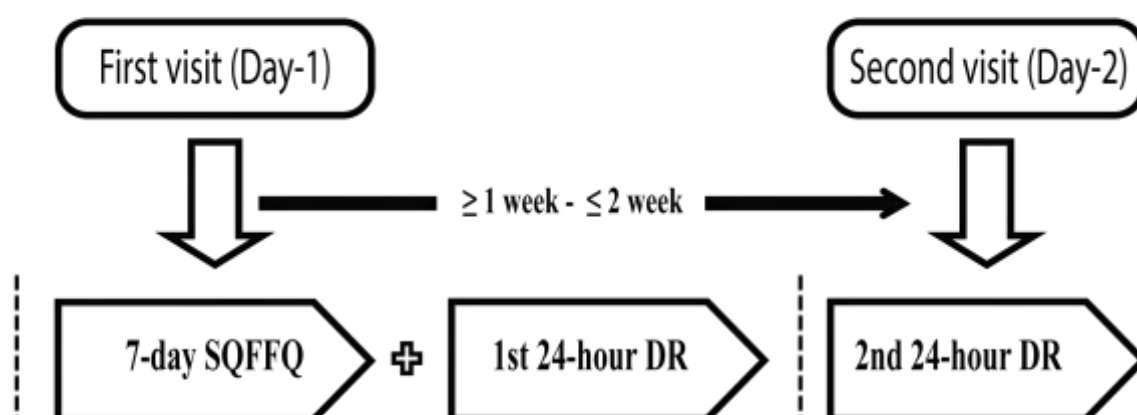
research ethics committees of the University of Dhaka, Bangladesh (Ref# 46/Biol. Scs. /2017–2018) and Griffith University, Australia ((Ref# 2017/467).

### Study design

There is no definitive ‘gold standard’ in dietary assessment, nor is there a ‘gold standard’ for assessing the validity of FFQ.<sup>9</sup> Therefore, estimation of a tool’s relative validity relies upon a comparison with a superior and preferably independent technique, known as comparative validation.<sup>15</sup> For a reference method, both weighed food record (WFR) and 24-hour DR- are commonly used due to their greater precision in the quantification of intake.<sup>15</sup> WFR is a suitable candidate for FFQ validation—but the need for good literacy and numeracy precludes its use in the rural Bangladesh context. Biochemical methods as the reference method to validate the SQFFQ, although less prone to errors involved with misreporting or poor memory, are expensive, invasive and nutrient-specific and hence not considered in the present validation study.<sup>9</sup> Considering the low literacy level of the respondents, 24-hour DR was chosen as the reference method.

The children’s food intake was measured by interviewing the mothers or caregivers, using a seven-day SQFFQ which was adopted from a national survey and a study in Bangladesh.<sup>7,8</sup> The validity of the nutrient intake measured by the SQFFQ was assessed by comparing to the average intake of the two 24-hour dietary recalls (24-hour DRs) as the reference method, administered on non-consecutive weekdays. The interval between the 24-hour DRs was  $\geq 1$  week -  $\leq 2$  weeks (Figure 1).

Figure 1: Design of the 7-day SQFFQ validation study



The respondents (mother or the primary caretaker of the child) were visited twice. On the first visit, first, the SQFFQ was administered, followed by the first 24-hour DR. During the second visit, after one week but within two weeks of the first visit, the second 24-hour DR was conducted. Data were collected by the trained interviewers. One of the researchers monitored the data collection to ensure the quality of data.

### Sample size

A total of 105 participants were included in the study. Bland-Altman plot is one of the most used statistical techniques to assess agreement in dietary validation studies. The sample size was considered on a recommendation that a minimum of 50 subjects is required if the Bland-Altman statistics are to be estimated, with a suggestion of 100 for the study.<sup>9</sup>

### Development of the SQFFQ

#### Selection of foods

The food frequency questionnaire was adopted from Bangladesh national micronutrient survey 2011–2012<sup>16</sup> and a recent dietary intake assessment study,<sup>8</sup> and pretested in the study population. The food list considered in the national micronutrient survey was referred from the comprehensive food consumption survey--a nationally representative dietary study.<sup>17</sup> It is

important to note that the dietary habit of Bangladeshi population is grossly homogenous with little diversity. The principles of the selection of the foods were as follows:

- a. Foods most commonly consumed in the Bangladeshi population.
- b. Foods rich in a particular nutrient. For example, several leafy vegetables were considered, because the foods are universally consumed across the population, and it is the largest source of a number of micronutrients in the setting.

Further, the processed foods were added to the list of the foods. Consumption of processed foods among children has increased over the last decade. We referred from the pretested list of the processed food from the study of Iqbal et al.<sup>8</sup> The processed foods are ready-to-eat, locally produced, energy-rich, fatty, and sugary with poor content of nutrients.

The SQFFQ used a total of 53 commonly consumed foods in the rural setting of Bangladesh. These foods were grouped as cereals, legumes, leafy and non-leafy vegetables, yellow/orange vegetables, fruits, small fishes, large fishes, meats, eggs, organ meats, and some ready-to-eat processed foods. Among the cereals were- rice, hand-made flat bread, sliced bread, oil-fried bread and puffed rice. Meats included were- chicken, beef, goat and liver. Fishes included small fishes which are eaten whole along with bones; and the most commonly eaten large fishes, e.g. carps and catfishes. Fruits included commonly eaten indigenous fruits e.g. mangoes, jackfruits, ripe banana, guava, plums and few others. The imported fruits included oranges, malta, apples, pomegranate. Non-leafy vegetables included commonly consumed items—sweet pumpkin, potatoes and gourds. The processed foods included were commonly preferred by the children—cakes, sweet biscuits, candies, juice-drinks, chocolates, fried flour-made snacks and few others. Portion size was one serving amount. We used portion-size reference of the Institute of Nutrition and Food Science, University of Dhaka which describes the serving size of the commonly eaten local foods.<sup>18</sup>

#### Development of the food album

The food album was intended to assist the respondents (i.e. mother) in assessing the amount of a particular food consumed by the children. It contained the principal foods listed in the SQFFQ. To develop the food album, for the cooked food we weighed the foods to a one-gram precision by a kitchen scale (SECA 852 digital diet scale); and kept in the plates/bowl in an amount of its serving size. The photos of the foods were captured in a standardized way, i.e.

at the same angle; at the same distance; and placed on a standard plate/bowl to standardize the relative size as it would appear when looked at. The raw food items and the ready-to-eat processed foods were photographed directly.

### Conduction of the SQFFQ

The mother of the child was asked about the food intakes of her child as per the following guidelines,

- a. Did the child consume a particular food (i.e. listed food) in the preceding 1 week?
- b. How many days in the last week did the child consume the food?
- c. How many times each day did the child consume the food?
- d. How much food (on average) each time did the child consume?

From questions b & c—the information derived was on the absolute number of times the particular food was taken over the week. From question d—information on the average amount of intake each time the food was consumed was gathered. The food album displayed the food items to their serving amount e.g. 1 half-plate full of leafy vegetables displayed amounting to its one serving. The enumerators explored the average amount of intake of a particular food by proportioning the amount displayed. In this way, they calculated both the serving amount and absolute amount (in grams/millilitres) taken over the week. The amount of the intake was also assessed by displaying standardized bowls, glasses, and spoons. These containers were pre-standardized by loading with the commonly eaten local food items, ink-marked at various levels and weighed by an electronic scale with 1 gram resolution (Seca Culina 852). At the interview, how much of the displayed container-load of the food the child consumed was inquired to the mother, and the amount was recorded. In the case of liquid foods, e.g. pulses, the measurement was done by asking the mother to pour plain water into the supplied graduated measuring beaker with 1 ml resolution from the bowls/glasses she used to feed the child. The amount was recorded in millilitres. The reported amount of weekly food consumption data was converted to the daily average intake by dividing by 7 (seven). For processed foods that were purchased, the inquiry was made into the brand names, how much money spent to purchase, and availability of the empty packets, to gather the information on the amount of the portion.

## 24-hour DR

For the 24-hour DRs, the preceding 24-hour was segregated into six time periods- breakfast, mid-morning, lunch, afternoon snacks, dinner, and bedtime. The amount of consumption of all food items over the period was assessed by the interviewers. The amount of consumption by the 24-hour DRs was measured following the same principle used for the SQFFQ. Weekends and special days, such as festivals and mourning were not considered for the 24-hour DRs.

## Nutrient estimation

An updated Food Composition Table (FCT) on Bangladeshi foods was used to calculate the nutrient intakes.<sup>19</sup> For a few nutrients which were missing in the FCT, the USDA database on the nutrient values was used.<sup>20</sup> The edible portion coefficients for Bangladeshi foods were used to derive the edible amount.<sup>19</sup> The cooked-food amounts were converted into the raw food weight, by dividing with the appropriate yield factors.<sup>19</sup> The nutrient values were calculated per 100 grams of the raw weight of the consumption as per the indication in the FCTs. For the non-cooked foods or the ready-to-eat processed foods, the nutrient values were directly gathered from the FCTs. For processed foods, information was also gathered from the nutrient facts labeled on the packets.

## Statistical analysis

Food and nutrients intake data from SQFFQ and average of two 24-hour DRs were tested for normality. Based on the Shapiro-Wilk testing (results not shown), the distribution of energy and nutrients intake were not reporting normal distribution. Thus, mean (SD) and median with interquartile ranges (IQR) were estimated for energy and nutrient intakes for the test and the reference methods.

We compared the food and nutrients intake data between SQFFQ and 24-hour DRs using the Wilcoxon signed-rank test. Spearman rank correlation coefficient ( $\rho$ ) was used to assess the strength and direction of the association between food and nutrients intakes measured by SQFFQ and 24-hour DRs.

The reference method (i.e. 24-hour DRs) can be imperfect and subject to within-person variation and/or day-to-day deviations, leading to the underestimated measures, i.e. the

correlation coefficient underestimating the degree of agreement. This underestimation is known as “attenuation bias”.<sup>21</sup> To minimize the attenuation bias, we computed the energy-adjusted correlation of the food and nutrients intakes obtained from the two methods. To adjust for energy intake, the nutrient density was calculated by dividing the mean nutrient value by the mean energy intake. The estimate was used in the Spearman rank correlational analysis instead of the original value of nutrient intake as recommended by Bingham et al.<sup>22</sup> Further, since the random within-individual variation in the measurement of any of the variables being compared tends to reduce correlation coefficients towards zero,<sup>23,24</sup> correlations with corrections for the attenuated effects of such measurement error in the two 24-hour DRs were calculated, by using the following formula:  $\gamma_t = \gamma_o (1 + \lambda/n)^{1/2}$

Where,  $\gamma_t$  is the true correlation coefficient;

$\gamma_o$  is the observed energy-adjusted correlation coefficient of the intakes recorded by the methods;

$\lambda$  is the ratio of the within-individual to between-individual variances of the daily intakes; and  $n$  is the number of replicates (here,  $n=2$  as two 24-hour DRs were administered).

To calculate the  $\lambda$ , One-Way Analysis of Variance (ANOVA) of foods and nutrients intakes measured by the two 24-hour DRs was computed to yield the variances (results not shown). Mean percent differences of all food groups and nutrients intake between the test and reference methods were used to assess the agreement at the group level (size and direction of error).<sup>25,26</sup> For calculation of the mean percentage difference, the reference value was subtracted from the test measure value, divided by the reference measure and multiplied by 100 for each participant.<sup>27</sup> Further, SQFFQ’s ability to rank the consumption correctly was examined by cross-quartile classification analysis. Participants whose intakes were ranked by the SQFFQ to the opposite extreme quintile of intakes as per their responses in the 24-hour DRs were considered grossly misclassified. The proportion of the measurements by both the methods falling in the same quintile was calculated, though the agreement may occur by chance.<sup>28</sup>

To assess the extent of the agreement by accounting for chance, we used the weighted kappa statistic ( $w$ ) with prerecorded weights, which assessed the inter-rater agreement of the measures estimated by the two methods while accounting for the possibility of the agreement occurring by chance.<sup>29</sup> The coefficient of Lin’s absolute agreement was estimated, which quantified the agreement of the two measurements of the same variable, i.e. nutrient

intakes.<sup>30</sup> Lin's coefficient which measured both the precision and the accuracy of the relationship between the methods has evaluated whether the observed data deviate significantly from the line of perfect concordance.<sup>31</sup>

Bland-Altman plots were used to illustrate the agreement between the measurements (test - reference measure) (y-axis) against the mean of the two measures [(test measure + reference measure / 2)] (x-axis) and identify the outliers and trends in bias for each subject.<sup>27,28,32</sup> The limits of agreements were estimated by using the mean and the standard deviation (SD) of the differences between the two measurements (mean difference  $\pm$  1.96 \* SD).<sup>33,34</sup> Since the histograms were not perfectly bell-shaped, log-transformation was done before the testing. Data analyses were done in STATA 14.0 (STATA Inc. College Station, Texas, USA).

#### Interpretation of statistical outcomes

A number of statistical tests were performed to provide a comprehensive assessment of the various aspects of validity. A correlation coefficient  $\geq 0.50$  was considered ~~good~~"; 0.20–0.49 was ~~acceptable~~", while  $< 0.20$  was ~~poor~~".<sup>35,36</sup> Percent difference of 0-10.9% was considered ~~good~~", between 11-20% was considered ~~acceptable~~" and  $> 20\%$  was regarded as ~~poor~~".<sup>26</sup> Cross-classification with  $\leq 10\%$  in opposite quintile was considered ~~good~~" and  $> 10\%$  was ~~poor~~".<sup>35</sup> Weighted Kappa statistics of 0.8-1.0 was considered ~~very good~~", 0.6-0.8 was ~~good~~", 0.4-0.6 was ~~moderate~~", 0.2-0.4 was ~~fair~~"; and  $< 0.2$  was considered as ~~poor~~".<sup>37</sup> Lin's concordance coefficient  $< 0.20$  was considered ~~poor~~", 0.20-0.80 was ~~acceptable~~", and  $> 0.80$  was ~~excellent~~".<sup>37</sup>

The quality of the validation study was evaluated as per the guidelines of the European Micronutrient Recommendations Aligned Network of Excellence (EURRECA).<sup>38</sup> The assessment was made on-- a. the sample and sample size; b. statistics: group means, correlations, agreements; c. data collection method; d. seasonality; and e. inclusion of supplements.

#### Results

##### General characteristics

Table 1 presents the socio-demographic characteristics of the study participants. The proportion of male children was 45.7%. On average, the children were  $37.3 \pm 0.9$  months old.

Mothers completed on average  $7.7 \pm 0.3$  years of schooling. “Improved” (built with cement and/or corrugated iron sheet) households according to the materials used for the construction was possessed by 36.1% of the respondents. Nearly half of the households (46.6%) reported having their own cultivable lands. On average, BDT.  $1823.5 \pm 953.1$  (US\$  $21.7 \pm 11.3$ ) was spent for purchasing food in the week preceding the interview.

Table 1: Some selected socio demographics of the participants (n105)

Traits	%
Age of child (month)	
Mean	37.3
SD	0.9
Sex (male)	45.7
Mother’s education (years)	
Mean	7.7
SD	0.3
Improved house <sup>a</sup>	36.1
Possession of cultivable land	46.6
Possession of cultivable land (decimals)	
Mean	39.5
SD	70.5
Last week’s spend on food <sup>b</sup> (BDT) <sup>c</sup>	
Mean	1823.5
SD	953.1

<sup>a</sup>Semi-pacca house (Floor: cement and bricks; walls and roof: corrugated iron sheet) & pacca house (whole parts cement and bricks built)

<sup>b</sup>Rice, flour, oil, fish, meat, eggs, vegetables etc.

