Dietary diversity through a mungbean meal for improving iron status in India

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Abstract

Background: Worldwide, iron deficiency anemia is more prevalent than any other health problem. Iron deficiency is for a large part preventable with appropriate and timely intervention (Kotecha, 2011). **Objective:** To assess the effect of an iron-rich meal combined with different doses of vitamin C on the iron status of primary school children between the age of 6 and 12 years old, who are iron deficient, in rural India. **Hypothesis**: The main hypothesis of this study is that eating guava, which is rich in vitamin C, in combination with mungbean, which is rich in iron, will improve the iron status of the children. **Study outcome: T**he primary outcome of this study is iron status, assessed by means of serum ferritin, hemoglobin and ZPP concentration. The secondary outcome is change in spontaneous physical activity measured by accelerometers in a sub-sample of the study population (n=30). This method is validated using observation methods. **Method:** A single-blind randomized controlled trial is set up with 3 treatment groups among 175 children. Each treatment receives Indian Dahl with 300 grams of mungbean in combination with either 0 grams of guava (control), 100 grams of guava or 200 grams of guava. **Analysis Plan:** A repeated measures ANCOVA test will be done to determine if there is a change in iron status between the different treatment groups over time for serum ferritin, hemoglobin and ZPP. Secondly, a binary logistic regression will be performed to compare the effects between the different treatment groups for iron deficiency anemia, iron deficiency and normal iron status. To measure changes in spontaneous physical activity, repeated measures ANCOVA will be conducted. **Results & Conclusion:** It is expected that the treatment effect is most pronounced in the group that receives 200 grams of guava since increasing doses of vitamin C counteracts the negative effect of phytate present in mungbean.

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Introduction

Worldwide, iron deficiency anemia is more prevalent than any other health problem, and affects health, education, economy, and productivity of an entire nation (Kotecha, 2011). Iron deficiency is for a large part preventable with appropriate and timely intervention (Kotecha, 2011).

Iron deficiency anemia is associated with poor cognitive and motor development, as well as with behavioral problems (Grantham-McGregor & Ani, 2001). Furthermore, longitudinal studies consistently indicate that children that were anemic during infancy continue to have poorer cognition, school achievement and more behavior problems into middle childhood (Grantham-McGregor & Ani, 2001). A study conducted in school-aged children and adolescents in the US showed lower standardized math scores among iron-deficient school-aged children and adolescents (Halterman, 2001). Iron deficiency can be defined as "a reduction in body iron beyond the point of depleted iron stores" (Skikne, Flowesr & Cook, 1990).

India is one of the countries with the highest prevalence of anemia in the world, with a prevalence of iron deficiency anemia of 75% in children under 5 years of age (Nutrition Foundation Of India, 2007). Different factors contribute to this high prevalence, like a "low dietary intake of iron rich foods, poor bioavailability of iron in the phytate and fiber rich Indian diet, and chronic blood loss due to infection" (Nutrition Foundation Of India, 2007).

To combine the intake of vitamin C and iron might be a promising strategy to increase the iron status of children in India. Vitamin C is shown to induce a marked increase of iron absorption in foods rich in phytates (Hallberg, 1989). Mungbean is the third most important legume crop of India and has a high iron content, but also contains anti-nutritional factors like phytate, that reduce the bioavailability of iron. Furthermore, guava is one of the three major tropical fruits produced in India, and has a high vitamin C content (FAO, 2005). To combine mungbean with a vitamin C rich food like guava (which are both locally produced food crops in Haryana, India) may therefore enhance iron uptake and contribute to a better iron status.

Objective

The main objective is to assess the effect of an iron-rich meal combined with different doses of vitamin C on the iron status of primary school children between the age of 6 and 12 years old, who are iron deficient, in rural India. The study is conducted in children living in the rural areas in the Haryana state in Northern-India. The secondary objective is to assess the effect of an iron rich meal combined with different doses of vitamin C on the level of spontaneous physical activity among school children.

Hypothesis

The main hypothesis of this study is that eating guava, which is rich in vitamin C, in combination with mungbean, which is rich in iron, will improve the iron status of the children. Mungbean has an iron content of 6.7 mg of Fe per 100g (United States Department of Agriculture, n.d.). However, mungbean is also high in anti-nutritional factors, such as phytic acid and polyphenol, which reduces the bioavailability of iron. Numerous studies have indicated that the phytate level present in a food product reduces the absorption of iron (Hallberg, Brune & Rossander, 1989) (Hurrell et al., 1992). In the study of Hallberg, Brune & Rossander (1989) it was indicated that the inhibition of iron absorption was strongly related to the amount of phytate present. The addition of 2 mg of phytate inhibited absorption by 18% ($p<0.001$), 25 mg by 64% ($p<0.001$) and 250 mg by 82% ($p<0.001$) (Hallberg, Brune & Rossander, 1989). However, the addition of vitamin C counteracted the inhibitory effect of phytate on iron absorption.

Due to these findings, it is expected that in the current study the combined consumption of mungbean and guava will have a positive effect on iron status in children between the ages of 6 and 12 in the Haryana state in Northern India, since the vitamin C present in guava is thought to counteract the negative influence of the phytate present in mungbean. A difference of 4% in hemoglobin concentrations is expected (Szymlek-Gay et al., 2009).

Primary and Secondary Outcomes

The primary outcome of this study is iron status, which is measured in terms of serum ferritin, hemoglobin and zinc protoporphyrin (ZPP) concentrations (see table 1). Iron deficiency occurs when serum ferritin levels are below 15 µg/L, hemoglobin levels are below 115 g/L and ZPP is above 40 µmol/mol heme. Iron status will indicate the effect of the intervention. Furthermore, C-reactive protein (CRP) will be measured to assess whether the children have any infections or inflammations. CRP is a highly conserved plasma protein that is involved in the systemic response to inflammation (Black, Kushner & Samols, 2004). CRP increases rapidly after tissue injury or infection and therefore contributes to host defense and is part of the innate immune response (Black, Kushner & Samols, 2004).

