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STUDY PROTOCOL: Effect of iron supplementation on psychomotor development of non-anaemic exclusively or predominantly breastfed infants: randomized, double-blind, placebo-controlled trial.

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STUDY PROTOCOL: EFFECT OF IRON SUPPLEMENTATION ON PSYCHOMOTOR DEVELOPMENT OF NON-ANAEMIC, EXCLUSIVELY OR PREDOMINANTLY BREASTFED INFANTS: RANDOMISED, DOUBLE-BLIND, PLACEBO-CONTROLLED TRIAL.

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ABSTRACT

Introduction

Uncertainty exists regarding the effects of iron supplementation during infancy on neurodevelopmental outcomes in the absence of anaemia. The aim of the study is to establish whether psychomotor and mental development is influenced by early iron supplementation in healthy, non-anaemic, exclusively or predominantly breastfed infants.

Methods and analysis

Healthy term infants will be recruited. If exclusively or predominantly breastfed (>50% of daily feedings) and not anaemic at 4 months, they will be randomised to receive either iron pyrophosphate (approximately 1 mg/kg) or placebo daily until 9 months of age. The primary outcome measure is neurodevelopment assessed with the Bayley Scales of Infant and Toddler Development (Bayley-III) at 12 months and repeated at 24 and 36 months of age. Haematological parameters of iron metabolism also will be measured.

Ethics and dissemination

The Bioethics Committee of the Medical University of Warsaw approved the study protocol before recruitment commenced. Study results will be submitted to peer-reviewed journals in the fields of paediatrics and nutrition and presented at relevant conferences.

Registration: clinicaltrials.gov (NCT02242188).

STRENGTHS AND LIMITATIONS OF THIS STUDY

- A precise clinical question has been posed to fill a gap in knowledge: Does iron supplementation in healthy, non-anaemic, breastfed infants influence their psychomotor and mental development?
- The study design (randomised controlled trial) is the methodology of choice to assess the effectiveness of such an intervention.
- The assessment of development at several time points up to 36 months of age will allow detection of possible long-term effects of iron supplementation.
- A longer follow-up period would be valuable, but probably will not be feasible due to attrition and cost.

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INTRODUCTION

Iron is a nutrient of essential importance to the human organism. It takes part in energy production, oxygen transportation, and DNA synthesis and is indispensable for the development of the central nervous system. Iron is required for the myelination and production of neurotransmitters. It has been well documented that iron deficiency anaemia (IDA) impairs child development.[1-3] If the diagnosis of IDA is delayed, the deficits may be irreversible.[4] Iron deficiency (ID) is the most common single nutrient deficiency and may affect up to 20% of children 1 to 3 years of age in Europe.[5] ID has the potential to negatively influence psychomotor development. However, a causal relationship is not as clear as for IDA.[6-8]

Previous studies have suggested that iron supplementation in healthy infants may enhance psychomotor development. A meta-analysis carried out by our group (Szajewska *et al*.[9]) evaluated the effects of iron supplementation in non-anaemic pregnant women and in non-anaemic healthy children aged <3 years on the mental performance and psychomotor development of the children. Seven randomised controlled trials (RCTs) were identified, 5 of which referred to supplementation during infancy.[10-14] The pooled results of 3 RCTs (n=561) showed significant improvement on the Psychomotor Development Index (PDI) of the Bayley Scales of Infant Development (BSID) at approximately 12 months of age in the iron-supplemented group compared with the control group (mean difference: 4.21; 95% confidence interval [CI] 2.31 to 6.12). No significant effect of iron supplementation on the Mental Developmental Index (MDI) or behaviour was found. A follow-up study of children aged 9 years who participated in a randomised trial during infancy (daily supplementation of iron, zinc, iron and zinc, or placebo at 4 to 6 months of age for 6 months) recently has been published.[15] Cognitive and school performance did not differ significantly between the 4 groups.[15]

Given that ID is a common problem in small children, measures to prevent it are being taken. According to the Committee of Nutrition of the European Society for Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN CoN), this should be achieved by delayed umbilical cord clamping, the use of iron-fortified