<sup>c</sup>USD  $21.7 \pm 11.3$

## Validity of the 7-day SQFFQ

### Comparative profile of the intakes

Table 2 shows the estimates of the daily food and nutrient intakes measured by the 7d SQFFQ and the reference method (24-hour DRs). The average of the two 24-hour DRs was computed as the reference value.

Table 2: Profile of intake estimates of food and nutrients in Bangladeshi children 24-59 months old as measured by the 7-day SQFFQ and 24-hour DRs

Food/Nutrients		Distribution of Daily Intakes						
		7- day SQFFQ			Average of two Dietary Recalls			
Food groups	Mean	SD	Median	IQR	Mean	SD	Median	IQR
Cereals <sup>a</sup> (g)	128.2	74.8	116.7	75.1,160.6	107.5	63.8	98.7	62.9,138.4
ASF <sup>a</sup> (g)	45.4	37.4	33.8	20.4,62.2	39.8	33.2	33.9	17.3,57.1
Milk <sup>a</sup> (ml)	350.2	418.2	111.6	15.6,781.2	307.9	377.2	129.7	0, 558.6
Legumes <sup>a</sup> (g)	6.4	12.7	2.8	0,5.5	5.1	11.1	0.0	0, 5.3
Fruits <sup>a</sup> (g)	48.6	65.2	28.5	5.3,68.1	32.3	58.1	0.0	0, 37.0
Vegetables <sup>a</sup> (g)	31.5	43.2	14.4	2.7,42.1	22.3	49.4	5.1	0, 30.3
Processed food (g)	25.5	23.8	18.5	6.4,37.2	23.4	26.7	14.0	5.0, 32.5
Nutrients								
Energy(kcal)	998.4	385.6	950.1	689.5,1259.4	875.9	321.7	910.6	601.2,1121.7
Carbohydrate(g)	157.2	60.2	148.1	119.6,198.6	130.6	52.6	122.0	94.9,163.9
Protein(g)	32.1	14.8	30.0	19.5,41.1	27.2	11.6	28.4	17.6,34.7
Fats(g)	23.5	17.0	19.7	11.1,33.7	24.0	12.5	23.2	14.7,31.1
Dietary fiber(g)	7.98	4.3	6.97	4.8,10.2	6.27	3.9	5.43	3.63,7.7
Iron(mg)	3.57	1.9	3.11	2.3,4.3	2.87	1.7	2.39	1.9,3.3
Zinc(mg)	5.3	2.3	5.1	3.5,6.8	4.4	1.9	4.3	2.8,5.7
Calcium(mg)	433.2	428.5	238.1	95.7, 827.4	369.3	378.5	191.5	67.9, 658.1
Magnesium(mg)	154.2	68.1	153.5	105.6, 195.6	124.6	54.1	116.8	81.1,165.2
Vitamin A(μg)	299.2	255.1	245.2	89.7, 417.1	258.8	336.7	146.7	53.9,327.5
Thiamine (mg)	0.67	0.29	0.66	0.44, 0.85	0.55	0.23	0.54	0.37, 0.73
Folates (mcg)	101.1	60.0	89.6	62.1, 129.9	82.8	65.2	65.8	40.8,102.3
Vitamin C	32.6	36.4	23.1	10.7,27.1	24.8	42.9	12.8	5.6,27.1

<sup>a</sup>Raw-food weight

By using the SQFFQ, the daily intakes of the cereals, animal source foods, milk and legumes appeared higher than that measured by the average of the two 24-hour recalls. Table 3 showed that the intakes were significantly higher ( $p<0.05$ ) for the food groups, measured by the SQFFQ compared to the average of two 24-hour DRs, except for the legumes ( $p=0.11$ ) and the processed foods ( $p=0.15$ ).

Table 3: Results of the statistical tests for assessing the validity of the 7-day SQFFQ and the interpretation of agreement for food, energy and nutrient intakes in Bangladeshi children 24-59 months old

Food/ nutrients	Unadjusted Spearman Rank correlation coefficient (rho) <sup>a</sup>	Energy adjusted Spearman Rank correlation coefficient <sup>a</sup>	Deattenuated correlation coefficient <sup>a</sup>	Wilcoxon Sign-ranked test <sup>b</sup>  (p value)	Percent difference (%) <sup>c</sup>	Cross-classification <sup>d</sup>		Weighted kappa statistics <sup>e</sup>	Lin's concordanc <sup>e</sup> coefficient of absolute agreement <sup>f</sup>  (rho_c)
						Same quintile  (%)	Extreme Quintile  (%)		
Food groups									
Cereals	0.76 (Good)	0.85 (Good)	0.93 (Good)	<0.001 (Poor)	16.0 (Acceptable)	80.0 (Good)	2.8 (Good)	0.56 (Moderate)	0.78 (Moderate)
ASF	0.64 (Good)	0.68 (Good)	0.77 (Good)	0.04 (Poor)	14.0 (Acceptable)	46.7 (Good)	0.0 (Good)	0.43 (Moderate)	0.56 (Moderate)
Milk	0.88 (Good)	0.88 (Good)	0.91 (Good)	0.0009 (Poor)	13.7 (Acceptable)	75.2 (Good)	0.0 (Good)	0.76 (Good)	0.90 (Excellent)
Legumes	0.39 (Acceptable )	0.38 (Acceptable )	0.51 (Good)	0.11 (Good)	20 (Acceptable)	57.1 (Good)	3.8 (Good)	0.28 (Fair)	0.46 (Moderate)
Fruits	0.50 (Good)	0.48 (Acceptable )	0.48 (Acceptable)	0.004 (Poor)	33.5 (Poor)	43.8 (Good)	8.5 (Good)	0.29 (Fair)	0.47 (Moderate)
Vegetable s	0.37 (Acceptable )	0.35 (Acceptable )	0.43 (Acceptable)	0.006 (Poor)	41.0 (Poor)	26.7 (Good)	6.7 (Good)	0.26 (Fair)	0.21 (Moderate)
Processed foods	0.46 (Acceptable )	0.65 (Good)	0.65 (Good)	0.15 (Good)	8.2 (Good)	40.9 (Good)	2.8 (Good)	0.28 (Fair)	0.31 (Moderate)
Nutrients									
Energy	0.68 (Good)	-		0.002 (Poor)	13 (Acceptable)	43.8 (Good)	2.8 (Good)	0.47 (Moderate)	0.62 (Moderate)
Carbohydrate	0.67 (Good)	0.68 (Good)	0.69 (Good)	<0.001 (Poor)	20 (Acceptable)	46.6 (Good)	3.8 (Good)	0.44 (Moderate)	0.61 (Moderate)
Protein	0.70 (Good)	0.50 (Good)	0.50 (Good)	<0.001 (Poor)	18 (Acceptable)	47.6 (Good)	1.9 (Good)	0.49 (Moderate)	0.63 (Moderate)
Fats	0.70 (Good)	0.74 (Good)	0.75 (Good)	0.25 (Good)	-2 (Good)	43.8 (Good)	0 (Good)	0.48 (Moderate)	0.70 (Moderate)
Dietary fiber	0.63 (Good)	0.74 (Good)	0.74 (Good)	<0.001 (Poor)	27 (Poor)	49.5 (Good)	4.7 (Good)	0.42 (Moderate)	0.62 (Moderate)
Iron	0.49 (Acceptable )	0.60 (Good)	0.60 (Good)	<0.001 (Poor)	24 (Poor)	40.9 (Good)	7.6 (Good)	0.30 (Fair)	0.48 (Moderate)
Zinc	0.69 (Good)	0.57 (Good)	0.57 (Good)	<0.001 (Poor)	20 (Acceptable)	48.5 (Good)	9.5 (Good)	0.46 (Moderate)	0.59 (Moderate)
Vitamin A	0.83	0.46	0.46	0.002	17	34.2	6.6	0.36	0.33

	(Good)	(Acceptable)	(Acceptable)	(Poor)	(Acceptable)		(Good)	(Fair)	(Moderate)
Calcium	0.53	0.84	0.85	0.003	10	51.4	0.0	0.60	0.87
	(Good)	(Good)	(Good)	(Poor)	(Good)		(Good)	(Good)	(Excellent)
Magnesium	0.60	0.39	0.39	<0.001	23.7	47.6	3.8	0.40	0.48
	(Good)	(Acceptable)	(Acceptable)	(Poor)	(Poor)		(Good)	(Moderate)	(Moderate)
Thiamine	0.70	0.56	0.56	<0.001	19	47.1	1.9	0.48	0.61
	(Good)	(Good)	(Good)	(Poor)	(Acceptable)		(Good)	(Moderate)	(Moderate)
Folates	0.55	0.46	0.47	<0.001	22	41.9	4.1	0.33	0.35
	(Good)	(Acceptable)	(Acceptable)	(Poor)	(Poor)		(Good)	(Fair)	(Moderate)
Vitamin C	0.35	0.30	0.30	<0.001	31	47.6	9.5	0.24	0.65
	(Acceptable)	(Acceptable)	(Acceptable)	(Poor)	(Poor)		(Good)	(Fair)	(Moderate)

\*Coefficients are significant at  $p < 0.001$  for all the foods, nutrients and energy

<sup>a</sup>To deattenuate the energy-adjusted coefficient for the within-individual variances in the intakes from the repeated recalls

<sup>b</sup>To assess the mean difference in the intakes recorded by the tools

<sup>c</sup> $[\text{SQFFQ} - \text{Avg. of two 24-hr recalls}] / \text{Avg. of two 24-hr recalls} \times 100$

<sup>d</sup>Ranking of the intakes measured by the SQFFQ in the same and to extreme opposite quintiles as measured by the 24-hour DR

<sup>e</sup>Cohen's weighted kappa statistic for quartiles ( $\frac{1}{2} \sum_{i,j} \delta_{ij} \frac{1}{k}$ ; where  $i$  and  $j$  index the rows and columns of the two ratings and  $k = 4$ ).

<sup>f</sup>Lin's concordance coefficient- a measure of accuracy and precision of the agreement

The intakes of energy, and the macronutrients (carbohydrate and protein) measured by the SQFFQ was significantly higher ( $p < 0.05$ ) than that measured by the 24-hour DRs, with an exception of fats ( $p = 0.25$ ) (Table 2, 3). Similarly, for the key micronutrients, such as iron, zinc, vitamin A, calcium and folic acid, the intakes measured by the SQFFQ were statistically significantly higher ( $p < 0.05$ ) compared to that measured by the 24 hour recalls (Table 2,3).

Test results and the assessment of the agreements of the compared methods

Table 3 presents the assessment of the validity of the SQFFQ and the interpretation of agreement of the measurements for food, energy and nutrient intakes derived from the 7-day SQFFQ and 24-hour DRs. The energy-adjusted correlation coefficients were “good” for the cereals (0.85), animal source foods (0.68), milk (0.88), and the processed foods (0.65); while, the coefficient was “acceptable” for legumes (0.38), vegetables (0.35), and the fruits (0.48).

The de-attenuated coefficients accounting for the within-subject variations of intakes showed the improvement of the association for cereals (0.93), animal source foods (0.77), milk (0.91), legumes (0.51) and vegetables (0.43); while it remained unchanged for other food groups. All the coefficients were significant at  $p < 0.001$ . The energy-adjusted coefficients for the nutrients were ~~–good~~” for the macronutrients (0.50-0.74,  $p < 0.001$ ) and for most of the micronutrients (0.56-0.84,  $p < 0.001$ ); while the coefficients were ~~–acceptable~~” for vitamin A, magnesium, folate and vitamin C (0.30-0.46,  $p < 0.001$ ). De-attenuated coefficients largely remained unchanged for all the macronutrients and micronutrients ( $p < 0.001$ ). The percent difference for the measurements between the SQFFQ and the reference tool was ~~–acceptable~~” for most food groups (cereals, animal source foods, milk, and legumes), ~~–good~~” for the processed foods, and ~~–poor~~” for fruits and vegetables. Regarding the nutrients, the percent difference was ~~–good~~” for fats and calcium; ~~–acceptable~~” for energy, carbohydrates, proteins, zinc, vitamin A and thiamin; and ~~–poor~~” for dietary fibre, iron, magnesium, folate and vitamin C.

Classification by the SQFFQ in the same quintile as measured by the 24-hour DRs was seen with  $>40\%$  of the respondents with 6 of the 7 food groups; with high proportions for cereals (80%) and milk (75.2%). Classification in the extreme opposite quintile was reported in  $<5\%$  of the respondents with 6 of the 7 food groups. Classification in the same quintile was observed in  $>40.0$ - $49.5\%$  of the respondents for energy and all the macronutrients and micronutrients, except for vitamin A, which was 34.2%. Classification in the extreme opposite quintile was observed in  $0$ - $<10\%$  of the respondents for all the nutrients. All the food groups and the nutrients were classified with a fair level of closeness, as depicted by the kappa estimates ranging from  $0.24$ - $0.76$  ( $p < 0.001$ ). Lin’s concordance correlation for absolute agreement showed the coefficient ( $\rho_c$ ) was ~~–excellent~~” for milk (0.90,  $p < 0.001$ ), and ~~–moderate~~” for other food groups ( $0.28$ - $0.76$ ,  $p < 0.001$ ). The absolute agreement was ~~–moderate~~” for all the macronutrients and micronutrients and energy ( $0.30$ - $0.70$ ,  $p < 0.001$ ), except for calcium which had an ~~–excellent~~” absolute agreement ( $0.87$ ,  $p < 0.001$ ).

Analysis of Bland–Altman plots (Figure 2a-2d, 3a-3d) showed that the key macronutrient and micronutrient intakes did not present significant proportional bias and most of the points fell within the 95% limits of agreement. Only a few of the points fell outside the agreement limits, which were between  $2.8$ - $7.6\%$ , for all the macronutrients and micronutrients. For example, for proteins, carbohydrates and fats the estimates were  $6.5\%$ ,  $4.7\%$  and  $2.8\%$

respectively. For iron, zinc and vitamin A, the estimates were 2.8%, 3.8% and 7.6% respectively.