Iron stores in the body exist primarily in the form of ferritin (World Health Organisation, 2011). In the body, small amounts of ferritin are secreted into the plasma, and the concentration of plasma ferritin is positively correlated with the total body iron stores, in the absence of inflammation (World Health Organisation, 2011). For this reason, it is very important to also measure CRP. The CRP concentration that invalidates the use of serum ferritin to diagnose iron deficiency is 10-30 mg/L (Zimmermann & Hurrell, 2007). The normal range of serum ferritin for children between the ages of 6-9 is 10-55 µg/L (Iron Disorders Institute, 2009). Iron deficiency and iron deficiency anemia occur when serum ferritin levels are below 15 µg/L (World Health Organisation, 2011) (Alton, n.d.). Furthermore, the normal range of hemoglobin levels for children between the age of 6 to 18 years it is 100 to 155 g/L (Iron Disorders Institute, 2009). When hemoglobin levels are below 115 g/L, iron deficiency occurs. Hemoglobin levels below 110 g/L indicate anemia (Macharia-Mutie, 2012). However, iron deficiency anemia is defined as the concurrent anemia and iron deficiency (Macharia-Mutie, 2012).

Finally, ZPP will be measured as a primary outcome to indicate iron status. The determination of ZPP is a screening method for the assessment of iron deficiency (Hastka et al., 1993). In iron deficiency, zinc is incorporated into proporphyrin IX instead of iron and ZPP is produced instead of heme (Hastka et al., 1993). Normal ranges of ZPP are below 40 µmol/mol heme, but in people with iron deficiency, ZPP levels are increased (Hastka et al., 1993). Iron deficiency anemia occurs when ZPP concentrations are above 80 µmol/mol heme in children older than 5 (Zimmermann & Hurrell, 2007).

The secondary outcome of this study is the change in spontaneous physical activity. Spontaneous physical activity includes all daily activity, ranging from walking, running or playing to conducting sports. Iron deficiency (or iron deficiency anemia) can lead to weakness, breathlessness and impaired aerobic capacity (Man, 2012) which could negatively affect the level of spontaneous activity. Therefore, spontaneous physical activity is expected to increase in iron deficient children when the iron status is improved. The difference in spontaneous physical activity (average of 7 days of measurements) between baseline and end-line will be measured in a sub-sample of all treatment groups.

Table 1: Age-specific cut-off points for iron deficiency and iron deficiency anemia

Age (years)	Indicator	Iron deficiency	Iron deficiency anemia
6-9	Serum ferritin	< 15 µg/L	<15 µg/L
6-18	Hemoglobin	<115 g/L	<110 g/L
> **5**	ZPP	>40 µmol/ mol heme	>80 µmol/ mol heme

Study Population & Sample Size Calculations

Study population

The target population of this study is primary school children between the age of 6-12 years old, living in rural areas in the Haryana state in Northern India. Inclusion criteria are iron deficiency and iron deficiency anemia, since iron status has an effect on iron absorption (Macharia-Mutie et al., 2012). Iron deficiency stimulates duodenal expression of DMT1, DCYTB, and ferroportin, and thereby increases iron absorption (Zimmermann & Hurrell, 2007). Furthermore, the study of Macharia-Mutie et al. (2012) indicated that iron absorption increases exponentially for iron-deficient individuals. The greater effect of treatment on hemoglobin and soluble transferrin receptor concentrations in iron deficient children may be due to their iron status, as newly available iron may be preferentially used for erythropoiesis (Macharia-Mutie et al., 2012). It has been indicated that iron deficiency up-regulates iron absorption, which means that individuals with a baseline iron deficiency or iron deficiency anemia absorb more iron than individuals with a normal iron status (Zimmermann et al., 2010). For this reason, children with a normal iron status are excluded. Iron deficiency occurs when serum ferritin levels are below 15 µg/L, hemoglobin levels are below 115 g/L and ZPP is above 40 µmol/mol heme. Iron deficiency anemia occurs when serum ferritin levels are below 15 µg/L, hemoglobin levels are below 110 g/L and ZZP is above 80 µmol/mol heme (Alton, n.d.)(Macharia-Mutie, 2012) (Zimmermann & Hurrell, 2007). Iron deficiency anemia occurs when iron deficiency and anemia occur at the same time.

An inadequate diet is not the only cause of iron deficiency since it also can be caused by heavy bleeding and intestinal inflammation (Man, 2012). Furthermore, it is shown that hookworm infections are quite common in India (Pal, 2007) and this kind of parasite is responsible for iron losses (Man, 2012). Lastly, menstrual bleeding can affect iron balance (Man, 2012). For these reasons, during the recruitment of the sample, subjects with a chronic medical problem, serious infection, significant blood loss and bleeding disorders will be excluded (Tayao, 2015). Menstrual bleeding is not expected to occur in the current sample, due to the age of the subjects. These exclusion criteria will be assessed by means of a questionnaire. The parents or caretakers will be asked to assess the prevalence of severe diarrhea and significant blood loss for a period of 1 months prior to the intervention. Weekly questionnaires will be provided to assess the prevalence of these conditions. Severe diarrhea is defined as more than 10 watery stools within 24 hours (Healthwise, 2012). Furthermore, significant blood loss is defined as blood loss exceeding 150 ml/min (Irita, 2011).
Parents and the general practitioner (if available) will also be asked to indicate whether the child suffers or has suffered from any chronic medical condition or bleeding disorder during 6 months prior to the intervention. The occurrence of serious infection is defined as CRP levels above 80 mg/L, which is assessed at baseline of the intervention (van den Bruel et al., 2011).

Also children with severe anemia, hemoglobin level below 80 g/L (Vinod Kumar, 2013), will be excluded from the study population. These children will be referred to a hospital if the condition is very severe. If the condition is less severe, children will be treated with supplements: 60 mg iron + 400 µg folic acid daily for 3 months (International Nutritional Anemia Consultative Group (INACG), 2000). Parents of children with iron deficiency or iron deficiency anemia will be informed about the current nutritional status of their children.