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formulas when formula feeding is needed, the postponement of the introduction of whole cow milk as the main drink until the end of the first year of life, and the promotion of consumption of complementary foods rich in iron.[5] Furthermore, the ESPGHAN CoN concluded that there is no convincing evidence that iron supplements should be provided to normal birth weight, exclusively breastfed infants during the first 6 months of life in populations with a low prevalence of IDA among 6-month-old infants. In contrast, the American Academy of Pediatrics (AAP) recommends iron supplementation (1 mg/kg/day) in exclusively breastfed infants beginning at 4 months of age that should be continued until iron from complementary foods is available.[16] As the level of iron intake is uncertain in partially breastfed infants, the AAP recommends that those who receive more than one half of their daily feedings as human milk also should be supplemented with 1 mg/kg/day of iron beginning at 4 months.[16]

This considerable difference between European and US guidelines is based on different interpretations of the scarce available evidence. The only study that has previously addressed this issue was performed by, among others, a member of our group (MD). In that study, 101 Swedish and 131 Honduran, non-anaemic, breastfed infants were randomised into 3 groups: placebo from 4 to 9 months, iron supplements (1 mg/kg/day) from 4 to 9 months, or placebo from 4 to 6 months and iron supplements from 6 to 9 months of age.[17] The study showed that iron supplements effectively decreased the risk of IDA at 9 months in Honduran infants, but not in the Swedish infants, who already had a low prevalence of IDA at 9 months (<3%). However, in the Swedish infants who received iron supplements, a negative effect was observed on growth.[18] Unfortunately, these infants were not followed up for longer than to 9 months and neurodevelopmental outcomes were not assessed.

There is, therefore, a clear need for interventional studies of good methodological quality to evaluate the role of iron supplementation in non-anaemic infants on their mental and psychomotor development, as well as on possible adverse effects on growth and infections.[19]

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Iron preparations in the form of syrup or drops, which are usually prescribed for the treatment of anaemia in children, have an unpleasant taste, may stain the teeth, and cause adverse effects mostly related to the gastrointestinal tract. Additionally, these preparations need careful measuring to obtain the desired dose.[20] Micronized ferric pyrophosphate is a novel form of microencapsulated iron, which can be packaged in easy-to-use sachets and added to milk or weaning foods. It has been generally recognized as safe (GRAS) as a nutrient supplement in food.[21] Ferric pyrophosphate, marketed as SunActive Fe, has been shown to be effective in infants with anaemia.[22] Its use was less often related to episodes of diarrhoea, vomiting, and teeth staining compared to ferrous glycine sulfate drops.[22] The easy-to-use form of the powder, which can be added to liquids or foods without changing the taste, makes long-term supplementation easier to pursue.[20]

Trial objectives and hypotheses

The main objective of this trial is to assess the effectiveness of low-dose iron (1 mg/kg/day) supplementation in healthy, term, breastfed infants from 4 to 9 months of age in regard to optimizing their developmental outcomes measured at 12, 24, and 36 months of age. The hypothesis being tested is that psychomotor and mental development will be superior in iron-supplemented children.

Secondary objectives are to assess the effects of iron supplementation on infant growth (weight, length, head circumference) and laboratory parameters of iron status (haemoglobin [Hb], mean corpuscular volume [MCV], haematocrit ([HCT], serum ferritin [s-Ferritin], reticulocyte Hb, hepcidin, and soluble transferrin receptor concentration [sTfR]). Behaviour at 36 months will also be assessed.

METHODS AND ANALYSIS

The trial is registered at www.clinicaltrials.gov (NCT02242188).

Study design

Randomised, double-blind, placebo-controlled trial.

Participants

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Healthy singleton infants will be considered for inclusion before completion of 4 months of age. Parents will be approached either shortly after birth at the obstetrics department or during well-baby visits at general practitioners' offices. Those who are eligible will be invited to participate in the study. A researcher will contact the caregivers by telephone at approximately 3.5 months of age to check for eligibility criteria again. Those exclusively breastfed or predominantly breastfed, i.e., receiving breast milk for over 50% of feedings at the age of 4 months, will be considered for inclusion and invited to the study site. After caregivers provide informed consent, an infant will be included and blood will be obtained. Those without anaemia, defined as Hb 105 g/L (< 10.5 g/dL)[5], will be randomised to receive either the iron supplement or placebo from the age of 4 completed months until 9 months of age. Apart from recruitment, all study procedures will be pursued in the Department of Paediatrics, Medical University of Warsaw, Poland.