Fig 2a: Bland Altman Plotting showing agreements between the SQFFQ vs. 24-hour DRs in measuring the intakes of energy

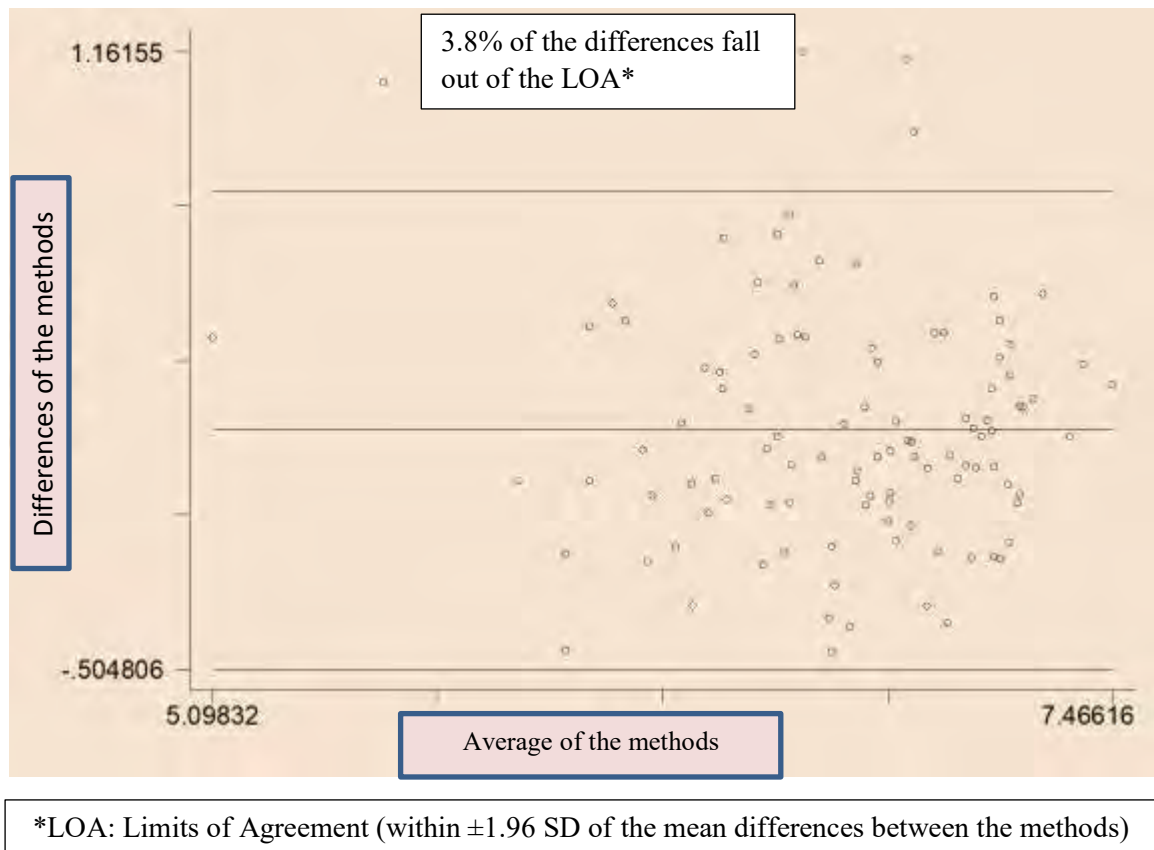
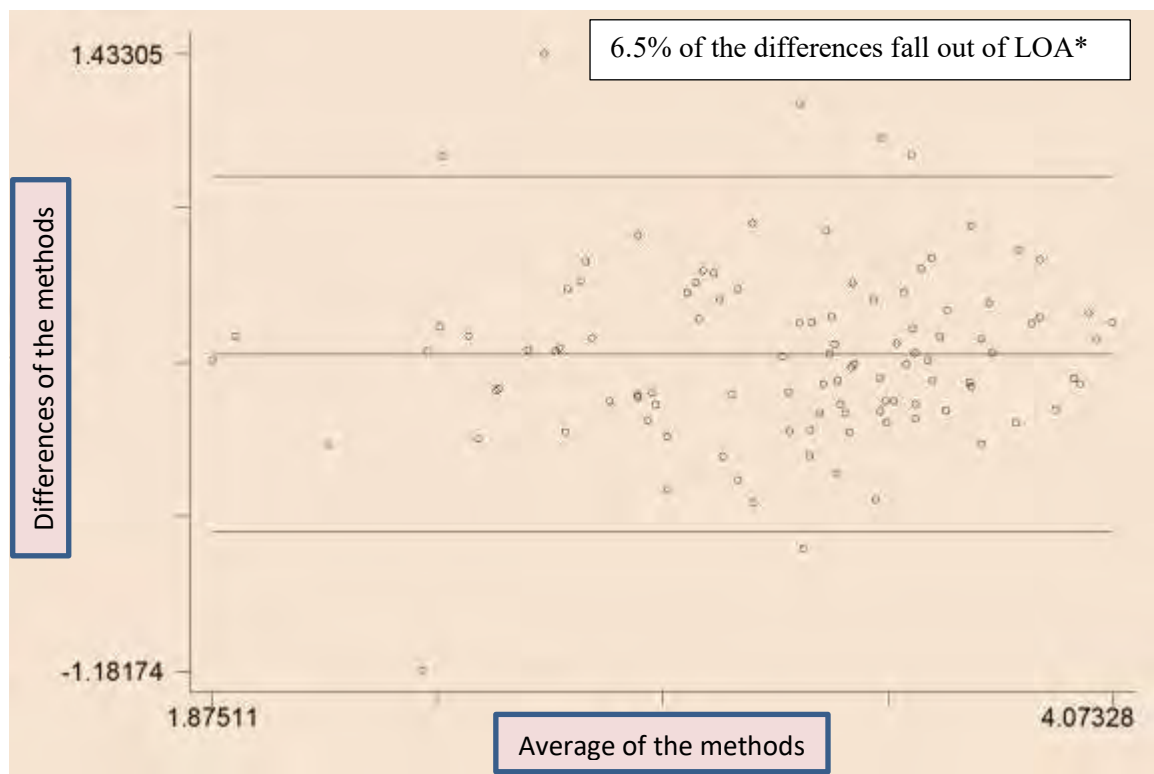
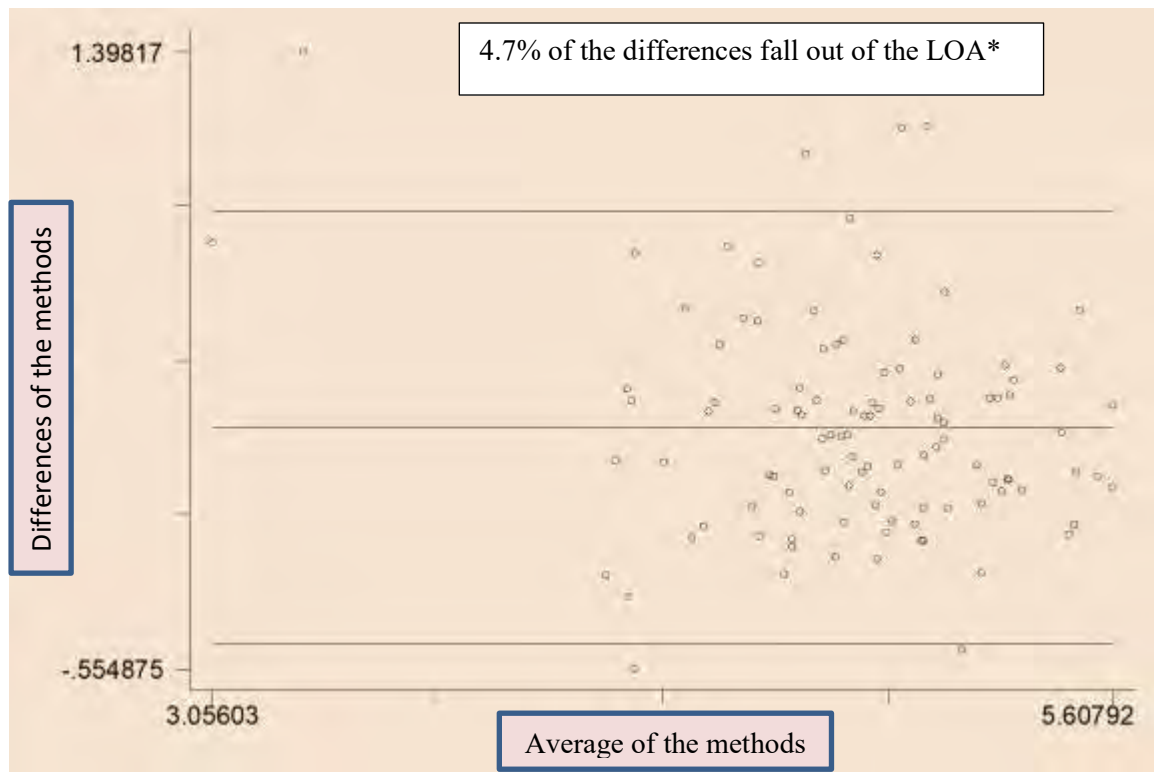


Fig 2b: Bland Altman Plotting showing agreements between the SQFFQ vs. 24-hour DRs in measuring the intakes of protein



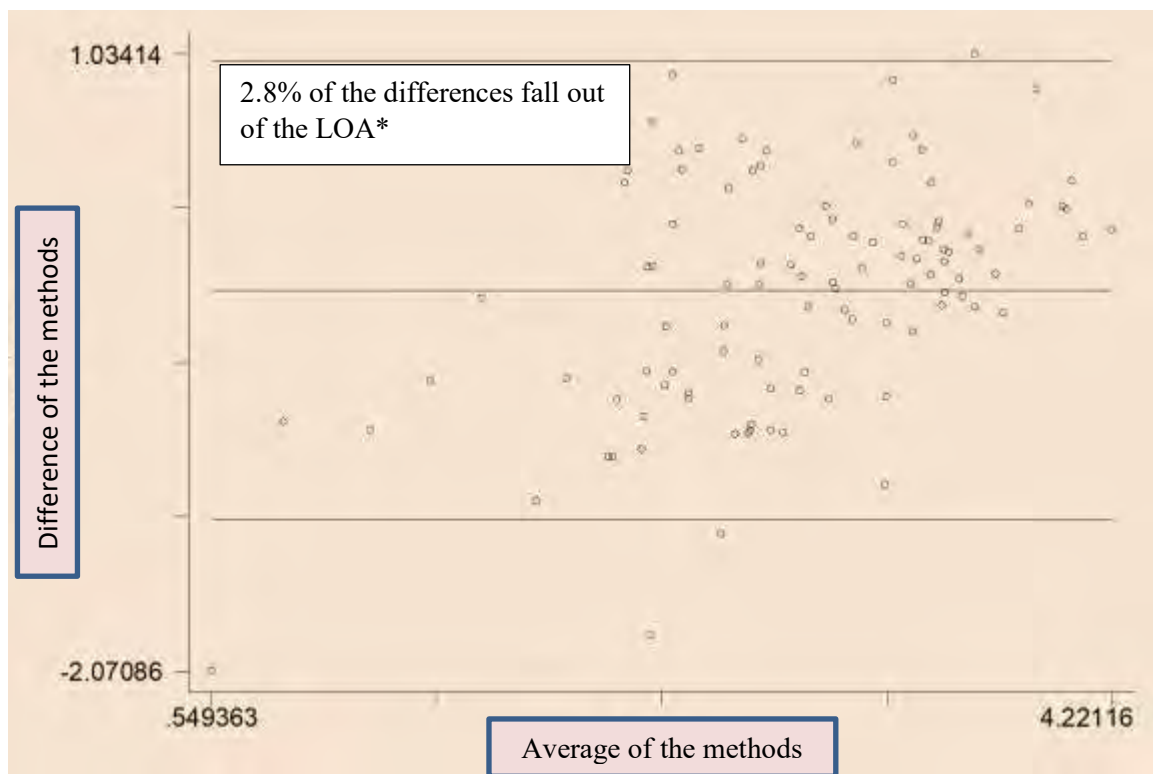
\*LOA: Limits of Agreement (within  $\pm 1.96$  SD of the mean differences between the methods)

Fig 2C: Bland Altman Plotting showing agreements between the SQFFQ vs. 24-hour DRs in measuring the intakes of Carbohydrate



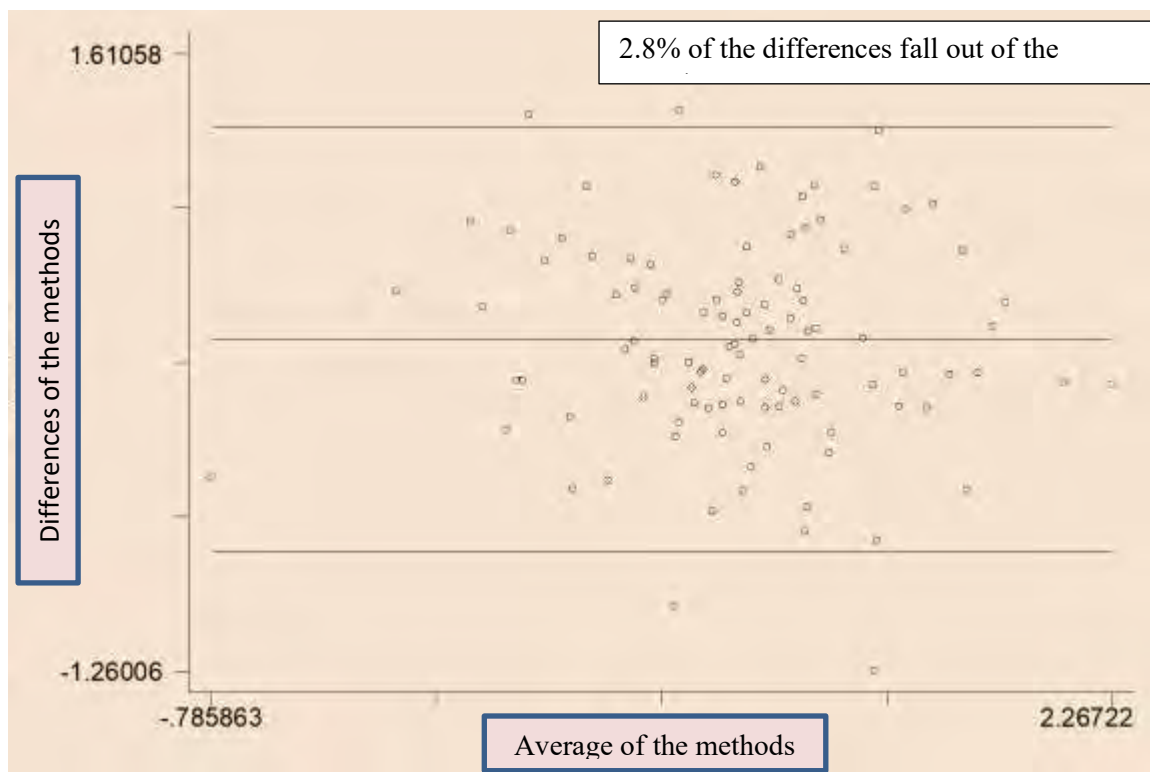
\*LOA: Limits of Agreement (within  $\pm 1.96$  SD of the mean differences between the methods)

Fig 2d: Bland Altman Plotting showing agreements between the SQFFQ vs. 24-hour DRs in measuring the intakes of Fats



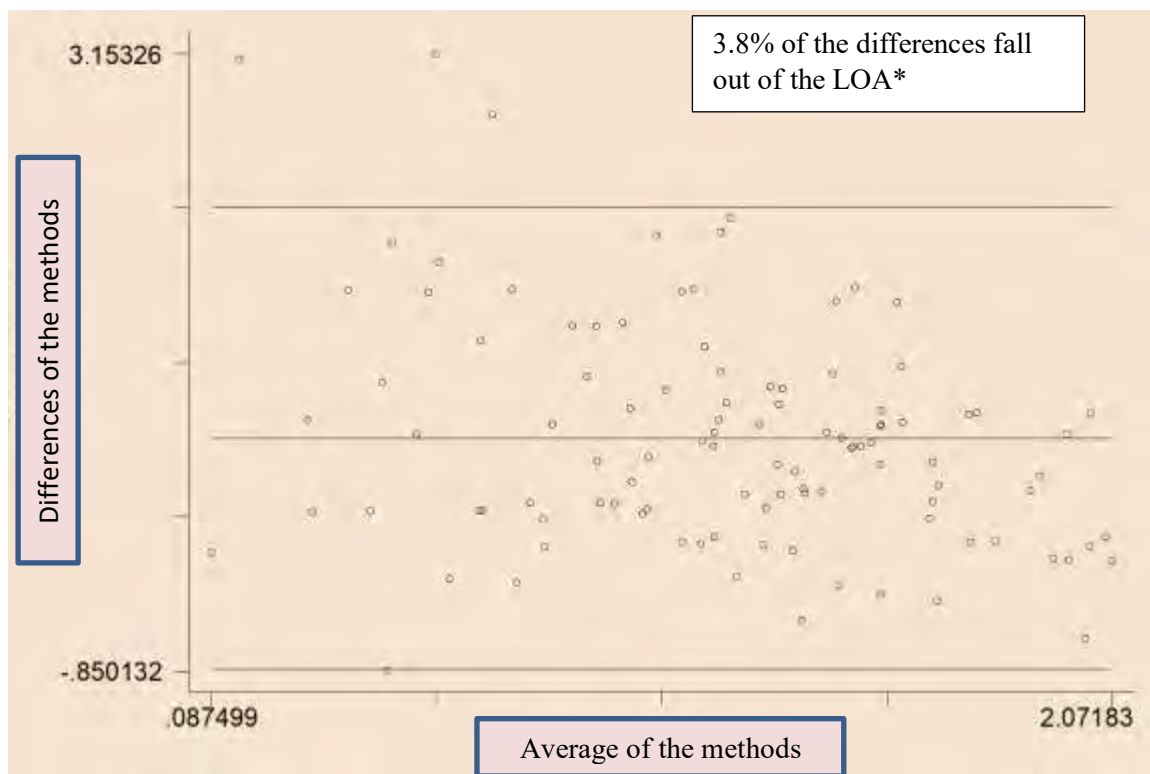
\*LOA: Limits of Agreement (within  $\pm 1.96$  SD of the mean differences between the methods)