Furthermore, parents and legal guardians will be informed about the study and measurements that will be conducted. Parents will have to give permission by means of an informed consent before children can be included in the study.

Below, an overview of the inclusion and exclusion criteria is given. **Figure 1** indicates the flowchart of the selection procedure.

Inclusion criteria

- Between 6 and 12 years of age
- Attend a primary school
- Serum ferritin level < 15 µg/L
- Hemoglobin level < 115 g/L (iron deficiency) or <110 g/L (iron deficiency anemia)
- ZZP > 40 µmol/mol heme (iron deficiency) or >80 µmol/mol heme (iron deficiency anemia)
- No chronic medical problems or bleeding disorders 6 months prior to the intervention
- Agreed on informed consent by parents or legal guardians

Exclusion criteria

- Serious infection: CRP >80 mg/L
- Severe diarrhea: more than 10 watery stools in 24 hours
- Significant blood loss > 150 ml/min
- Severe anemia: hemoglobin < 80 g/L

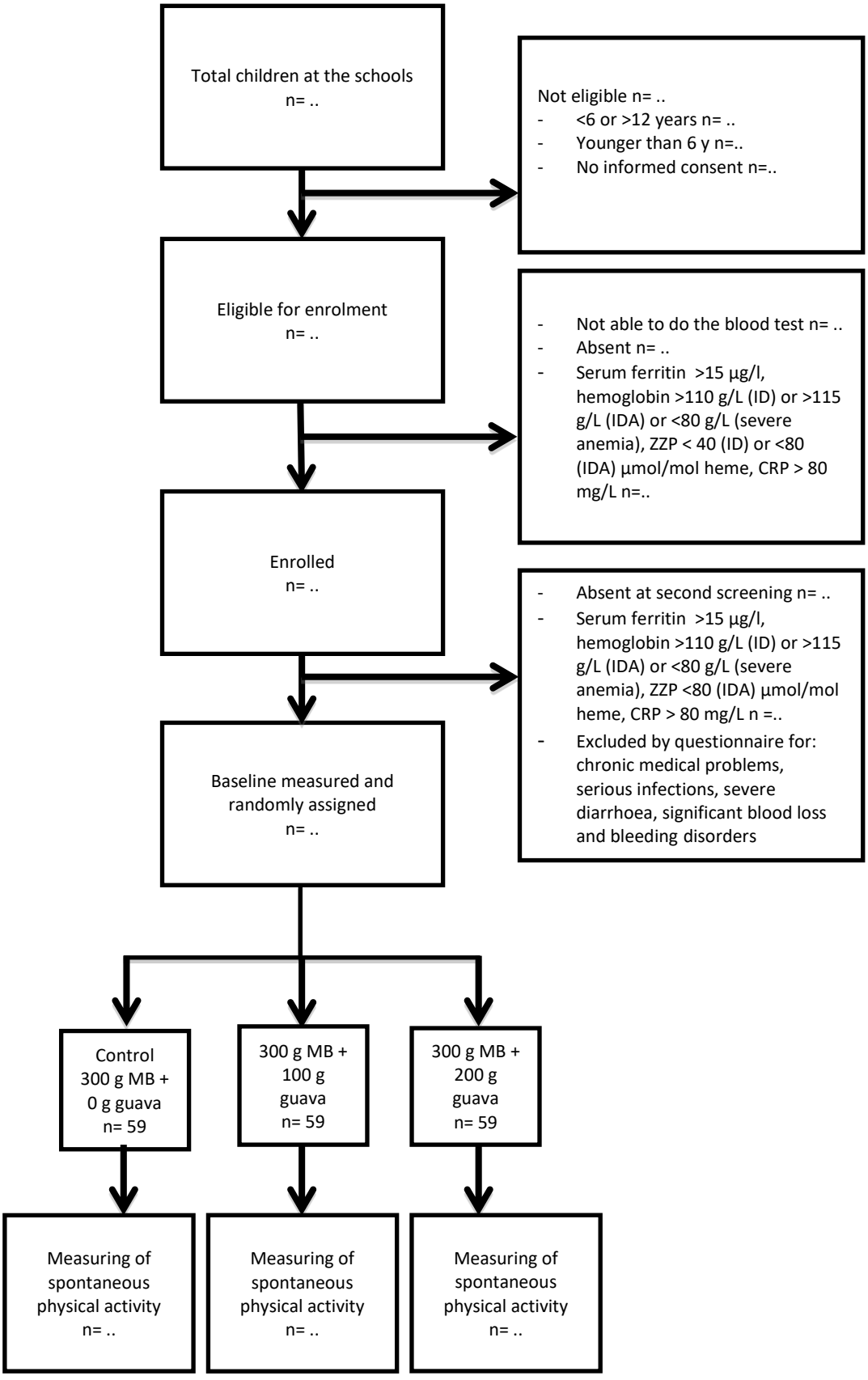

Figure 1: Flow chart study population

Sample size

Tayao (2015) conducted a study to assess the effect of iron supplementation or iron and vitamin C supplementation on hemoglobin, hematocrit, reticulocyte count and red cell indices in anemic undernourished children. The study indicated a 3.6% increase in hemoglobin concentrations after the combined supplementation, namely from 12.79 g/dL to 13.25 g/dL (Tayao, 2015). Furthermore, Kapur et al. (2002) conducted a study to assess the magnitude/severity and possible etiology of anemia and iron deficiency among children 9-36 months of age in Delhi. Based on a pilot study to assess the prevalence of anemia, the standard deviation of the prevalence of anemia was estimated at 1.9. Based on this literature, in the current study, an expected difference of 4% in hemoglobin concentrations is expected (Szymlek-Gay et al., 2009). The sample size calculation is based on an expected difference of 4% between the control group and the intervention group which receives 200 grams of guava, with a power of 80% and an alpha of 0.05 (Szymlek-Gay et al., 2009). Furthermore, the standard deviation used in the current study is based on the research of Kapur et al. (2002) and is therefore equal to 1.9. Even though the study of Kapur et al. (2002) was conducted in an urban area and the current study is conducted in a rural area, this standard deviation is taken as this literature best represents the current study. Due to the different study area used in the current study, the prevalence of anemia and iron deficiency may deviate from the results of Kapur et al. (2002). The allocation ratio used to calculate the sample size is 1, since equal amounts of children will be present in each treatment group. Based on the formula present in figure 1, the sample size for independent samples is calculated.