Inclusion criteria

To be included in the study, a child must be a healthy singleton infant aged 1 day to 4 months old, born at term (37 to 42 weeks of gestation), with a normal birth weight of >2500 g. If approached shortly after delivery, the mother of a child must express the intention to breastfeed. If recruited at an older age, an infant must be breastfed either exclusively or predominantly (>50% feedings). A caregiver must provide written informed consent.

Exclusion criteria

The following exclusion criteria will be applied: preterm delivery (<37 weeks of gestation), birth weight < 2500 g, multiple pregnancy, major illness or congenital anomaly, being <50% breastfed at the time of inclusion, food allergy, anaemia (Hb <105 g/L [10.5 g/dL]) at inclusion, lack of informed consent, and difficult communication with caregivers.

Randomisation criteria

The participants will be randomised at the age of 4 completed months, after re-checking the inclusion and exclusion criteria, under the condition of being

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exclusively breastfed or receiving breast milk for at least 50% of their daily feeds at the time of randomisation.

Interventions

Infants in the experimental group will receive the powder preparation of iron pyrophosphate (Actiferol, SunActive Fe, Sequoia, Poland) in a single daily dose of approximately 1 mg per kilogram of body weight from 4 months to 9 months of age (dose in line with the AAP recommendations). Three doses will be used: 7 mg for infants up to 7 kg of body weight, 10 mg for infants from 7 to 10 kg of body weight, and 15 mg for those exceeding the weight of 10 kg. Caregivers will be instructed to administer the daily dose at the same time of a day, after mixing the content of the sachet with little amount of breastmilk, water or adding to small portion of a solid food. Infants in the placebo group will receive maltodextrin.

Allocation concealment and blinding

A computer-generated randomisation list will be used to allocate subjects to the study groups (A or B). Blocks of ten will be applied. Consecutive randomisation numbers will be given to participants. The study products will be delivered to the centre in boxes labelled with the letters A and B (meaning of A and B blinded, information deposited in a sealed envelope in a safe at the administrative part of the department). The boxes will also be carry the information on the specific dose. Subsequently, the letters A and B will be removed from the boxes by an independent person unrelated to the study planning and conduct, and replaced with numeric codes corresponding to the randomisation numbers, e.g., 001, 002 etc. Sachets containing the study product will be packed in small packages of 30 pieces each. Neither the collective packages nor the sachets will carry any labelling. The active product and placebo will be packed in identical sachets and the content will look and taste the same. Researchers, caregivers, outcome assessors, and a person responsible for the statistical analysis will be blinded to the intervention until a statistical report for the 12-months' developmental assessment is available. After that, both the caregivers and main outcome assessor will remain blinded until the completion of the study. The information on intervention assignment will be stored in a sealed envelope in a safe in the administrative part of the department.

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Compliance

The caregivers will be asked to bring the remaining study product to the study site each time they bring the infant for a check-up visit during the intervention, i.e. at 6 and 9 months of age. Compliance with the study protocol will be checked by counting the number of sachets left unused. Those subjects receiving less than 70% of the recommended doses will be considered noncompliant.

Primary outcome

Psychomotor development at the age of 12 months will be the primary outcome measure. Additional assessments will be performed at 24 and 36 months. The Bayley Scales of Infant and Toddler Development (Bayley-III or BSID-III) will be used.[23] Fine and gross motor, cognitive, language, and social-emotional development scores will be measured with the Bayley-III, as derivatives and equivalents of the PDI and the MDI of the BSID-II, the previous edition of the test. A psychologist qualified and experienced in assessment with use of the Bayley-III will perform the test within one month of the moment a child reaches 12, 24, and 36 months of age.

Secondary outcomes

Behaviour

At 3 years, additional screening for behavioural and emotional problems will be performed with use of Child Behaviour Checklist (CBCL), a version for preschool children.[24]

Laboratory tests

Haematological status will be assessed at 4, 12, and 24 months. Samples will be analysed for Hb concentration, MCV, reticulocyte Hb concentration, s-Ferritin concentration, hepcidin concentration, and soluble transferrin receptor concentration (sTfR). The C-reactive protein (CRP) level also will be measured. Iron deficiency anaemia will be defined as Hb concentration <105 g/L (i.e. <10.5 g/dL) and s-Ferritin concentration of <45 pml/L (i.e. <20 mcg/L) at 4 months or <27 pml/L (i.e. <12 mcg/L) for 12 and 24 months. Non-anaemic iron deficiency will be defined as Hb concentration >105 g/L and s-Ferritin concentration <45 pml/L at 4 months or

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<27 pml/L for subsequent measurements.[5] Children diagnosed with anaemia will be offered clinical evaluation and treatment within the department or referred to a pediatrician.