Fig 3a: Bland Altman Plotting showing agreements between the SQFFQ vs. 24-hour DRs in measuring the intakes of iron



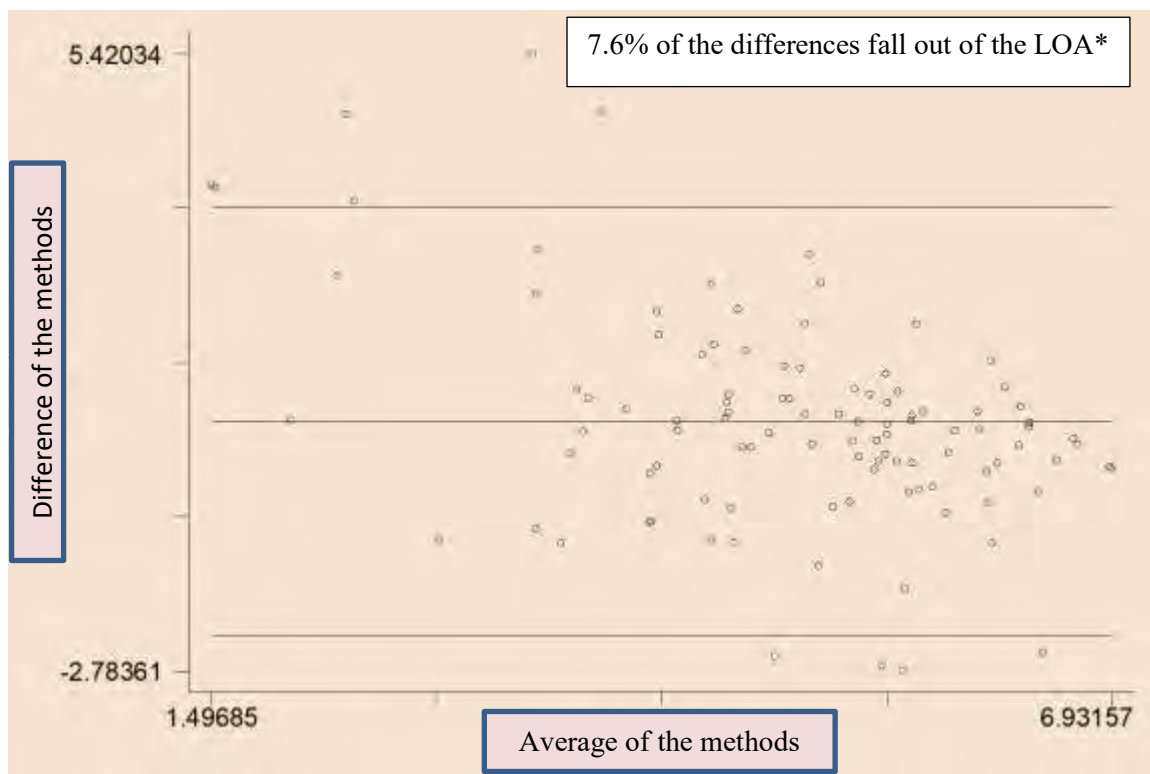
\*LOA: Limits of Agreement (within  $\pm 1.96$  SD of the mean differences between the methods)

Fig 3b: Bland Altman Plotting showing agreements between the SQFFQ vs. 24-hour DRs in measuring the intakes of zinc



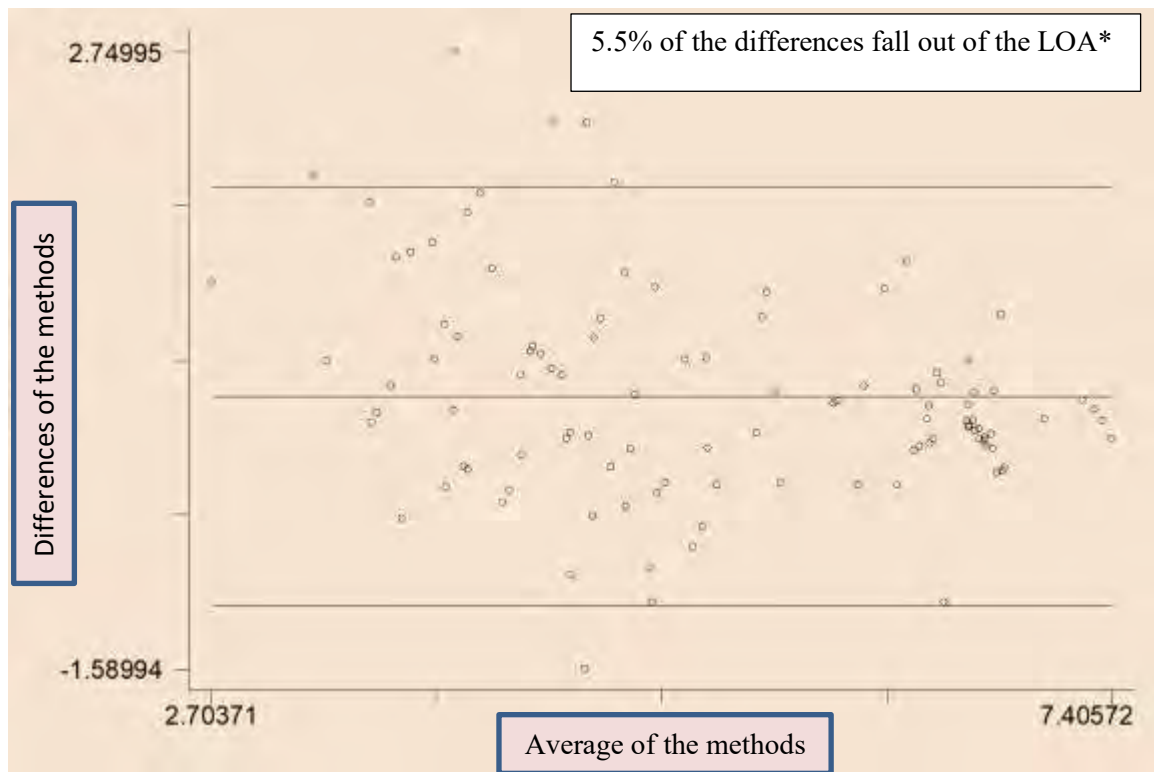
\*LOA: Limits of Agreement (within  $\pm 1.96$  SD of the mean differences between the methods)

Fig. 3c: Bland Altman Plotting showing agreements between the SQFFQ vs. 24-hour DRs in measuring the intakes of Vitamin A



\*LOA: Limits of Agreement (within  $\pm 1.96$  SD of the mean differences between the methods)

Fig. 3d: Bland Altman Plotting showing agreements between the SQFFQ vs. 24-hour DRs in measuring the intakes of calcium



\*LOA: Limits of Agreement (within  $\pm 1.96$  SD of the mean differences between the methods)

## Overall appraisal of the results

Table 4: The evaluation<sup>a</sup> of the study in the framework of the European Micronutrient Recommendations Aligned Network of Excellence (EURRECA)<sup>38</sup>

Domains	Standard elements	Designated points	Elements included in the present study	Scored Points	EURRECA classification
Sample and sample size	Non-homogenous sample (sex, obesity)	0.5	Homogenous sample	0.0	–Good”
	Sample size>100	0.5	Sample size>100	0.5	
Statistics					
Group level	Compare/test mean or median or difference	1.0	Test means	1.0	
Correlations	Unadjusted	0.5			
	Energy-adjusted	1.0			
	De-attenuated or intra-class correlations	1.5	De-attenuated	1.5	
Agreement	Classification or Bland-Altman plots	0.5	Classification, Bland-Altman plots, Lin’s concordance etc	0.5	
Data collection	Gathered by face to face interview	1.0	Gathered by face-to-face interview	1.0	
Seasonality	Considered	0.5	Not considered	0.0	
Supplements information	Included and data considered in analysis	1.5	Not included	0.0	
Total points		7.0		4.5	

<sup>a</sup>The domains of the assessment are- a. the sample and sample size, b. statistics: group means, correlations, agreements, c. data collection method, d. seasonality, and e. inclusion of supplements

Interpretation:

- (1) Very good/excellent:  $\geq 5.0$ -7.0
- (2) Good: 3.5-<5
- (3) Acceptable/reasonable: 2.5- <3.5
- (4) Poor: <2.5

After appraisal of the domains of the assessment as per the EURRECA guidelines, the overall score of the study was 4.5, consistent with the rank of –good” (Table 4).

## Discussion

In the present study, we assessed the validity of a 7-day SQFFQ used in a randomized control trial to examine the efficacy of MNP supplementation in Bangladeshi rural children aged 24-59 months. Unlike the traditional way of conduction of FFQ, the 7-day SQFFQ was interviewer-administered; had an open-ended actual number of times of consumption; and the reference time of food intake of seven days. The present study compared the daily intakes of foods and nutrients measured by the 7-day SQFFQ with the average of the two 24-hour DRs using a battery of seven statistical tests. The results showed that this SQFFQ demonstrated good/acceptable validity against the 24-hour DRs for most food groups and nutrients. Classification in the extreme opposite quintile was observed in 0-<10% of the respondents for all the nutrients. All the food groups and the nutrients were classified with a fair level of closeness, as depicted by the kappa estimates.

Overall, the SQFFQ overestimated the intakes of the foods and nutrients compared to that estimated by the 24-hour DRs. This finding is expected and consistent with other studies.<sup>39-43</sup> The possible reason for overestimated intakes is believed to be due to the fact that parents may not adequately assess the small portion sizes consumed by their children sometimes without consuming full portions, leading to the overestimation of the portion size for some foods.<sup>39, 42</sup> Despite some overestimation, the SQFFQ provided acceptable estimates of the intakes in the young children with good agreement with the 24-hour DRs.

There is a paucity of studies in Bangladesh which validated FFQ with an open-ended frequency of consumption option and considered a similar comparator method as used in the present study. In an assessment of the external validity of the present SQFFQ, we compared its measured energy intake to a dietary assessment study in rural Bangladeshi children aged 24-48 months using 12-hour recall and 12- hour of weighing observation.<sup>44</sup> Daily intake of energy was 998.4 kcals and 889 kcals by the present study and by Arsenault et al,<sup>44</sup> respectively. Considering, a slight mismatch of age group (24-59 months in the present study vs. 24-48 months in the Arsenault et al study) and a considerable difference in the assessment methods, the energy estimated by the present tool appears to be reasonable.

The deattenuated correlation coefficients marked an increase from the energy-adjusted coefficients for food groups- cereals, animal source foods, legumes and vegetables. This implies that there is some degree of the within-subject variance of intakes measured over the

repeated recalls. The within-subject variance of intake was small for rice (results not shown). The day-to-day variances in the intakes of bread (flat bread, sliced bread), which are consumed less consistently in this setting, might have contributed to some within-subject variance for the cereals group. Hence, the de-attenuation of the variance has improved the coefficient in the cereal group. Animal sourced food is expensive for rural families, and the within-subject variance of intake was common (results not shown), which led to the larger de-attenuated coefficient. Legumes and vegetables were consumed sparsely in this age group, with some within-subject variances over the 24-hour DRs and therefore, resulting in larger de-attenuated coefficients. Regarding the nutrients, there was hardly any difference between the energy-adjusted and the de-attenuated coefficients. This is difficult to explain; however, we observed that the within-subject variances of the intakes of the nutrients over the two 24-hour DRs were very small leading to the negligible within-subject to between-subject ratios (results not shown). The underlying reason for this could be the distribution of the nutrients in the foods commonly consumed were largely homogenous, and the children's dietary pattern was less- diversified throughout assessments.

Consistent with the study of Lovell et al,<sup>38</sup> we observed an increasing magnitude of the coefficient of association of the SQFFQ and the reference method, as the frequency of consumption increased. In this setting, the consumption of cereals- e.g. rice; and the consumption of milk was frequent and consistent; and very large energy-adjusted and de-attenuated correlation coefficients were observed regarding those foods. However, much smaller de-attenuated coefficients were observed for vegetables and fruits, since the intakes of those foods were less consistent. The smaller coefficients with the episodically consumed food items are consistent with the other studies<sup>45</sup> due to the high day-to-day variability of the intakes.

A “poor” percent-difference between the intake estimates measured by the methods was observed for some of the nutrients, such as dietary fibre, iron, magnesium, folate and vitamin C. Of this methodological difference of estimates, iron and magnesium were marginally outside the 20% cut-off (i.e. magnitude of difference above which suggesting a “poor” agreement). However, for vitamin C (31%), dietary fibre (27%), the difference was on the higher side. One of the explanations for this is that children in this age and setting are less likely to consume vegetables and fruits consistently. This is supported by the observation that the SDs for the mean intakes was larger for the 24-hour DRs compared to the SQFFQ regarding vegetables, fruits and vitamin C. This is suggestive of infrequent consumption of

these food items in the children. Hence, while the SQFFQ might have captured the intakes but the 24-hour DRs failed, leading to the widening of differences between the methods.

The correlation coefficients and the level of agreement of the present SQFFQ with the reference method were larger than the agreements observed in other FFQs validated in Bangladeshi populations,<sup>10,11</sup> and it was larger than the median coefficients reported in a systematic review of FFQ validation studies.<sup>46</sup> The possible reasons for the difference in the coefficient estimates are - the tool itself, the reference tool and the methods of administration. Lin et al<sup>10</sup> used an interviewer-administered dish-based SQFFQ with up to a one-year reference time, and the respondent-administered Food Diaries (FD) was the reference method. Chen et al<sup>11</sup> used an interviewer-administered FFQ with open-ended consumption options but with a one-year reference time and the interviewer-administered two 7-day FDs as the reference method. The reason for the larger coefficient estimates and a higher level of agreement observed in the present study than the above-referred studies is perhaps the short reference time. With the short time span, the respondents could report the actual intake with higher precision, rather than reporting habitual consumptions over a long reference time recorded in the other studies. The other reason for the larger coefficients observed in the present SQFFQ is the time of administration of the 24-hour DRs. After taking the SQFFQ, the two non-consecutive recalls were completed within two weeks. Within this short interval, the pattern of intake was largely unchanged. Both the test and the reference methods were interviewer-administered, who could record the amount of intakes with good precision, which might have contributed further to the high levels of agreement.

The present study findings have implications on the dietary intake assessment of the children recruited in a trial assessing the effect of an MNP supplementation on hemoglobin and iron parameters. The nutrients of particular interest to the trial were iron, zinc, vitamin A, vitamin C and folic acid. The de-attenuated correlation coefficients were consistent with “good” to “acceptable” for the nutrients. Lin’s concordance of absolute agreement between the methods for these nutrients was “moderate” justifying the usage of the SQFFQ for a valid estimate of the children’s intake of the micronutrients in the concerned trial.