$$n = \frac{(r+1)^2 (z_\alpha + z_\beta)^2 \sigma^2}{\delta^2 r} \quad \rightarrow \quad \frac{\left((1+1)^2 \times (1.96+0.84)^2 \times (1.9)^2\right)}{\left((0.96)^2 \times (1)\right)}$$

Figure 2: Formula to calculate sample size (Van 't Veer, Souverein & Geelen, 2014)

Based on this formula, a total sample size of 122 is required, which means that 41 children will be allocated to each treatment group. Due to the fact that drop-out will occur, a larger sample population is initially required. To account for these factors, the following formula is used to calculate the adjusted sample size: N = n/(1-(z/100)), where N is the adjusted sample size, n is the initial sample size and z is the expected percentage that will drop-out (Prasanth, Rajani & Mathai, n.d.). Z is estimated at 30% (Prasanth, Rajani & Mathai, n.d.). For these reasons, the adjusted sample size calculation is: N =122/(1-(30/100)) = 175 participants in total, what means 59 children per treatment group.

Selection Procedure

Three different primary schools in the Haryana state in Northern India will be included in this study. The districts in Haryana state in Northern-India will be randomized and one district will be selected. Within this district, one village will be randomly chosen. Due to the fact that India is a highly populated country, it is assumed that several schools will be present in one village. Furthermore, one village is randomly chosen to minimize any confounding factors which may be present in different parts of one district. All schools within this village will be screened on facilities needed for the preparation and consumption of the meals. These facilities include a kitchen with adequate kitchen utensils, a school canteen for the consumption of lunch meals, access to all necessary ingredients and school staff have to be present to prepare the meals. The schools that meet these criteria will be randomized, after which three schools will be selected for the study. The schools that do provide all the facilities needed might be a bit richer than schools which do not. This might also mean that a lower percentage of the children has iron deficiency or iron deficiency anemia, but it is assumed that

the prevalence of these deficiencies will still be high since 75% of Indian children under five suffer from iron deficiency anemia (Nutrition Foundation Of India, 2007). Prior to the study, all children of the different primary schools between the age of 6 and 12 will be asked to come to the first screening when their parents or legal guardians agreed on the informed consent. At this screening a blood test will be done to measure serum ferritin, hemoglobin, ZPP and CRP concentrations, which indicates whether the children meet the inclusion criteria of iron deficiency or iron deficiency anemia. Based on these criteria, questionnaires will be provided to the parents or caretakers of the children, to assess the occurrence of severe diarrhea, significant blood loss, chronic medical problems, bleeding disorders or serious infection. Based on these exclusion criteria, a selected group of children will be asked to come to the second screening at the start of the intervention period. During this screening, serum ferritin, hemoglobin, ZPP and CRP concentrations will be measured. Parents or caretakers are asked to fill in the questionnaires for the full intervention period.

It is expected that the treatment will have a different effect depending on the iron status of the children; therefore, this will be taken into account in the data analysis by analyzing the data of iron deficient and iron deficient anemic children separately. Furthermore, randomization will ensure equal amounts of children with iron deficiency and iron deficiency anemia.

Study Design

Type of study and treatment groups

A single-blind randomized controlled trial is implemented to study the effect of different doses of vitamin C upon iron bioavailability and subsequently iron status. The schools within the selected village which meet the facility criteria will be randomized and three schools will be chosen. The study population in each school will receive a different treatment, which means that each school gets only one of the three treatments. Therefore, the subjects are blinded, since they do not know the other treatments. It is assumed that minimal differences are present within the selected schools since schools are selected from one village and therefore the same habits will be present. A professional supervisor will teach the school staff the correct method of preparation of the meals, including the use of proper kitchen utensils, using the right ingredients in the right quantities, meal preparation and serving methods. Furthermore, periodically (two times per week), the supervisor will check whether the proper conduct of preparation is still conducted in the schools.

A randomized controlled trial will be implemented with 3 different treatment groups, namely: 300 grams of mungbean in combination with either 0 grams of guava (0 mg vitamin C) (control group), 100 grams of guava (228.3 mg vitamin C) or 200 grams of guava (456.6 mg vitamin C) (United States Department of Agriculture, n.d.). In the study of Cook & Monsen (1977) it was found that an average intake of supplemental vitamin C of 280 mg daily during breakfast was associated with a nearly 2-fold increase in the amount of iron absorbed in men. If this was divided between different doses, the increase in iron could be more than 3-fold (Cook & Monsen, 1977). For this reason, the above-mentioned doses of guava are given.