Growth

Growth parameters (weight, length/height, and head circumference) will be recorded at 4, 6, 9, 12, 24, and 36 months of age. Measurements will be performed in a standardized way with use of periodically calibrated scales and measuring boards, according to the World Health Organization (WHO) recommendations.[25]

Dietary iron intake

Dietary assessment in terms of iron intake will be performed at 9 months by means of a 3–day, prospective food diary, which will be prepared on a standardized form by the caregiver(s) and delivered to health provider at the 9-month follow-up visit.

Adverse events

Parents will be asked to fill out a form of possible adverse events of the intervention daily. The symptoms listed in the form will include diarrhoea, vomiting, constipation, discolouration of the stool, fever, and respiratory tract infections. The forms will be collected at each check-up visit during the intervention.

Timeframes of activities during the study

Recruitment for the trial will start in August 2015 and the whole study, including the 36-month follow-up, will last until mid-2018. The time points of all participant-related actions to be taken during the study period are presented in **Table 1.**

Table 1. Timetable of activities planned during the course of the study directly related to participants.

Age (months) / Activity	1	4	5	6	7	8	9	10	11	12	24	36
Enrolment	+											
Randomisation		+										

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Intervention			+	+	+	+	+					
Dietary assessment							+					
Anthropometry		+		+			+			+	+	+
Blood withdrawals		+								+	+	
Neurodevelopmental assessment										+	+	+
Behaviour												+

Retention of participants in the study

Retaining participants in a clinical trial of long-term duration represents a substantial problem for research teams. To make the obtained results the most credible, a high percentage of subjects should complete the study and their data should ideally be available for the assessment of main outcomes.[26] The current study is a trial with a relatively long intervention period and a long follow-up. To avoid high attrition rate, actions recommended in the literature are planned.[27] The caregivers of participating children will be free to contact a researcher (paediatrician) or a nurse by telephone or email at any time. The researchers and study nurse will contact parents by telephone during the intervention period to ensure that the infant receives the supplement. Visits will be scheduled by telephone 2 weeks in advance, and the parents will receive a reminder text message on the day before the scheduled visit. Transportation cost refund will be offered.

Power calculation

The sample size was calculated for the main outcome of fine and gross motor, cognitive, language, and social-emotional development scores to be measured using the Bayley-III at the age of 12 months.[23] In the previous editions of the test, these elements of the assessment were combined into the PDI and the MDI. To detect a difference of 5 points in the PDI between the study groups with a power of 80% and α =0.05, a sample of 91 infants is needed in each study group. This sample size is based on the assumption that the standard deviation would be 12 points for the PDI in each study group. To account for 20% of loss to follow up, we aim to recruit a total of 220 infants for this study.

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Statistical analysis

The SAS System (SAS System 9.4, SAS Institute Inc., Cary, NC, USA, 2013) will be used for calculations: Power, NPAR1WAY, TTEST and MIXED Procedure. Results will be analysed on an intention-to-treat (ITT) basis. Per-protocol (PP) analysis will be applied to compliers only (>70% of doses taken). The experimental and control groups will be compared in terms of developmental scores by means of the Student t test or the Mann–Whitney test. Repeated–measures analysis of variance will be performed for developmental scores at 12, 24, and 36 months. For categorical variables, Fisher's exact test will be used. In exploratory analysis, 2 factors will be taken into account: gender and birth weight (2500-3000 g vs. >3000 g). Psychomotor development as the main outcome of the study will be adjusted for gestational age, parental education, and socioeconomic status. A two–tailed p–value of the test statistics < 0.05 will be considered significant.

Practical importance of the project

Iron supplementation in infants at risk of iron depletion is an established practice and one of the main objectives of this project is to optimise neurodevelopment by means of preventing anaemia. Exclusive breastfeeding increases the risk of iron deficiency, but controversies exist about whether iron supplementation in this population should be recommended. The results of our project may shed light on these uncertainties and will contribute to optimising child healthcare. If repeated by other research teams, these results may substantially influence early feeding recommendations and practices.

ETHICS AND DISSEMINATION

The Bioethical Committee of The Medical University of Warsaw issued the study approval before recruitment commenced. The findings of this RCT will be submitted to a peer-reviewed journal (paediatric, nutrition, or gastroenterology). Abstracts will be submitted to relevant national and international conferences.