### Strength and limitations

The strength of our SQFFQ is the fact that it recorded the actual number of intakes of foods over seven days preceding the interviews, unlike the habitual frequency of consumption

captured in other types of FFQs. Cade et al <sup>9</sup> reported that the commonly employed statistical methods in the SQFFQ validation studies are—correlation, percent-difference, cross-classification, kappa estimates of agreement and Bland Altman plots. A recent review by Lovell et al <sup>38</sup> reported that the mean comparison, correlations, cross-classifications and kappa statistics are commonly reported statistical workup in dietary validation studies. Lin et al <sup>10</sup> in the validation of a dish based SQFFQ in the Bangladesh context have performed all the above-stated tests. The present study in addition to the above stated statistical tests reported the concordance agreement—hence the robustness of the assessment is the strength of the study. The method will be useful in the epidemiological dietary assessment in pre-school children by providing a convenient alternative to the standard methods, e.g. 24-hour DRs, which typically needs multiple non-consecutive day administrations posing logistical challenges. The tool is generalizable for dietary assessment of Bangladeshi children aged 2-5 years, since, the foods of the FFQ are derived from a nationally representative dietary assessment survey as the foods commonly consumed.

However, this study has some limitations. Due to logistical difficulties, we could not repeat the SQFFQ. The single-time administration of the method failed to test the reproducibility and seasonality. Despite the SQFFQ captured the most commonly consumed foods in a largely homogenous longitudinal intake pattern of rural Bangladeshi setting; not testing the seasonality may compromise its long-term validity for some micronutrients, such as vitamin A, which is consumed in higher amount in the summer fruit season. Secondly, we used 24-hour DR, as the reference method. Both 24-hour DRs and FFQs are prone to measurement error associated with recall bias and awareness of portion size. Errors associated with these methods are not mutually independent, and the correlation coefficients might have been overestimated. <sup>15</sup> Thirdly, data on continuing breastfeeding was not gathered due to difficulties in measuring the breast milk quantity. <sup>47</sup> However, as breastfeeding was not recorded by either of the competing methods it is unlikely to affect the comparability about the relative validity. Nonetheless, not inquiring about the data constitutes a limitation. Fourthly, the possible recall bias for accommodating both the methods—SQFFQ and the first 24-hour recall in the same interview may not be ruled out. We attempted to minimize it by orienting the interviewers about such possibility and to conduct the communication to allay this as much as possible. The methods being administered by the interviewers and not being self-reported aided in minimizing the issue. Traditionally FFQs tend to overestimate the measure when compared to 24-hour recalls. <sup>39-43</sup> The estimates of the present FFQ reported

somewhat higher values than that reported by the recalls; hence it was consistent with the general trend of the relative measure between these methods.

Fifthly, for most of the non-processed foods, the nutrient intake was calculated as per its content in 100 grams of raw food as per the updated Food Composition Table of Bangladesh. Some nutrients, such as folate and vitamin C are lost to some extent during the cooking process. Not accounting for such losses is a limitation of the study. Lastly, the FFQ had the limitation to assess vitamin C intake as the low agreement levels were observed in terms of kappa statistics, cross-classification and percent differences. The fruits and vegetables, the rich sources of vitamin C are eaten sparsely by the children in this setting. Hence, validating with a low number (n=2) of the recalls might have led to the recall-days when the child did not take a vitamin C-rich food; and thus the poor agreement is expected.

The study performed well among the FFQ validation studies in light of the assessment over some standard metric parameters, <sup>38</sup> and ranked high among the studies tagged as “good” classification. The study did not consider the intake of supplements, thus it just fell short off the “excellent” ranking according to Lovell’s appraisal. <sup>38</sup> However, the usage of supplements among pre-school age rural Bangladeshi children is rare.

## Conclusion

In conclusion, the interviewer-administered, seven-day SQFFQ with an open-ended actual number of times of intake is a valid tool in assessing the food and nutrient intakes in 24-59 months old Bangladeshi children. The tool can be used for assessing children’s short-term intake of food in the epidemiological studies in Bangladesh, where the respondent’s literacy is suboptimum.

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## Conflicts of interest

The authors declare no competing interests.

Authorship: S.R., F.A., P.L.: Study design; S.I., S.R.: Tool development; S.R.: Data collection; F.A., M.R.K.: Supervision of data collection; S.R.: Data analysis and writing the first draft of the manuscript; F.A., P.L.: Guiding in the data analysis and critical review of the manuscript to finalise; F.A.: Had primary responsibility for final content. All authors read and approved the final manuscript.

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### **8.3 ANNEX 3**

#### **8.3.1 OVERVIEW OF PREPARATORY PAPER 3**

Title: Temporal Effect on Iron Concentration of the Extracted Groundwater Samples in

Bangladesh: Potential Implication on Iron-status in the Population

Status: Submitted to a journal

Journal: Journal of Water and Health [Impact Factor: 1.7]

#### **8.3.2 INTRODUCTION**

Iron in groundwater exists in a soluble ferrous state. If the water is left for consumption at a later time, the ferrous iron might be oxidized to a ferric insoluble state and precipitated. Hence, one purpose of this substudy was to examine the decay of iron concentration in stored groundwater samples, which could potentially adjust iron intake from groundwater in the trial participants.

The other intent of the study was to examine a perception or myth long held among nutritional practitioners that if groundwater is left and stored for later consumption, it loses all the iron being precipitated as ferric form due to oxidation from contact with air. Thus, there is an alleged prospect of not acquiring any iron from groundwater which might be ingested.

In this study, a groundwater sample for iron concentration was measured using a colorimeter at six time points. A mixed-effect regression was done comparing the adjusted temporal iron concentrations. The post-6 hour's concentration remained consistent with the *high* amount of iron in groundwater. The findings intimated that the decay of iron upon storing is functionally inconsequential in regard to its contribution to the build-up of iron status in the trial participants.

### 8.3.3 SUBMITTED PAPER

Temporal Effect on Iron Concentration of the Extracted Groundwater Samples in Bangladesh: Potential Implication on Iron-status in the Population

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## Abstract

Ferrous iron in groundwater is believed to be rapidly oxidized and precipitated upon air exposure leading to a debate about the prospect of drinking groundwater ameliorating iron deficiency. Groundwater iron concentration was measured using a colorimeter at six time points. A mixed-effect regression was done comparing the adjusted temporal iron concentrations. The mean concentrations did not differ statistically up to the fourth time-point (2 hours), except for the last two time points. The post-six hours concentration remained consistent with the “high” amount of iron in groundwater. The findings consolidate the potential of groundwater iron contributing to population iron status.

Keywords: Groundwater iron, Temporal concentrations; Iron status; Bangladesh

## 1. Introduction

Groundwater is the principal source of drinking water in Bangladesh (BGS/DPHE 2001). The groundwater contains a predominantly high concentration ( $\geq 2$  mg/L) of iron in many parts of the country (BGS/DPHE 2001). Highlighting its public health implication, several studies including a nationally representative survey showed a positive association between high groundwater iron concentration and iron status in different population groups (Merrill 2011, Rahman 2016, Ahmed 2018). A recent study reported that in the children aged 2-5 years residing in a high groundwater iron setting, the mean concentration of iron in the drinking groundwater was high ( $\sim 8$  mg/L). The prevalence of iron deficiency (% ferritin  $< 12$  ng/ml) was very low at 1.8%, despite their intake of dietary iron was grossly suboptimum (Rahman 2019). These findings suggest that iron in groundwater has a positive role in the buildup of the iron status of the Bangladesh population.

High content of iron in groundwater is imparted to predominantly reducing conditions [ferrous ( $\text{Fe}^{2+}$ ) state] existing in the aquifers of Bangladesh (BGS/DPHE 2001). For absorption in human, iron requires to be in the ferrous ( $\text{Fe}^{2+}$ ) state (Ems 2021). On the other hand, iron in ferrous (soluble) form in groundwater after pumping off the tube-wells may oxidize by the oxygen in air into ferric (insoluble) form (Daniel 2011). Hence, there is a speculation among the health academics and nutrition specialists that, freshly pumped groundwater if not consumed immediately or left for drinking later, might lose the iron content as the precipitation induced by the ferric iron; diminishing the potential for acquiring

iron from groundwater to improve the iron status in population despite consuming groundwater regularly. There is no nationally representative data on the water drinking behavior of the Bangladesh population. However, Ahmed et al (2018) in a trial of assessing the effect of iron-supplements on the pregnant women reported that 18.7% women collected water in a holding pot and drank later. Merrill et al (2011) reported that in a northern district, 60% of the studied women reported drinking within 5 minutes of pumping and another 30% reported drinking within 30 minutes of the extraction. Merrill et al furthermore reported that the rural people of Bangladesh habitually prefer freshly pumped water because it is cool, tastes good and quenches thirst (Merrill 2010). Put the above information together, it is apparent that a modest proportion of the population might prefer drinking after storing in containers.

Hence, the objective of the study is to assess the concentrations of iron in the stored groundwater samples over the temporal time points after pumping off the tube-wells up to 6 hours. This sub study was nested in a randomized controlled trial to examine the efficacy of a low-iron supplement (low-iron MNP) in preventing anemia in young children drinking from the groundwater with a high concentration of iron. The trial received ethical approval from the Faculty of Biological Science, the University of Dhaka, Bangladesh (Ref# 46 /Biol. Scs. /2017-2018), and the Griffith University Human Ethics Committee, Australia (Ref# 2017/467). Therefore, the other rationale for conducting this study is to assess the effect of the holding time on the decay in the iron level in groundwater in order to get an understanding of the actual consumption of iron from water in the children participating in the parent trial.

## 2. Methods

A total of 111 tube-wells were assessed for temporal measurement of concentration of iron in the water samples.

### 2.1 Selection of tube-wells

Selection of the tube-wells was purposive to account for different subgroups with different level of groundwater iron concentrations. At first after receiving the verbal consent of the household head, an adult member (household head/wife) of the randomly encountered households were asked how they feel about the level of iron present in the tube-wells they drink the water from. At next, a novel sensorial non-device based tool e.g. “taste rating” for

iron of the groundwater sample was used to have an initial assessment of iron content in the groundwater (Rahman 2020, Rahman 2018). Finally, the concentration of iron of the well-water sample was measured by a handheld colorimetric device (Hannathai.com). This process of selection of the tube-wells was continued to fulfill the desired number of the wells per subgroup. At first, two groups were considered --- low-iron ( $<2$  mg/L) and high-iron ( $\geq 2$  mg/L) wells taking into consideration of the cut-off (2 mg/L) consistent with the upper limit (UL) of iron in water that does not lead to adverse health issues (WHO 2006). We arbitrarily subdivided the latter group of the wells into “high” (2-10 mg/L) and “very high” ( $\geq 10$  mg/L) sub-groups. The sub-group assessment was performed to observe if the pattern of the changes in the temporal iron concentration in the water samples remain consistent at the differing levels of the initial concentration.

## 2.2 Sample size

We considered that 30 tube-wells per sub-group would provide valid estimates of iron concentration considering the fact that the analysis would approximate a Gaussian distribution to provide a valid mean with standard deviation as per the central limit theorem (Fischer 2012). However, we could assess 26, 46 and 39 tube wells in the “low”, “high” and in the “very high” sub groups respectively. The reason for slightly lower sample number than the requirement in the “low” subgroup is the difficulty in finding the wells with low iron ( $<2$  mg/L) in the study area, which is a high groundwater iron setting (BGS/DPHE 2001). In the process of the well selection, the number in the other two subgroups went somewhat higher than the requirement.

## 2.3 Considerations for a 6-hour follow-up time

As stated elsewhere, rural people of Bangladesh prefer freshly extracted groundwater for drinking. On another note, the average distance of the tube-wells is short; as most of the shallow wells of the country are located within  $<45$  meters from the households (Escamilla 2011). Another study reported the average distance of the shallow-wells from the households was  $27.6 \pm 37.1$  meters (Goel 2019). The above information summed up implies that the storing groundwater for a long time occurs less commonly. Anecdotal experience suggests that people in many areas collect water from their wells at bed time to store overnight inside the dwelling room for any required late-night consumption. At the dawn next day the freshly pumped water is collected. Hence, in the present study we considered a period of 6 hours for follow up to assess the changes in the iron concentration in the water samples. In support of

this time-interval another study reported the duration of ~ 7 hours for storing of the shallow well water among the practitioners who prefer to store drinking water (Goel 2019).

#### 2.4 Collection of groundwater sample and measurement of iron

After pumping the selected tube-well water for 5 minutes, the sample water was taken in a 1 liter plastic-mug. The mug was devoid of any lid to aid in free mixing of air with the sample water as the container was held open in air over the examination period. The examination period was 6 hours. Concentration of iron in the water sample was measured at six time-points following collection of water in the container-- first (0.0 hours), second (0.5 hours), third (1 hour), fourth (2 hours), fifth (3 hours) and the sixth (6 hours).

#### 2.5 Assessment of iron in the water samples

We used a portable handheld colorimeter (Hannathai.com) to measure concentration of iron in the sample water. The device transforms the soluble and insoluble iron present in the water sample in ferrous form, which then reacts with the reagent 1, 10 phenanthroline to turn the color of the resultant solution reddish (Hannathai.com).

#### 2.6 Statistical analysis

Concentration of groundwater iron was estimated as mean  $\pm$  SD and median with inter-quartile ranges (IQR) sorted by – the whole samples and the sub-samples-- at concentration <2 mg/L (low), 2-10 mg/L (high), and >10 mg/L (very high). A generalized mixed-effect multilevel linear regression was used considering the fixed effects e.g. “temporal time points”, subgroups of tube wells for the relative magnitude of iron level and the area of residence (i.e. Unions); and the concentrations of iron in groundwater as the dependent variable. The pertinent other covariates in the model were -- pH, temperature and the oxidation-reduction potential (ORP) of the water samples. Covariates, temperature, pH and ORP were considered since these variables are usually associated with concentration of iron (Zhang 2020). Coefficient plot with 95% CIs of the model was prepared for a visual depiction.

### 3. Results

The temporal concentrations of iron in groundwater samples were estimated. In the whole samples, at the start of the assessment (0.0 hours) the mean and median concentrations of iron in the water samples were  $9.1 \pm 7.9$  mg/L and 7.88(2.66, 12.65) mg/L respectively. The concentrations gradually had a downward trend with the 6-hours concentrations being  $4.4 \pm 4.1$  mg (mean) and 3.47(1.05, 9.85) mg/L (median) respectively. The 6-hour concentration was ~44% of the initial concentration. In the “low” subgroup (<2 mg/L), the initial (0.0 hours) concentrations of iron in the water samples were  $0.83 \pm 0.53$  mg/L (mean) and 0.85(0.38, 1.28) mg/L (median). With a gradual decreasing trend, at 6 hours the respective concentrations were  $0.53 \pm 0.39$  mg/L and 0.60(0.12, 0.87) mg/L. The 6 hour average concentration was 70.6% of the initial concentration. Similarly, a gradual decreasing trend of groundwater iron concentration was observed in the “high” and “very high” sub groups. The 6-hour concentrations were 47.1% and 44.7% of the initial concentration in the “high” and “very high” sub groups respectively (Table 1).