The average amount of iron in 100 grams of mungbean is 6.7 mg (United States Department of Agriculture, n.d.). Furthermore, boiled mungbean contains 1 mg of vitamin C (United States Department of Agriculture, n.d.). The daily requirement of iron for children between the age of 4-8 years old is 10 mg and for children between the age of 9-13 years old is 8 mg (National Institutes of Health, 2015). Furthermore, the RDI for vegetarians is 1.8x higher compared to people who consume meat. Since the Hindi diet of the children contains limited amounts of meat, fish and poultry, this must be taken into account. The bioavailability of iron in a mixed diet is 14-18% (which contains a substantial amount of meat, seafood, and vitamin C), however, for a vegetarian diet this is 5-12% (National Institutes of Health, 2015). 300 grams of mungbean will be given to the children since this

is a sufficient portion size for children as it contains 318 calories (Selfnutritiondata, 2014). Based on the previously mentioned values, 300 grams of mungbean contain 20.1 mg of iron and the daily requirements for children range between 8-10 mg. Based on the fact that many people in India have a vegetarian diet, RDI is increased to 14.4-18 mg. The bioavailability of iron in a vegetarian diet ranges between 5-12%. This means that 0.72-2.16 mg of iron is absorbed if it is consumed without guava. Based on the study conducted by Cook & Monsen (1977), it is expected that 100 grams of guava (228.3 mg vitamin C) will approximately double iron absorption, meaning iron absorption of 1.44-4.32 mg. The consumption of 200 grams of guava (456.6 mg vitamin C) will increase iron absorption even further, approaching the daily requirement of iron for children, without reaching the upper limit of iron intake of 40 mg (U.S. Department Health & Human Service, n.d.). Even though this intervention does not reach the daily iron requirements of children completely, it is assumed that children also consume iron during other meals throughout the day.

Normally, school lunches in India are provided by the government. This is a program called 'Mid Day Meal (MDM) scheme'. There are guidelines for the content of these meals (Ministry of Human Resource Development, 2006). A lunch for children in primary school has to consist of the next quantities: 100 g of foodgrains, 200 g of pulses, 50 g of vegetables, 5 g of oil & fat. The guidelines for the nutritional contents of these meals are: 450 calories, 12 g of protein and adequate quantities of micronutrients like iron, vitamin A, etc. The children in the study will receive a whole dhal meal. Because this meal contains, besides mungbean, also vegetables and other ingredients, this will probably be comparable to a normal school meal, but with more iron. With the normal school lunches no fruits are given, so this is only the case for the study meals.

High ingestion of iron could accumulate in the body and result in toxic effects on organs. Children are most vulnerable to iron overload with abdominal pain, vomiting, metabolic acidosis and cardiovascular collapse as a result (Man, 2012). For a child, these symptoms occur when the daily iron intake exceeds 40 mg (U.S. Department Health & Human Service, n.d.). However, illness due to iron overload is mostly due to a long-term intake of high amounts of iron (Man, 2012). Due to the fact that the upper limit of iron intake is not reached in this study, this is not a problem. The possibility of having a vitamin C overload during the study is quite difficult as the maximum of vitamin C that will be dosed (with 200 g of guava) is 456.6 mg/day and a safe daily dose for a children between 6 to 12 years old is about 800 mg/day (U.S. Department Health & Human Service, n.d.).

Duration of the Study and Procedure

The children will receive a daily meal of mungbean in combination with guava for a period of four months, during their school lunch meals. This study duration is chosen based on a study done in vegetarians, in which twenty-eight strict vegetarians received 500 mg of vitamin C twice a day after lunch and dinner for two months. This study was conducted to assess the effect on hemoglobin and certain iron status parameters (Sharma & Mathur, 1995). Since the doses of vitamin C in the present study are lower and the iron is given through a mungbean meal rather than by supplementation, a study duration of four months is chosen, to be able to show a significant effect. Appendix I (page 19) contains a visualization of the study design.

The study will start with a screening to identify the children that meet the inclusion criteria. This will be followed by a preparation period. The preparation period is included to make sure that the procedure works effectively, and no systematic errors are made, such as errors during meal preparation. Possible errors may include incorrect quantities of the ingredients or incorrect preparation methods. Furthermore, the kitchen utensils will be checked during this period, to make sure the right utensils are used during the study. Besides that, the vitamin C content in prepared guava will be measured. During the preparation period, a smaller quantity of the meals will be prepared, however, they will not yet be given to the children. This cannot be done as it may intervene with the study outcomes.

Children that passed the first screening will be invited to a second screening. At this screening the baseline serum ferritin, hemoglobin levels, CRP and ZPP in red blood cells will be measured with blood tests. Since it will take some time to analyze these tests, the screening will take place two weeks before the start of the intervention. At mid-term and end-term of the study, the measurements will be repeated. CRP will be measured since concentrations between 10-30 mg/L invalidate serum ferritin concentrations as a valid procedure to diagnose iron deficiency (Zimmermann & Hurrell, 2007). Therefore, when children have CRP values within this range, they will be excluded. Furthermore, at mid-term and end-term, a questionnaire will be handed out to the parents or caretakers of the children, which will assess the prevalence of severe diarrhea episodes and blood loss. If these questionnaires indicate repeated episodes of severe diarrhea and blood loss, these children will be excluded from data analysis.

The parents or legal guardians of the children that are invited to the second screening will receive a Food Frequency Questionnaire (FFQ) while being at the second screening. The Food Frequency Questionnaire will focus on the intake of iron and vitamin C rich foods and whether these are consumed together. For iron rich foods they will be asked whether and how much their children consumed different kinds of meat, vegetables, legumes and other food sources the last two months. (see table 2 (United States Department of Agriculture, n.d.)). For vitamin C rich foods they will be asked whether and how much their children consumed different kinds of fruits, fruit juices and vegetables during the last two months (see table 3 (United States Department of Agriculture, n.d.)). These FFQs will be repeated at the mid-term and end-term of the intervention when the blood levels of the children are measured again, to keep the 2 months factor practically constant.