Table 1: Unadjusted groundwater iron concentrations over the temporal time-points compared to the initial level (0.0 hours) sorted by the sub-groups and in the whole sample

Combined sample				% of 0.0 hour median value
Whole sample	n	Mean $\pm$ SD	Median(IQR)	
0.0 hours	111	9.1 $\pm$ 7.9	7.88(2.66,12.65)	100
0.5 hours	111	9.37 $\pm$ 8.3	7.58(2.33, 13.0)	96.19
1.0 hours	111	9.19 $\pm$ 8.27	7.3(1.72, 15.15)	92.63
2.0 hours	111	8.59 $\pm$ 7.7	6.8(2.14,13.6)	86.3
3.0 hours	111	7.83 $\pm$ 6.8	6.15(2.05,11.95)	78.04
6.0 hours	97	4.40 $\pm$ 4.14	3.47(1.05, 9.85)	44.03
Sub-sample(<2 mg/L): Low-iron groundwater				
00 hours	26	0.83 $\pm$ 0.53	0.85(0.38,1.28)	100
0.5 hours	26	0.74 $\pm$ 0.49	0.775(0.22, 1.04)	91.17
1.0 hours	26	0.73 $\pm$ 0.48	0.78(0.2, 1.12)	91.76
2.0 hours	26	0.695 $\pm$ 0.49	0.745(0.15,1.07)	87.6
3.0 hours	26	0.70 $\pm$ 0.46	0.745(0.26,1.04)	87.6
6.0 hours	25	0.53 $\pm$ 0.39	0.60(0.12,0.87)	70.58
Sub-samples (2-10 mg/L): High-iron groundwater				
00 hours	46	6.48 $\pm$ 2.4	7.22(3.81,8.55)	100
0.5 hours	46	7.63 $\pm$ 5.6	6.9(3.82,8.04)	95.56
1.0 hours	46	7.64 $\pm$ 5.7	6.74(4.22,8.0)	93.35
2.0 hours	46	7.35 $\pm$ 6.04	6.15(3.74,7.25)	85.18
3.0 hours	46	6.84 $\pm$ 5.8	5.36(3.52,7.35)	74.23
6.0 hours	41	4.12 $\pm$ 3.73	3.4(2.3,4.25)	47.1
Sub-samples (>10 mg/L): Very high-iron groundwater				
00 hours	39	17.71 $\pm$ 6.5	16.75(12.1,20.1)	100
0.5 hours	39	17.19 $\pm$ 6.7	16.0(12.0,22.5)	95.52
1.0 hours	39	16.66 $\pm$ 7.1	15.9(11.8,21.35)	94.92
2.0 hours	39	15.33 $\pm$ 6.1	13.85(10.15,21.15)	82.68
3.0 hours	39	13.75 $\pm$ 5.3	12.15(9.85,18.1)	72.53
6.0 hours	31	7.87 $\pm$ 3.3	7.5(5.55,9.85)	44.75

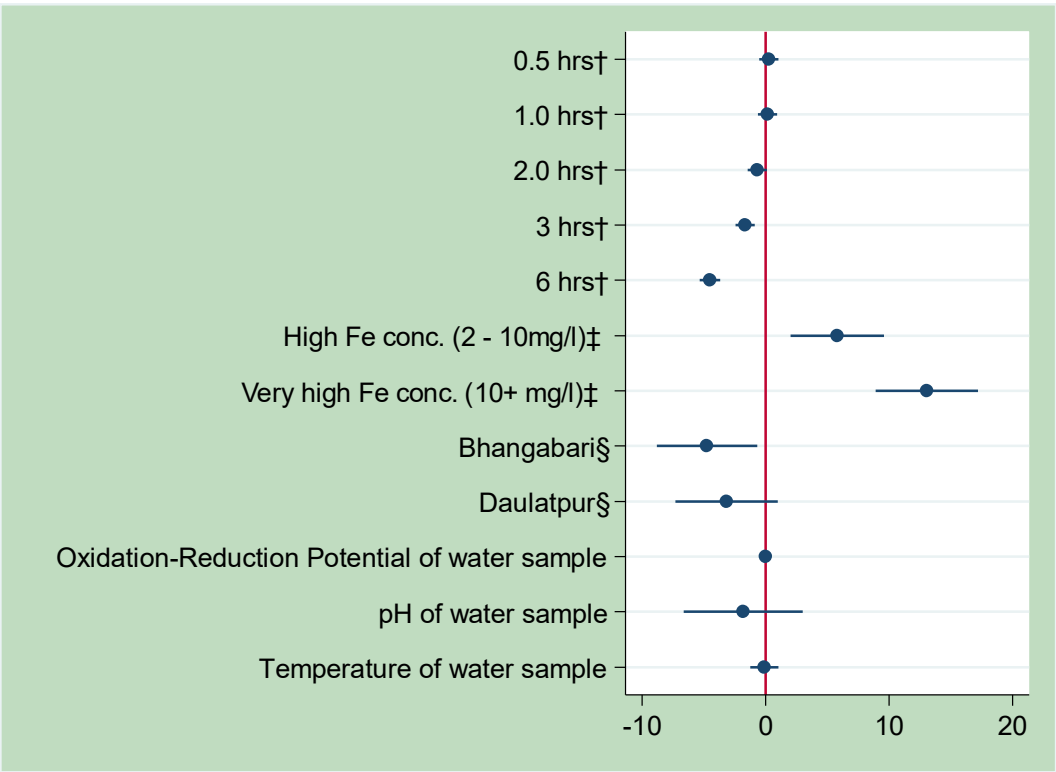
Table 2: Generalized linear mixed effect multilevel regression comparing the groundwater iron concentrations over the temporal time-points relative to the initial iron estimate (0.0 hours)

A. Estimates of Fixed Effects						
<i>Parameter</i>	<i>Estimate</i>	Std Error	z value	p-value	95% CI <i>Lower</i>	Upper
Intercept	21.2	25.4	0.84	0.40	-28.5	71.12
Time-points (Ref: 0.0 hrs)						
0.5 hrs	0.24	0.39	0.62	0.54	-0.53	1.02
1.0 hrs	0.18	0.39	0.45	0.65	-0.60	0.96
2.0 hrs	-0.66	0.39	-1.68	0.09	-1.45	0.11
3.0 hrs	-1.65	0.39	-4.14	0.00	-2.43	-0.87
6.0 hrs	-4.49	0.42	-10.75	0.00	-5.31	-3.67
Iron concentration by subgroups (Ref: Low<2 mg/L)						
High (2- 10 mg/l)	5.80	1.94	2.99	0.003	1.99	9.60
Very high (> 10 mg/l)	13.1	2.11	6.18	0.00	8.94	17.23
Union (Ref: Subornosara)						
Bhangabari	-4.76	2.07	-2.29	0.02	-8.83	-0.68
Daulatpur	-3.15	2.11	-1.50	0.13	-7.29	0.97
ORP	0.003	0.008	-0.39	0.70	-0.02	0.01
pH	-1.89	2.46	-0.73	0.46	-6.62	3.02
Temperature	-0.1	0.58	-0.18	0.85	-1.25	1.04
B. Estimates of Covariance Parameters						
Random-effects Parameters	Estimate	Std Error	Wald Z	p-value	95% CI <i>Lower</i>	Upper
Residual	6.59	0.46	14.19	<0.001	5.74	7.57
Intercept	20.46	3.36	6.08	<0.001	14.82	28.23

\*Covariates in the model

The Table 2 using the generalized linear mixed effect multilevel regression illustrates the effect of the temporality of the time on the follow-up groundwater iron concentrations after adjusting for subgroups of iron level and the area of residence. The adjusted baseline estimate (i.e. intercept) of iron was 21.2 mg/L at the 0.0 hours. Over the next three time points the estimates of the concentration of iron in groundwater were +0.24 mg/L ( $p=0.54$ ; 0.5 hours) and +0.18 mg/L ( $p=0.65$ ; 1.0 hours) and -0.66 mg/L ( $p=0.09$ ; 2 hours) relative to the baseline which were statistically non-significant. The concentration of groundwater iron after 3-hours and 6-hours were lower by 1.65 mg/L ( $p<0.001$ ) and 4.49 mg/L ( $p<0.001$ ) relative to the baseline value. The 3 and 6-hours post-measurements have shown the reduction of iron concentration by 7.7% and 21.1% respectively relative to the baseline estimate. The pH, temperature and ORP of the water samples did not influence on the temporal iron concentrations. Figure 1 shows the relative distribution of the coefficients of the temporal concentration of groundwater iron with 95% confidence interval after adjusting for the area of residence, sub-group level initial iron concentrations, pH, temperature and ORP.

Figure 1: Coefficient plot of the temporal concentrations of groundwater iron with 95% CIs.



†Relative to the reference time (0.0 hrs)  
‡Relative to the low-groundwater iron subgroup (<2 mg/L)  
§Relative to Subornopara Union

#### 4. Discussion

We measured the concentration of groundwater iron at the time of extraction off the wells and at 0.5, 1.0, 2.0, 3.0 and 6.0 hours after. The temporal concentration of iron was estimated in the whole samples; and in the three sub-groups—low iron (<2 mg/L), high iron (2-10 mg/L) and very high iron (>10 mg/L). In all the subgroups and in the whole sample, the unadjusted estimates showed a gradual decrease of iron concentration over time. However, after 6 hours, a considerable amount of the soluble iron was remaining in the samples—44-47% for the whole, high and very high groups; and ~71% for the low-iron group. Interestingly, after 6 hours the median concentration of iron remained 7.5 mg/L (very high iron group), ~3.4 mg/L (high iron group & in the whole samples) which were considerably higher than the FAO/WHO defined level of iron in drinking water ( 2 mg/L) consistent with the upper

tolerable intake from water sources. Furthermore, the multilevel linear modeling of the temporal iron concentrations after adjusting for the fixed effects—subgroups of iron level, area, and the physical parameters of water revealed that significant decline of iron concentration was observed at 3 hours and 6 hours following the extraction. Nonetheless, the declines were modest by 7.7% and 21.1% of the initial iron level respectively. After 6 hours, 79% retention of the iron content in the water samples contravene the popular perception that the iron in drinking groundwater rapidly oxidizes and precipitates soon after it is extracted off the wells.

Aquifer of Bangladesh is inherently rich in soluble iron in reducing form. The underlying reason is a high level of dissolved organic mass (DOM) in the groundwater (Ayres 2016). Groundwater iron and dissolved organic matter (DOM) cycling is microbially mediated via Fe oxide reduction. Mladenov et al explained that DOM at shallow depths of Bangladesh groundwater was characterised by terrestrial (plant/soil) signatures. The study provided evidence to support a dual role of natural DOM in Bangladesh aquifers--- (1) as a labile substrate for Fe- and humic-reducing bacteria and (2) as an electron shuttle via humic substances to enhance microbial iron reduction, and thus enabling solubility of iron (Mladenov 2010).

The colorimetric method uses conversion of ferric iron present in the water sample to ferrous iron; then the ferrous iron complexes with phenanthroline to impart reddish hue to the water sample. Degree of coloring is proportional to the degree of the soluble iron (Hannathai.com). As stated above, the presence of ferric iron in Bangladesh groundwater is negligible at the outset. However, as our samples were kept in open air for a long time, it is assumed that oxidation of the ferrous iron to ferric form had occurred resulting in some precipitation of iron out of the solution. Given the fact that a considerable concentration of iron (which is

consistent with soluble ferrous iron) was measured after several hours, we assume that a substantial amount of oxidized iron (i.e. ferric iron) was made to remain soluble in the water sample over that period. This possibly allowed the reducing agent present in the colorimetric reagents to reduce the ferric into the ferrous state and enabling the measurement of the iron level. It is difficult to understand how the ferric iron possibly remained in the sample solution. The following facts about groundwater characteristics of Bangladesh might shed some lights.

During the transition from ‘confined’ (i.e. underground) to ‘unconfined’ (i.e. expressed off the wells), the change in composition and the redox conditions favors the oxidation of ferrous ( $\text{Fe}^{2+}$ ) to ferric ( $\text{Fe}^{3+}$ ). Thermodynamically,  $\text{Fe}^{3+}$  is expected to precipitate—first, with phosphates/arsenates, and then as ferrihydrite. However, it can still remain within the  $0.4\ \mu\text{m}$  filterable fraction provided that precipitation results in the formation of nanoparticles or colloids, perhaps stabilized by the dissolved organic matter (DOM). For example, fulvic and humic substances can effectively maintain  $\text{Fe}^{3+}$  nanoparticles in solution (or suspension) through a combination of the electrostatic and steric effects of their negatively charged macromolecules. Depending on the Fe/organic-carbon ratio, a fraction of the total Fe may also bind the humic macromolecules and form truly soluble complexes. Equilibrium constants for the formation of  $\text{Fe}^{3+}$  complexes with humic groups are much larger than the corresponding constants for  $\text{Fe}^{2+}$ . Therefore, once these complexes are formed—either by direct interaction between  $\text{Fe}^{3+}$  and DOM or by oxidation of a pre-existing  $\text{Fe}^{2+}$  - DOM complex; the  $\text{Fe}^{3+}$  can resist precipitation and contribute to the elevated concentration of Fe (Jirsa 2013, Muller 2015, Krachlera 2016). This is a possible reason for detecting high concentration of iron after the hours of observation.

This has got implication on the status of iron in populations. In Bangladesh water is predominantly drunk within a short period of pumping (Merrill 2012). Anecdotal experience

suggests that the reason for such habit of water-drinking is the fact that freshly pumped water feels “cool”, “tastes good” and “quenches thirst”. Furthermore, the other factor which might discourage drinking of the stored water is the fact that, if the water is kept in containers for long hours, significant deterioration of the organoleptic properties occur due to oxidation of iron, rendering the water unpleasant to drink. For most of the residents, the tube-wells are located within the vicinity of the household, enabling them not to travel a long distance to collect water. Despite these, in absence of any robust survey on water drinking behavior, we presume that a fair proportion of the population do not drink immediately following the express of the water from the wells; and drink from preserved water in containers over several hours. In the present study, the detection of a high concentration of iron (i.e. soluble in water) after several hours is indicative that a substantial amount of iron has not precipitated out and can be still ingested through drinking of groundwater.

After the ingestion in the gastro-intestinal tracts, iron exists in ferric form under physiologic pH. Iron must be in the ferrous ( $\text{Fe}^{2+}$ ) state to be absorbed. The low pH of gastric acid in the proximal duodenum allows a ferric reductase enzyme, duodenal cytochrome B (Dcytb), on the brush border of the enterocytes to convert the insoluble ferric ( $\text{Fe}^{3+}$ ) to absorbable ferrous ( $\text{Fe}^{2+}$ ) ions (Ems 2020). So, from the findings and discussion, it is explicit that the long time held groundwater sample has not lost all its iron to precipitation; instead the substantial amount of the remaining soluble iron still classify as “high” amount of water iron and can still be consumed and absorbed in the body.

The findings of the study revealed that a tangible effect on the consumption of iron upon the probable storage time, in the children participating in the parent trial was unlikely (Rahman 2019). The parent trial has shown that 85% of the day’s total volume of water was drunk

immediately or within 30 minutes of expressing off the tube wells. The adjusted model of the temporal iron decay showed hardly any changes in the concentration of iron in the water samples within the first 120 minutes. Hence, the effect of holding time on the concentration of iron in water was minimal if any; and thus the intake of the iron from groundwater in the children of the parent trial was hardly affected.

The parent trial (Rahman 2019) studying the efficacy of a low-iron MNP, reported high baseline level of serum ferritin and the reserve of the body iron in the 2-5 year-old children. The trial noted that the median concentration of groundwater iron was 4 mg/L; and the reserve of the body-iron at baseline was 560 mg (Standard MNP group) and 548.8 mg (low-iron MNP group). Compared to the net cost of 580 mg iron during the duration of pregnancy (Bothwell 2000), the reserve of iron in the children appears substantial for their age. This adequacy of the reserve of body-iron was observed despite the dietary intake of iron was below the EAR for the age; and being the cereal based diet had a poor absorption potential. Similarly, the present study reported after 6 hours of holding, the median concentration of groundwater iron of ~3.5 mg/L in the whole sample which is nearly similar to the parent trial. Woorwood et al (1996) suggested that the average absorption of iron from iron-rich natural water was ~23%. Therefore, it is apparent from the discussion that the remaining level of iron at 6- hours is consistent with the build up a good body iron status.