Table 2: Food Sources of Iron

Description	Weight(g)	Measure	Iron, Fe(mg) Per Measure
Beef, variety meats and by-products, spleen, cooked, braised	85.0	3.0 oz	33.46
Lamb, variety meats and by-products, spleen, cooked, braised	85.0	3.0 oz	32.87
Seaweed, spirulina, dried	112.0	1.0 cup	31.92
Soybeans, mature seeds, raw	186.0	1.0 cup	29.20
Goose, liver, raw	94.0	1.0 liver	28.70
Winged beans, mature seeds, raw	182.0	1.0 cup	24.46
Rice bran, crude	118.0	1.0 cup	21.88
Mothbeans, mature seeds, raw	196.0	1.0 cup	21.27
Beans, white, mature seeds, raw	202.0	1.0 cup	21.09
Seeds, sesame seeds, whole, dried	144.0	1.0 cup	20.95
Pork, fresh, variety meats and by-products, spleen, cooked, braised	85.0	3.0 oz	18.90
Beans, kidney, california red, mature seeds, raw	184.0	1.0 cup	17.20
LOMA LINDA Redi-Burger, canned, unprepared	85.0	1.0 slice , 5/8"	17.00
Soy meal, defatted, raw, crude protein basis (N x 6.25)	122.0	1.0 cup	16.71
Soy meal, defatted, raw	122.0	1.0 cup	16.71
Beans, small white, mature seeds, raw	215.0	1.0 cup	16.62
Cowpeas, catjang, mature seeds, raw	167.0	1.0 cup	16.62
Beans, kidney, royal red, mature seeds, raw	184.0	1.0 cup	16.01
Beans, black turtle, mature seeds, raw	184.0	1.0 cup	16.01
Mungo beans, mature seeds, raw	207.0	1.0 cup	15.67
Pork, fresh, variety meats and by-products, liver, cooked, braised	85.0	3.0 oz	15.23
Beans, kidney, all types, mature seeds, raw	184.0	1.0 cup	15.09
Natto	175.0	1.0 cup	15.05
Chicken, broilers or fryers, giblets, cooked, fried	145.0	1.0 cup, chopped or diced	14.96
Teff, uncooked	193.0	1.0 cup	14.73
Amaranth grain, uncooked	193.0	1.0 cup	14.69
Yardlong beans, mature seeds, raw	167.0	1.0 cup	14.38
Beans, pink, mature seeds, raw	210.0	1.0 cup	14.22

Table 3: Food Sources of Vitamin C

Description	Weight(g)	Measure	Vitamin C, total ascorbic acid(mg) Per Measure
Acerola juice, raw	242.0	1.0 cup	3872.0
Acerola, (west indian cherry), raw	98.0	1.0 cup	1644.0
Orange juice, frozen concentrate, unsweetened, undiluted, with added calcium	262.0	1.0 cup	379.4
Orange juice, frozen concentrate, unsweetened, undiluted	262.0	1.0 cup	379.4
Guavas, common, raw	165.0	1.0 cup	376.7
Guava sauce, cooked	238.0	1.0 cup	348.4
Peppers, sweet, yellow, raw	186.0	1.0 pepper, large (3-3/4" long, 3" dia)	341.3
Juice Smoothie, BOLTHOUSE FARMS, BERRY BOOST	252.0	1.0 cup	273.7
Grapefruit juice, white, frozen concentrate, unsweetened, undiluted	207.0	1.0 can (6 fl oz)	248.0
Peaches, frozen, sliced, sweetened	250.0	1.0 cup, thawed	235.5
Peppers, sweet, red, cooked, boiled, drained, without salt	135.0	1.0 cup, strips	230.8
Pokeberry shoots, (poke), raw	160.0	1.0 cup	217.6
Peppers, sweet, green, sauteed	115.0	1.0 cup chopped	203.6
Currants, european black, raw	112.0	1.0 cup	202.7
Kiwifruit, gold, raw	186.0	1.0 cup, sliced	196.0
Mustard spinach, (tendergreen), raw	150.0	1.0 cup, chopped	195.0
Peppers, sweet, red, raw	149.0	1.0 cup, chopped	190.3
Apple juice, frozen concentrate, unsweetened, undiluted, with added ascorbic acid	211.0	1.0 can (6 fl oz)	187.6
Peppers, sweet, red, sauteed	106.0	1.0 cup chopped	172.6
Tomato juice, canned, with salt added	243.0	1.0 cup	170.3
Tomato juice, canned, without salt added	243.0	1.0 cup	170.3
Kiwifruit, green, raw	180.0	1.0 cup, sliced	166.9
Drumstick pods, raw	100.0	1.0 cup slices	141.0
Vegetable juice cocktail, low sodium, canned	254.0	1.0 cup	137.9
Vegetable juice cocktail, canned	253.0	1.0 cup	137.4
Litchis, raw	190.0	1.0 cup	135.8
Pokeberry shoots, (poke), cooked, boiled, drained, with salt	165.0	1.0 cup	135.3

To measure the secondary outcome of this study, spontaneous activity, accelerometer bracelets will be used. In studies with frail elderly (Tieland et al., 2012) spontaneous physical activity was measured by an Actigraph GT3X accelerometer. Subjects wore the Actigraph during daytime for seven consecutive days and the uniaxial data was analyzed by a MAHUffe Analyzer program. Actigraph GT3X was already used in a study among children in Australia (Ridgers, 2013), where children were instructed to wear the accelerometer during all waking hours except during water-based activities (e.g., swimming and bathing) and were provided with information concerning the correct wear and care of the accelerometer.

These studies indicate that the accelerometer is an adequate and economically sustainable method to measure physical activity levels, and the cost of these tools is about 200 euros (Actigraphcorp). Measurements will be taken for 7 days at the beginning of the intervention and for 7 days at the end of the intervention according to the protocol of Ridgers's (2013). An average of these 7 days will be taken as the outcome measure. However, a sub-sample is taken to measure the secondary outcome, as providing an accelerometer to 175 children is too expensive. Furthermore, the proper use of such a tool in a developing country like in India could be problematic and it is never done before. Thus, in each treatment group, 10 children will be randomly chosen and the mean of their physical activity will be measured at baseline and the end of the intervention. For half of the sub-sample (5 children), direct observations of their physical activity are made by using the children activity rating scale (CARS) (Hands,2006). This is done to validate the results of the accelerometer. The sub-sample of 5 children will be chosen randomly. Per primary school, 3 PhD students from Wageningen University will be trained to conduct the observations of spontaneous physical activity, to allow proper validation. Considering the fact that accelerometers might dysfunction or stop working during the intervention period, an initial amount of 50 accelerometers will be taken to the study site, whereas 30 accelerometers are needed. Children will be instructed to be very careful with the equipment; to make sure minimal dysfunction will occur.

The measurement variables of the accelerometer are: mean counts/minute; time spent in sedentary activity (0-199 counts/minute, METs < 1.5), light activity (200-1951 counts/minute, METS >1.5 and

<3), moderate activity (1952-5724 counts/minute, METs>3 and <6) and vigorous intensity (over 5724 counts/min, METs>6). MET stands for metabolic equivalent of task, which is a physiological measure expressing the energy cost of physical activities (Jetté, Sidney & Blümchen, 1990). It is defined as the ratio of metabolic rate during specific physical activity compared to a reference metabolic rate (Jetté, Sidney & Blümchen, 1990). The mean METs over 7 days will be calculated and used to classify the children into different groups. Children with a mean METs of <1.5 will be classified as sedentary active, children with a mean METs between 1.5 and 3 will be classified as light active, children with a mean METs between 3 and 6 will be classified as moderate active and children with a mean METs over 6 will be defined as vigorous active. The possible change from activity group at the end of the intervention indicates the change in spontaneous physical activity which can indicate possible positive effects of the intervention, concerning an improved iron status.

Intervention agent

As described earlier, the treatment will be implemented in 3 different treatment groups. The control group will receive 300 grams of mungbean, where the 2 intervention groups will receive 300 grams of mungbean combined with either 100 or 200 grams of guava. The children who will not receive guava or who receive 100 grams of guava will not receive a control. This choice is made, as a substitute for guava of which it is known that it will not interact with the iron in the mungbeans, is not present. Therefore, a possible confounder within this study could be the different energy contents of the meals provided to the children. However, since guava only contains 68 kcal per 100 grams, this effect is expected to be small or not existing (United States Department of Agriculture, n.d.).

The study of Oliveria et al. (2010) has shown that the amount of vitamin C in guava is very stable in different preparation and storage methods. Mahanom et al. (2010) showed that the amount of vitamin C in the leaves of the guava declines during drying. It is not shown that this also occurs in the fruit itself, but to make sure that this does not happen fresh guava will be used which will be bought at the nearest local market available. The guava will be prepared by slicing the fruit with peel in cubes of 1.5 cm, because research has shown that this does not significantly affect the total vitamin C content of the guava (Oliveria et al. 2010). The guava will be served to the children immediately after preparation, to prevent possible losses of vitamin C during storage (Uddin, 2002). The vitamin C content in the guava, prepared as described above, will be measured once during the preparation period, to ensure that the assumed vitamin C content (228.3 mg vitamin C per 100 gram) is correct. For this measurement, HPLC with Electrochemical Detection (HPLC/EC) will be used to determine the amount of ascorbic acid in the guava. (Washko, 1992) The detector potential for this analysis is set at, or above, the midpoint potential of ascorbic acid. By this way, electrochemically active compounds with higher midpoint potentials won't be converted by the detector. By coupling this electrochemical detection technique to HPLC, interference is substantially decreased. (Washko, 1992). For ascorbic acid, HPLC/EC has many advantages over other methods in providing both sensitivity and specifity. (Washko, 1992)

Mungbean is widely grown and consumed in India (Bains, Yang & Shanmugasundaram, 2003). Subramanian and Yang (1998) have prepared improved mungbean and vegetable recipes based on simple cooking methods, locally available and inexpensive ingredients, and consumer acceptability (as cited in Bains, Yan & Shanmugasundaram, 2003). Furthermore, the recipe book of Bains, Yan and Shanmugasundaram (2003) incorporates many mungbean recipes especially applicable to Northern India, such as Mung Amaranth Saag, Mung Dhal Khichri and Parantha. A study conducted by Dahiya (2014) showed that whole dhal was one of the most liked products produced with mungbeans among different population groups including children in the Haryana state; for this reasons there should not be problems about acceptability of the meals. Furthermore, whole dhal is cooked (Dahiya, 2014) and the study of Barakoti et al. (2007) showed that cooking reduces the amount of phytate and therefore enhances the bioavailability of iron. Therefore in the current study whole dhal

will be made as the mungbean compartment of the meal. The study of Yu-Wei Luo et al. (2013) showed that germinated mungbeans have the highest bioavailability of iron, however due to a long germinating time it is decided that the mungbeans will not be germinated. The meals will be prepared by the staff of the school. The meal will consist of fresh guava with whole dhal. The long period of the study could cause some complications, children could get tired of eating the same food every day, but this will probably rarely happen because the acceptability of this product is quite high (as cited in Bains, Yan & Shanmugasundaram, 2003) and also children will be served whole dhal with guava only in the school days so during their free days (two days per week) they do not have to eat the same food as served at school.

Analysis Plan

Before starting the analysis, children that experienced significant blood loss defined as a loss of more than > 150 ml/min or repeated episodes of severe diarrhea with more than 10 watery stools within 24 hours (Healthwise, 2012) during the intervention will be excluded from data analysis. Besides that children with CRP values between 10-30 mg/L will also be excluded for analysis, because they probably might have had serious infection during the intervention period. For analyzing the data serum ferritin, hemoglobin and ZPP will be used as the three outcome measures. The cut-off values for serum ferritin are >15 µg/L up to 55 µg/L for normal iron status and <15 µg/L for iron deficiency and iron deficiency anemia (World Health Organisation, 2011) (Alton, n.d.). For hemoglobin these values are 100 to 155 g/L for normal iron status (Iron Disorders Institute, 2009), <115 g/L for iron deficiency and <110 g/L for iron deficiency anemia (Macharia-Mutie, 2012). For ZPP the cut-off values are <40 µmol/mol heme for normal iron status, >40 µmol/mol heme for iron deficiency and >80 µmol/mol heme indicates iron deficiency anemia (Hastka et al., 1993)(Zimmermann & Hurrell, 2007). Taking these cut-off values into account, a child will be classified as iron deficient when the serum ferritin level is below 15 µg/L, the hemoglobin level is between 110-115 g/L and when the ZPP value is above 40 µmol/mol heme. A child will be classified as iron deficient anemic when the serum ferritin level is below 15 µg/L, hemoglobin levels below 110 g/L and when ZPP value is above 80 µmol/mol heme.