In conclusion, the temporal concentrations of groundwater iron samples were statistically indifferent over the two-hour period after the extraction. The temporal concentrations of water iron at the 3-6-hours post-extraction were significantly lesser than the 0.0 hour samples. However, the concentrations were maintained higher than the level consistent with “high” level of groundwater iron. This level of iron is consistent with a fair amount of intake

of iron from the groundwater sources. The study consolidates the potential of the contribution of groundwater iron in developing the population's iron status irrespective of some time lag of the consumption after the extraction off the wells.

#### Author's contributions

Sabuktagin Rahman: Conceptualization, Methodology, Data curation, Data analysis, Original draft preparation; Patricia Lee: Conceptualization, Data analysis, Writing-Reviewing and Editing; Moudududur Rahman Khan: Data Supervision; Faruk Ahmed: Conceptualization, Methodology, Data analysis, Writing- Reviewing and Editing.

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## 8.4 ANNEX 4: ETHICAL CLEARANCE

**Professor Dr. M. Imdadul Hoque**

**Dean**

Faculty of Biological Sciences

The University of Dhaka

Dhaka-1000, Bangladesh



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**Fax: (+880-2)-8615583**

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Ref. ...~~46~~...../Biol.Sc./2017-2018

Date: 16.07.2017

### **Ethical Review Committee**

**Professor Dr. Khan Moududur Rahman**

Institute of Nutrition and Food Science

University of Dhaka

**Sub: Ethical Clearance.**

**Dear Professor Moududur Rahman,**

With reference to your application on the above subject, this is to inform you that your research proposal entitled "**Efficacy of micronutrient powder formulation with low-dose iron in Bangladeshi children living in areas of high iron in groundwater**" has been reviewed and approved by the Ethical Review Committee of the Faculty of Biological Sciences, University of Dhaka.

I wish for the success of your research project.

A handwritten signature in black ink, likely belonging to Professor Dr. M. Imdadul Hoque.

**Professor Dr. M. Imdadul Hoque**

Dean, Faculty of Biological Sciences

University of Dhaka

Dhaka-1000.

## 8.5 ANNEX 5: CONSENT FORM



### Efficacy of Micronutrient Powder (MNP) with a low-dose of iron in children living in the areas with high level of iron in groundwater

Institute of Nutrition and Food Science, University of Dhaka

Griffith University, Australia

Greetings. My name is..... I am here to conduct a research on behalf of the Institute of Nutrition and Food Science, University of Dhaka and Griffith University, Australia.

What research and why it is important?

Childhood anemia is a major public health problem of Bangladesh. As the consequences, the child's development of intelligence is hindered; the child gets tired readily and cannot concentrate in studies. In order to have a remedial of this problem, the government of Bangladesh has taken various initiatives. Under such initiative, the University of Dhaka and Griffith University have jointly undertaken a research to test the usefulness of a nutrient-formulation containing a low dose of iron. If this research project turns out to be successful, it will benefit the national childhood anemia control policy. This might improve the health of millions of young children of Bangladesh.

What will we do if you allow your child to take part in this research?

If you consent us to allow your child to take part in the research,

- a. We shall collect some pertinent data regarding you and your child—such as your occupation, employment, education, wealth status of household, household expenses, dietary intake of your child, household food security etc.
- b. Your child's weight and height will be taken twice—during baseline and the endpoint data collection. Information about the common illnesses suffered by her will be collected.
- c. If your child is selected for iron status assessment and thalassemia screening, we shall collect 3.5 ml of blood from the vein (equivalent to less than a teaspoonful) during baseline and the endpoint. We shall ensure standard aseptic arrangement. The piercing of the vein will cause some pain. The phlebotomy will be done by an experienced medical technologist who will attempt to minimize the pain and comfort the child.

- d. If your child is selected for gut bacteria (microbiota) assessment, a small amount of stool sample will be collected at baseline and endpoint after the child defecates. The process will not cause any discomfort.
- e. The tube well you use for drinking water shall be tested for iron and arsenic at free of cost
- f. The child will be provided with 60 sachets of nutrient-powder for consumption over 60 days. You will be taught how the nutrient powder is mixed with your child's food (rice) and fed.
- g. Every week, your child will be visited by our field personnel for assessment of the consumption and top up the additional sachets. She will inquire the health of your baby. Should your child experience any common illnesses, she will be referred to our project physician who will treat your baby free of cost.

If you do not participate in the research

You have the right not to participate in the research. After the enrollment you may withdraw your child from the research at any point if you wish.

Is there any benefit to your child for participating in the research?

You will know the nutritional status of your child. If selected for assessment, you will immediately know if your child is suffering from anemia. If your child suffer from illnesses, such as fever, cough and cold, diarrhea, loose stool and common pediatric illnesses, she will be provided treatment free of cost by our project physician.

Confidentiality of information

We shall try to the best of our efforts to keep the information you provide confidential. The data you provide will be disseminated in aggregated form. The name of you and your child will never be revealed to anyone except a few key researchers of the project. All data will be preserved in a safe almirah and be kept under lock and keys.

Use of data

The results of this research will be disseminated to the national policymakers, academics and researchers which might pave the way for an improved national program to control childhood anemia. Nonetheless, the confidentiality of your child's identity will be maintained.

For inquiries

If you want to know further about this research or you have any concern, you are free to contact Dr. Faruk Ahmed Assoc. Professor, Griffith University (email: [f.ahmed@griffith.edu.au](mailto:f.ahmed@griffith.edu.au)) or Prof. Moududur Rahman Khan, Institute of Nutrition and Food Science, University of Dhaka (email: [khan.moudud@gmail.com](mailto:khan.moudud@gmail.com); phone: 96611772).

I have been adequately informed about the research. I consent to allow my child's participation in the research and permitting the collection of the pertinent data including the blood and/or stool samples.

---

Signature/thumb impression of the mother/father/legal guardian of the child

Date.....

---

Signature/thumb impression of the witness

Date.....

---

Signature of the interview-taker/representative of the site-PI

Date.....

## 8.6 ANNEX 6: QUESTIONNAIRE (RCT)



### Study Questionnaire

<b>Efficacy of micronutrient powder formulation with low-dose of iron in Bangladeshi children living in areas of high level of iron in groundwater.</b>	
Study questionnaire (baseline=1, end line=2) <span style="float: right;"><input type="checkbox"/></span>	
<b>Household Identification</b>	
Upazilla..... <input type="checkbox"/>	Union/Ward..... <input type="checkbox"/>
Village..... <input type="checkbox"/>	Para..... <input type="checkbox"/>
Household Identification: <span style="float: right;"> <input type="checkbox"/><input type="checkbox"/><input type="checkbox"/><input type="checkbox"/><input type="checkbox"/><input type="checkbox"/> </span>	
Name of the Household Head.....	Date of data collection: DD/MM/YY <span style="float: right;"> <input type="checkbox"/><input type="checkbox"/> / <input type="checkbox"/><input type="checkbox"/> / <input type="checkbox"/><input type="checkbox"/> </span>
Cell Phone No.....	Name of the cell phone bearer.....
Name of the Interviewer.....	Signature:.....
Name of the Supervisor.....	Signature:.....
Written informed consent: <span style="margin-left: 100px;">1=Yes, 0=No</span> <span style="float: right;"><input type="checkbox"/></span>	
Result code: Complete=1, Incomplete=0 <span style="float: right;"><input type="checkbox"/></span>	
Reason, the data collection was not completed..... ..... .....	

	QUESTIONS	CODING CATEGORIES	Code box
1a	What is your name (respondent)?		
1b	Relationship with household Head? 0=Wife, 1= Daughter-in-law, 2= Daughter, 3=Sister, 4=Self, 5=Others (Please specify.....)		<input type="text"/>
2	What is the religion practiced by most of the people who live in this household?  (Mark only one answer)	Islam=0 Hinduism=1 Christianity=2 Buddhism=3 Others=5 Don't know =99  (Please specify.....)	<input type="text"/> <input type="text"/>
	QUESTIONS	CODING CATEGORIES	Code box
3	What is the main occupation of the head of the household?	Professional/technical=0 Business=1 Factory worker=2 Service=3 Skilled labour/service=4 Unskilled labour=5 Farmer/agricultural worker=6 Poultry/cattle raising =7 Home based manufacturing=8 Domestic help=9 House wife=10 Others=77 (Specify)	<input type="text"/> <input type="text"/>
4	Which stream of education did you obtain?	General=0, Madrasha=1	<input type="text"/> <input type="text"/>
5	How many years of formal education did you attain?		<input type="text"/> <input type="text"/>
6	What is the main source of drinking water for the members of your household?	Supply tap=0 Tube well=1 Well=2 Overhead tank=3 Surface water (River/dam/lake/pond/ stream/canal/irrigation channel) =4 Others=77 (Please specify.....)	<input type="text"/> <input type="text"/>
7	What kind of toilet facilities do the members of your household usually use?  (Observe yourself)	Flushed to piped sewer system=0  Flush to septic tank=1  Pit latrine with slab=2  Pit latrine without slab/open pit=3  Bucket toilet=4  Hanging toilet=5	<input type="text"/> <input type="text"/>

		No facility/bush/field=6  Others=77  (Please specify.....)	
8	What type of household do you live in?	Kachcha=0, <i>[floor: soil; wall: soil/jute-stick/straw/shrub/bamboo; roof: tin/straw]</i>  Kachcha-tin=1, <i>[floor: soil; wall: tin; roof: tin]</i>  Semi-pacca=2 <i>[floor: cement; wall/roof: tin]</i>  Pacca=3 <i>[cement all]</i>	<input type="checkbox"/>
9	Does the Household owned by the household head?	Yes=1  No=0	<input type="checkbox"/>
10a	Does the Household own any cultivable land?	Yes=1  No=0	<input type="checkbox"/>
10b	If yes, how much cultivable land does the household possess (cultivable/fallen)?	(Decimals)	<input type="text"/> <input type="text"/> <input type="text"/>
11	Does your household possess the following assets?		
	a. Electricity?	Yes=1, No=0	<input type="checkbox"/>
	b. Radio/ cassette player/DVD/CD player?	Yes=1, No=0	<input type="checkbox"/>
	c. Television?	Yes=1, No=0	<input type="checkbox"/>
	d. Mobile phone?	Yes=1, No=0	<input type="checkbox"/>
	e. Land phone?	Yes=1, No=0	<input type="checkbox"/>
	f. Electric fan	Yes=1, No=0	<input type="checkbox"/>
	g. Refrigerator?	Yes=1, No=0	<input type="checkbox"/>
	h. Almirah/wardrobe?	Yes=1, No=0	<input type="checkbox"/>

	i. Table?	Yes=1, No=0	<input type="checkbox"/>
	j. Chair?	Yes=1, No=0	<input type="checkbox"/>
	k. Watch?	Yes=1, No=0	<input type="checkbox"/>
	l. Bicycle?	Yes=1, No=0	<input type="checkbox"/>
	m. Motor cycle/scooter/tempo?	Yes=1, No=0	<input type="checkbox"/>
	n. Jewelry?	Yes=1, No=0	<input type="checkbox"/>
	o. other?	Yes=1, No=0	<input type="checkbox"/>
12	In the last week, how much money did you spend on food?  (rice, wheat, oil, sugar, salt, fish, egg, meat, grocery etc)	(In BDT.)	<input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/>
	Hand washing behaviour		
13a	Before feeding the child, do you wash your hands?	Yes=0, no=1	<input type="checkbox"/>
13b	If yes, what you use to wash hands?	Soap and water=0, Ash and water=1 Soil and water=2, Only water=3 Others=77,.....specify Not applicable=88, don't know=99	<input type="text"/> <input type="text"/>
14a	After the toilet, do you wash your hands?	Yes=0, no=1	<input type="checkbox"/>
14b	If yes, what you use to wash hands?	Soap and water=0, Ash and water=1 Soil and water=2, Only water=3 Others=77,.....specify Not applicable=88, don't know=99	<input type="text"/> <input type="text"/>
15a	Will you show me where the members of this household wash their hand?	Observed=1 Not observed as permission not obtained=2 Not observed for other reasons=3	<input type="checkbox"/>
15b	Is water available here?	Yes=1, no=0	<input type="checkbox"/>

15c	Is soap, detergent or other cleaning agent available here?	Soap(bar, liquid, paste)=1, Detergent(bar, liquid, powder)=2 Ash/mud=3 None=4	<input type="checkbox"/>
15d	How much money do you spend on soap/detergents per month?	.....(BDT.)	
Household food insecurity I would now like to ask you some questions about the amount of food available for members of your household.			
16	In the past four weeks, did you worry that your household would not have enough food?	Yes=1 No=0	<input type="checkbox"/>
16a	How often did this happen?	Once or twice in the past four weeks (Rarely)=1 Three to ten times in the past four weeks (Sometimes)=2 More than ten times in the past four weeks (Often)=3	<input type="checkbox"/>
17	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?	Yes=1 No=0	<input type="checkbox"/>
17a	How often did this happen?	Once or twice in the past four Weeks( Rarely)=1 Three to ten times in the past four weeks (Sometimes)=2 More than ten times in the past four weeks (Often)=3	<input type="checkbox"/>
18	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?	Yes=1 No=0	<input type="checkbox"/>
18a	How often did this happen?	Once or twice in the past four weeks (Rarely)=1	<input type="checkbox"/>

		Three to ten times in the past four weeks (Sometimes)=2  More than ten times in the past four weeks (Often)=3	
19	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?	Yes=1 No=0	<input type="checkbox"/>
19a	How often did this happen?	Once or twice in the past four weeks (Rarely)=1  Three to ten times in the past four weeks (Sometimes)=2  More than ten times in the past four weeks (Often)=3	<input type="checkbox"/>
20	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?	Yes=1 No=0	<input type="checkbox"/>
20a	How often did this happen?	Once or twice in the past four weeks (Rarely)=1  Three to ten times in the past four weeks (Sometimes)=2  More than ten times in the past four weeks (Often) =3	<input type="checkbox"/>
21	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?	Yes=1 No=0	<input type="checkbox"/>
21a	How often did this happen?	Once or twice in the past four weeks (Rarely)=1  Three to ten times in the past four weeks (Sometimes)=2  More than ten times in the past four	<input type="checkbox"/>

		weeks (Often) =3	
22	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?	Yes=1 No=0	<input type="checkbox"/>
22a	How often did this happen?	Once or twice in the past four weeks (Rarely)=1  Three to ten times in the past four weeks (Sometimes)=2  More than ten times in the past four weeks (Often) =3	<input type="checkbox"/>
23	In the past four weeks, did you or any household member go to sleep at night hungry because there was not enough food?	Yes=1 No=0	<input type="checkbox"/>
23a	35a. How often did this happen?	Once or twice in the past four weeks (Rarely)=1  Three to ten times in the past four weeks (Sometimes)=2  More than ten times in the past four weeks (Often) =3	<input type="checkbox"/>
24	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?	Yes=1 No=0	<input type="checkbox"/>
24a	How often did this happen?	Once or twice in the past four weeks (Rarely)=1  Three to ten times in the past four weeks (Sometimes)=2  More than ten times in the past four weeks (Often) =3	<input type="checkbox"/>

Particulars of the child		
25	Child's ID	<input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/>
26	Name of the child.....	
27	What is the date of birth of the child (name)? Day/month/year	<input type="text"/> <input type="text"/> <input type="text"/> / <input type="text"/> <input type="text"/> <input type="text"/> / <input type="text"/> <input type="text"/>
28	How old is s/he? (completed months)	<input type="text"/> <input type="text"/>
29	Sex of the child	Male=1, Female=2 <input type="text"/> <input type="text"/>
30	What is your relationship with the child?	Mother=1, Grandmother=2, Sister=3, Aunt=4, Other=5(specify..... ...) <input type="text"/>