The data will be analyzed using SPSS for Windows. Before analyzing, the data will be checked for normality by using the Kolmogorov-Smirnov test. If the data is not normally distributed this will be corrected by applying log transformation to the data. To analyze the continuous values a repeated measures ANCOVA test will be done to determine if there is a change in iron status between the different treatment groups over time for serum ferritin, hemoglobin and ZPP. ANCOVA is a method used to assess whether there are significant differences between two or more independent groups (Laerd Statistics, n.d.). ANCOVA is different from one-way ANOVA since it allows statistical control for a covariate, in this case the baseline measurements of iron status (Laerd Statistics, n.d.). Secondly, a binary logistic regression will be performed to compare the effects between the different treatment groups for iron deficiency anemia, iron deficiency and normal iron status. Binary logistic regression can be used to assess the probability that a characteristic is present, given the explanatory variables (Pennsylvania State University, 2015). Therefore the children will be classified by iron status according to the cut-off values. In the binary logistic regression iron status will be defined as 2 for iron deficiency anemia, 1 for iron deficiency and 0 for a normal iron status. The values will be reported as means ± SD and a significance level of $p < 0.05$ will be used on all tests.

The change in physical activity level will be measured after the intervention period. Data obtained from the accelerometers will be validated with the data of the observations, to conclude whether the accelerometers accurately measure physical activity. Validation will be done by using modified Bland Altman plots to check for systematic errors in data from the observations compared to data from the accelerometers (Sullivan, 2012). Data will be analyzed using repeated measures ANCOVA, where the physical activity level at the start of the intervention is the baseline measurement.

Ethical considerations

Ethical approval will be asked from the medical ethical committee of the Wageningen University & Research Centre and from the Institutional Ethics Committee for Human Research from the Indian Council of Medical Research. Ethical approval is necessary for the blood tests that need to be conducted. Results of these blood tests might indicate that some children suffer from severe iron deficiency anemia (hemoglobin levels below 80 g/L) (Vinod Kumar, 2013). Children with severe iron deficiency anemia will be excluded from the study and will be treated with iron supplements or are provided with hospital care.

All the children that are part of the study will be seated in one area during their lunch, to allow minimal contact with children who do not participate in the study and therefore have different meals during the school lunch. The children in the study will receive fruit with their meal, but the children receiving a normal lunch will not. This is not a big problem because they normally did not receive fruit either. Lastly, if the results of the study indicate the treatment to be effective, advice will be given to the schools to improve school lunch meals by using mungbean and guava, in order to improve the iron status of the children. Schools will be highly encouraged to provide meals high in iron and vitamin C, in order to maintain the possible positive outcomes of this study. This will be done because it might be unethical to let the iron status decline again after the intervention.

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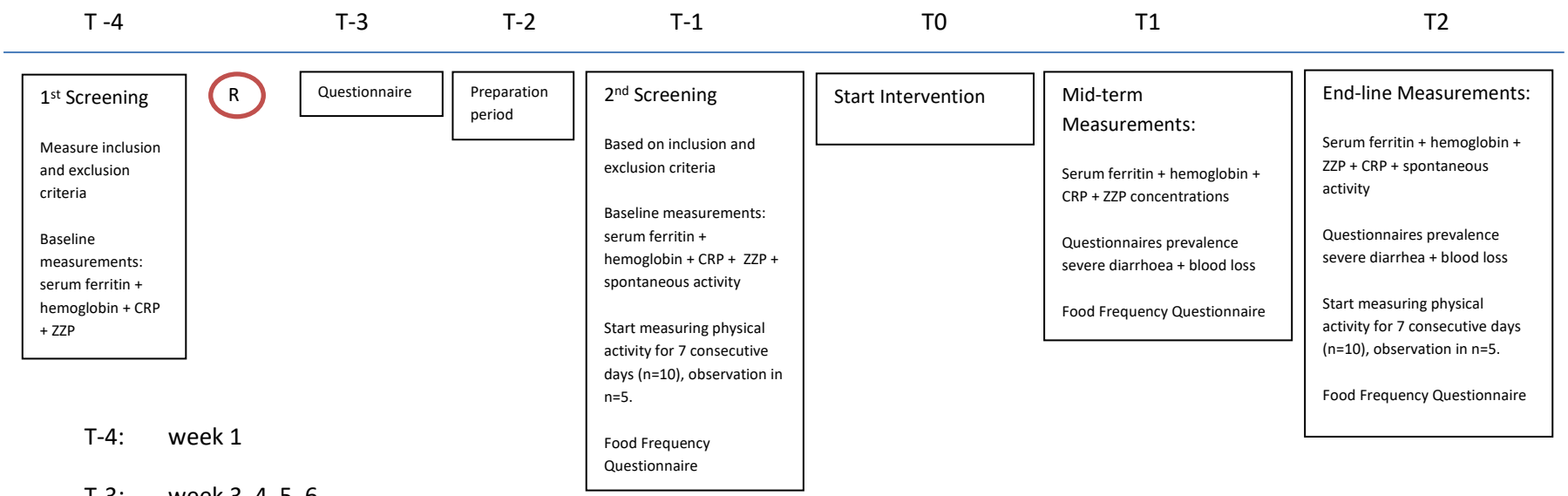
Appendix I: Visualization of the study



T-4: week 1

T-3: week 3, 4, 5, 6

T-2: week 7

T-1: week 9

T0: week 11

T1: week 19

T2: week 27

R = randomization