Morbidity of the child			
31	Did the child (Name) have diarrhoea in last 2 weeks?	Yes=1, No=0, Don't know =99	<input type="text"/> <input type="text"/>
<i>Diarrhoea: 3 or more watery or loose/liquid stool in last 24 hours</i>			
32	"Has (Name) been ill with a fever at any time in the last 2 weeks?"	Yes=1, No=0, Don't know=99	<input type="text"/> <input type="text"/>
33	In the past two weeks has (Name) had an illness with a cough, and fast breathing or difficult breathing?	Yes=1, No=0, Don't know=99	<input type="text"/> <input type="text"/>
<i>Difficult breathing: chest depressed and/or peculiar sound while breathing.</i>			
34	Was the fast or difficult breathing due to a problem in the chest or to blocked or runny nose?	Yes=1, No=0, Don't know =99	<input type="text"/> <input type="text"/>
<i>Inquire whether the nose was blocked or there was watering from nose.</i>			
35	Did (Name) have measles in last 6 months?	Yes=1, No=0, Don't know =99	<input type="text"/> <input type="text"/>
36	Does (Name) have an EPI card?	Yes=1, No=0, Don't know =99	<input type="text"/> <input type="text"/>
37	( Ask this question if the child is 2 years or more)  Did the child (Name) receive anti-helminthic tablet/syrup in last 6 months?	Yes=1, No=0, Don't know=99 N/A= 88	<input type="text"/> <input type="text"/>
38	Did the child receive vitamin A over last 6 months?	Yes=1, No=0, Don't know=99	<input type="text"/> <input type="text"/>
39	Did the child receive any antibiotics for illness since the last interview? (Observe the prescription, medicine bottles/packs/strips)	Yes=1, No=0, Don't know=99	<input type="text"/> <input type="text"/>
40	Did the child receive any MNP or iron supplementation since last interview? (Show the mother the sample of MNP/supplements)	Yes=1, No=0, Don't know=99	<input type="text"/> <input type="text"/>

Breastfeeding status			
41	Has (Name) ever been breastfed?	Yes=1, No=0, Don't know=99	<input type="checkbox"/> <input type="checkbox"/>
42	How long after birth did you first put (name) to the breast?	If Immediately (check 00)  If hours (check exact number of hours)  If days( check exact number of days)	<input type="checkbox"/> <input type="checkbox"/>
43	Did you give colostrum to the child (name)?	Yes=1, No=0, Don't know=99	<input type="checkbox"/> <input type="checkbox"/>
44	Did you put anything in (child's name) mouth before first breast milk?  (E.g. sugar or honey)?	Yes=1, No=0, Don't know=99	<input type="checkbox"/> <input type="checkbox"/>
45	Did you put anything in (name's) mouth within 3 days after birth?	Yes=1, No=0, Don't know=99	<input type="checkbox"/> <input type="checkbox"/>
46	Did you put any food or drink in (name's) mouth within 6 months after birth?  (probe for water)	Yes=1, No=0, Don't know=99	<input type="checkbox"/> <input type="checkbox"/>
47	At what age did you first put anything (food/drink) in (name's) mouth outside breast milk	Days (if) [put actual days in box]  Months(if)[put actual months in box]	<input type="checkbox"/> <input type="checkbox"/>  <input type="checkbox"/>
48	For how many days/months did you breast-feed the baby?	Days(if) [put actual days in box]  Months(if) [put actual months in box]	<input type="checkbox"/> <input type="checkbox"/>  <input type="checkbox"/>
49	What was the type of breastfeeding?	1=Exclusive 2=Predominant	<input type="checkbox"/>
50	Is the child still breastfed?	Yes=1, No=0	<input type="checkbox"/>
50a	If no, at what age did you stop breastfeeding? (Days)	Days:..... 99=NA	<input type="checkbox"/> <input type="checkbox"/>

# Dietary intakes assessment using a seven-day semi-quantitative

Now I would like to ask you some questions about the food eaten by your child. I know this is sometimes hard to remember, but please give me the best answer you can.

51	During the past 7 days, on how many days did your child (Name) eat the following foods?					
	Foods	Serving size	# of days in last 7 days	# of servings in last 7 days	# of servings per day	gm/ml
	1. Rice?	1 cup	<input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>
	Breads					
	2. Chapatti?	2 pieces	<input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>
	3. Bread?	2 slices	<input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>
	4. Parata?	1 piece	<input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>
	Fish					
	5. Small fish (with bones)?	60 gram	<input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>
	6. Big fish (boneless)?	30 gram	<input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>
	7. Egg?	One	<input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>
	8. Pulse?	½ cup	<input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>
	9. Potato	75 gm or 1/2 of medium sized	<input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>
	Green Leafy Vegetables (Shak):					
	10. Puishak?	½ cup	<input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>
	11. Palongshak ?	½ cup	<input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>
	12. Lal shak	½ cup	<input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>
	13. Kalmi shak	½ cup	<input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>

14. Paatshak?	½ cup	<input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>
15. Kochushak?	½ cup	<input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>
16. Shorishashak?	½ cup	<input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>
17. Moolashak?	½ cup	<input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>
18. Others (specify.....)	½ cup	<input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>
Yellow/orange vegetables/fruit					
19. Carrots	½ cup	<input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>
20. Ripe mango	½ cup	<input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>
21. Sweet Pumpkin	½ cup	<input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>
22. Ripe jackfruit	½ cup	<input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>
23. Ripe papaya	½ cup	<input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>
24. Tomato	½ cup	<input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>
25. Sweet potato	½ cup	<input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>
26. Orange	½ cup	<input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>
27. Water melon	½ cup	<input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>
28. Banana	½ cup	<input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>
29. Others (Specify.....)		<input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>
Meats					
30. Chicken	60 gram	<input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/>

						<input type="checkbox"/> <input type="checkbox"/>
	31.Beef	60 gram	<input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>
	32.Mutton	60 gram	<input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>
	33.Liver	60 gram	<input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>
	Milk and milk products					
	34.Milk	1 cup	<input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>
	35.Yogurt	½ cup	<input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>
	36.Cheese	Measure of a thumb	<input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>
	37.Sugar, honey, molasses	1 tbsf	<input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>
	38. Beans, nuts	½ cup	<input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>
	Junk foods					
	39.Candy	28 gm	<input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>
	40.Chocolate	30 gm	<input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>
	41.Cake	58 gm	<input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>
	42.Potato chips	100 gm	<input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>
	43.Biscuit (sweet)	100 gm	<input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>
	44.Mango juice	½ packet	<input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>
	45.Ice cream	100 gm	<input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>

Water analysis for iron and water usage behaviour				
52. Tube well ID			<input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/>	
53. Who owns the tube well?	0=Public/Institute (.....) 1=Personal 2=Shared 3=Relative 4=Neighbour 5=NGO (.....) 6=Other (Specify.....) 99=Don't know		<input type="text"/> <input type="text"/>	
54. What year was it installed?	<input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/>	year	9999 (Don't know)	
55. What is the depth of the tube well in feet?	<input type="text"/> <input type="text"/> <input type="text"/>	feet	9999 (Don't know)	
56. How much iron do you think is in the water that you pump from this drinking tube well?	0=None, 1=A little, 2=A medium, 3= A lot, 99=Do not know		<input type="text"/> <input type="text"/>	
57. Temperature of the water[C]	<input type="text"/> <input type="text"/> . <input type="text"/>	99.9 (Not read)		
58. Ph of the water	<input type="text"/> <input type="text"/> . <input type="text"/>	99.9 (Not read)		
59. Oxidation-Reduction Potential	(-)=0, (+)=1, Not read=9 <input type="text"/>	<input type="text"/> <input type="text"/> <input type="text"/>	Eh=0-998 Not read=999	
Iron concentration in water				
60. At the tube well spout	<input type="text"/> <input type="text"/> . <input type="text"/>			
61. For how long have your household used this tube well for drinking water?	<input type="text"/> <input type="text"/>	year	<input type="text"/> <input type="text"/>	month
62. I will now ask you about the container you use to drink your child water. Can you show me what container you usually used in the past 7 days to drink your child water collected from your tube well?	0=No, 1= Hand, 2= Glass, 3= Mug, 4= Bowl, 5= Jug, 6= Plate, 8= Other, Specify..... 9= Don't know		<input type="text"/> <input type="text"/>	
62a. Record the material.	1= Glass, 2= Plastic, 3= Melamine, 4= Aluminium, 5= Brass, 6= Steel, 8= Other, Specify :..... 9= Don't know		<input type="text"/> <input type="text"/>	
62b. Record the size of the container [Measure with the aid (container) provided with you]	001 - 500 = mL 501= 501 or more mL 555 = 1 L / l kg 999 = Unmeasured		<input type="text"/> <input type="text"/> <input type="text"/>	

<p>I will now ask you about how many usual CONTAINERS of water did your child drink, when s/he woke up yesterday until today when s/he woke up.</p> <p>[Guideline for the enumerator: Please guide the respondent in the following manners to respond in order to facilitate her in the recall of the drinking of water by her child].</p> <p><i>a) Miss.....please, think about when your child first woke up yesterday. Did she drink water at that time? If yes: how many of the containers? If she drank a portion of or a multiple of containers, report as such, i.e. 0.5 or 0.75 or 1.5 containers. Did she drink water at the time of breakfast? If yes, how many of the containers? Did she drink water between breakfast and lunch? If yes, how many containers? Continue asking so until the last time prompt between dinner and waking up the next morning. Also, inquire if the child drank direct, short-stored or a long-stored water.</i></p>					
<p>I will now ask you about how many USUAL CONTAINERS your child drank yesterday, when s/he woke up yesterday until today when s/he woke up.</p>					
<p>63. Yesterday TIME (a - f) how many USUAL CONTAINERS did your child drink?</p>					
	Total	Direct <sup>3</sup>	Filtered	Short stored <sup>1</sup>	Long stored <sup>2</sup>
	00-99=number 66.6=refused 99.9=don't know				
a. With the morning meal	<input type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>
b. Between the morning meal and lunch	<input type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>
c. With lunch	<input type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>
d. Between lunch and dinner	<input type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>
e. With dinner	<input type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>
f. After dinner until morning meal	<input type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>
<sup>1</sup> Short stored: stored for 5-<30 minutes after pumping off the well <sup>2</sup> Long stored: stored for >=30 minutes after pumping off the well <sup>3</sup> Direct: consumed within 5 minutes of pumping off the wells					
64. Do you filter the water you drink	0=Yes, 1=No		<input type="text"/>		
64a. When you filtered your water, what type of filter did you <i>usually</i> use? (observation)	1= Home made 2= Purchased, Specify :.....);brand 3= Other, Specify		<input type="text"/>		

	:.....) 99= Don't know		
65. Can you show me the jug, pot or whatever you usually used in the past 7 days to store drinking water collected from your tube well for a long period of time?	00= No show,01= Clay/mud,02= Glass,03= Plastic, 04= Melamine,05= Aluminum,06= Brass; 07= Stainless steel, 08= Iron  09= Tin, 10= Cement, 88= Other, specify:_  99= Don't know	□□	
Anthropometry of the child			
	1 <sup>st</sup> time[a]	2 <sup>nd</sup> time[b]	Average[c]
66. Weight of the child (kg)?	□□.□□	□□.□□	□□.□ □
67. Height of the child (cm)?	□□□.□	□□□.□	□□□. □
Biological parameters of the child			
		Stipulated for sample collection	Sample collected
68. Haemoglobin(mark √/× as appropriate)		a.□	b.□
	Sample ID	c.□□□□□□□	
	Haemoglobin (gm/l)	d.□□.□	
69.Congenital Hb disorders(mark √/× as appropriate)		[Applies only at baseline]	
	Sample ID	a.□	b.□
	Sample ID	c.□□□□□□□	
70. S. ferritin, CRP, AGP, sTfR(mark √/× as appropriate)		a.□	b.□
	Sample ID	c.□□□□□□□	
71.Gut microbiome (stool) (mark √/× as appropriate)		a.□	b.□
	Sample ID	c.□□□□□□□	



Biological Sample Collection Form



# Biological Sample Collection Form

[illegible]

## 8.8 ANNEX 8: BIOLOGICAL SAMPLE TRANSFER FORM



Biological Sample Transfer Form



Efficacy of micronutrient powder with a low dose of iron in children  
living in the areas with a high level of iron in groundwater

Biological Sample Transfer Form

Form- A

Date:

Unique Sl.#	Child ID	Sex	Age	Sample ID	Sample type	Remark

Form- B		
Medical Technologist (MT)		
Collection date	Collection time	
Delivery to FA	Time	Date
Name of MT	Signature	
Field Attendant (FA)		
Travel date	Delivery time	
Name of FA	Signature	
Laboratory personnel 1		
Time of receipt	Date of receipt	
Name	Signature	
Laboratory personnel 2		
Time of receipt	Date of receipt	
Name	Signature	

#### Directives

- Form A will be filled in by the field medical technologist.
- Form B will be filled in by the field medical technologist, sample transport personnel and the laboratory representative in the respective spaces.
- Two copies of the forms will be prepared. One copy will remain at the field office. The other copy after obtaining the acknowledgement of receipt of the laboratory representative will be returned and stored at the field office.

## 8.9 ANNEX 9: DATA FORM ON GROUNDWATER PARAMETERS

Efficacy of Micronutrient Powder (MNP) with a low-dose of iron in children living in the areas with high level of



iron in groundwater



[Data on Groundwater Parameters]

S l. #	Household ID	Name of the child	Child's ID	Name of the father	Tube-well ID	Iron ( mg/l )	Arsenic ( ppm )	ORP ( mV )	pH	Temperature ( C )	Comment

## 8.10 ANNEX 10: DATA FORM ON THE TEMPORAL IRON MEASUREMENT OF GROUNDWATER



Efficacy of Micronutrient Powder with a low-dose of iron in children living in the areas with high level of iron in groundwater: Serial Iron Measurement Sub Study

Log Sheet

Date:

00 min	time	Feconc.	30 min	time	Feconc.	1 hr	time	Feconc.	2hr	time	Feconc.	3 hr	time	Feconc.	6 hr	time	Feconc.

## 8.11 ANNEX 11: WEEKLY MNP CONSUMPTION MONITORING



### Efficacy of Micronutrient Powder (MNP) with a low-dose of iron in children living in the areas with high level of iron in groundwater

#### Weekly MNP Consumption Monitoring

Child Name:

Child ID:

Category:

Start Date:

Ending:

SL	Date of Disbursement	Top Up		Consumed		Return of MNP Empty		If not consumed, Note	Remarks
		week	Cumulative	week	Cumulative	week	Cumulative		
01									
02									
03									
04									
05									
06									
07									
08									
09									
	Total								

Name of Monitor:

Name of RC:

## 8.12 ANNEX 12: FIELD PERSONNEL



Efficacy of Micronutrient Powder (MNP) with a low dose of Iron  
children living in the areas high level of iron in ground water

### Staff Information

Name	Designation
Mr. Sabuktagin Rahman	Site-Principal Investigator
Mr. Faruk Hossain	Field Coordinator
Ms. Mehnaz Pervin	Research Assistant
Mr. Sajjad Hossain	Medical Technologist
Mr. Imrul Hassan	Research Assistant
Ms. Nigat Sultana	Research Assistant
Mr. Siddiqur Rahman	Data Management Assistant
Ms. Ety Khatun	Research Assistant
Ms. Minara Akter	Research Assistant
Ms. Sumiaya Akter	Research Assistant
Mr. Nazrul Islam	Research Assistant
Mr. Dulal Gazi	Field Attendant
Mr. Raihan	Field Attendant
Mr. Zahidul Islam	Field Attendant
Ms. Taslima Khanom	Research Assistant
Mr. Iqbal Hossain	Research Assistant
Mr. Sujoy Mondol	Field Attendant