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AUTHORS' CONTRIBUTIONS: AC and HS conceptualised the study. MD and GC contributed to the study design. ZL and AC planned the statistical analysis. AC wrote the first draft of the protocol. All authors read and approved the final version.

FUNDING STATEMENT: This work will be supported by The Medical University of Warsaw. At the time of submission of this protocol for publication, no specific grant from any funding agency in the public, commercial, or not-for-profit sectors has been awarded to this project. The study product will be manufactured by Sequoia Ltd., Poland, free of charge. The company had no influence on the study design and will have no authority over any of the study activities.

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COMPETING INTERESTS STATEMENT: None declared.

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STUDY PROTOCOL: EFFECT OF IRON SUPPLEMENTATION ON PSYCHOMOTOR DEVELOPMENT OF NON-ANAEMIC, EXCLUSIVELY OR PREDOMINANTLY BREASTFED INFANTS: RANDOMISED, CONTROLLED TRIAL.

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ABSTRACT

Introduction

Uncertainty exists regarding the effects of iron supplementation during infancy on neurodevelopmental outcomes in the absence of anaemia. The aim of the study is to establish whether psychomotor and mental development is influenced by early iron supplementation in healthy, non-anaemic, exclusively or predominantly breastfed infants.

Methods and analysis

Healthy term infants will be recruited. If exclusively or predominantly breastfed (>50% of daily feedings) and not anaemic at 4 months, they will be randomised to receive either iron pyrophosphate (approximately 1 mg/kg) or placebo daily until 9 months of age. The primary outcome measure is neurodevelopment assessed with the Bayley Scales of Infant and Toddler Development (Bayley-III) at 12 months and repeated at 24 and 36 months of age. Haematological parameters of iron metabolism also will be measured.

Ethics and dissemination

The Bioethics Committee of the Medical University of Warsaw approved the study protocol before recruitment commenced. Study results will be submitted to peer-reviewed journals in the fields of paediatrics and nutrition and presented at relevant conferences.

Registration: clinicaltrials.gov (NCT02242188).

STRENGTHS AND LIMITATIONS OF THIS STUDY

- A precise clinical question has been posed to fill a gap in knowledge: Does iron supplementation in healthy, non-anaemic, breastfed infants influence their psychomotor and mental development?
- The study design (randomised controlled trial) is the methodology of choice to assess the effectiveness of such an intervention.
- The assessment of development at several time points up to 36 months of age will allow detection of possible long-term effects of iron supplementation.
- A longer follow-up period would be valuable, but probably will not be feasible due to attrition and cost.

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INTRODUCTION

Iron is a nutrient of essential importance to the human organism. It takes part in energy production, oxygen transportation, and DNA synthesis and is indispensable for the development of the central nervous system. Iron is required for the myelination and production of neurotransmitters. It has been well documented that iron deficiency anaemia (IDA) i.e. decreased concentration of haemoglobin and depleted iron stores, is associated with impaired child development.[1-3] If the diagnosis of IDA is delayed, the deficits may be irreversible.[4] A recent Cochrane review stated that iron treatment may improve developmental outcomes in young children with IDA, but the existing evidence is scarce and there is an urgent need for trials assessing long-term effect of this therapy.[5] Iron deficiency (ID) is the most common single nutrient deficiency and may affect up to 20% of children 1 to 3 years of age in Europe.[6] ID has the potential to negatively influence psychomotor development. However, a causal relationship is not as clear as for IDA.[7-9] Exclusive breastfeeding increases the risk of ID, but controversies exist about whether iron supplementation in this population should be recommended.[6,10].

Previous studies have suggested that iron supplementation in healthy infants may enhance psychomotor development. A meta-analysis carried out by our group (Szajewska *et al.*[11]) evaluated the effects of iron supplementation in non-anaemic pregnant women and in non-anaemic healthy children aged <3 years on the mental performance and psychomotor development of the children. Seven randomised controlled trials (RCTs) were identified, 5 of which referred to supplementation during infancy.[12-16] Although the included trials were of relatively good methodological quality (all were blinded, most of them randomized, with proper allocation concealment), the major limitation was incomplete outcome data (>20% lost to follow-up) and risk of selective reporting (study protocols were not available). The pooled results of 3 RCTs (n=561) showed significant improvement on the Psychomotor Development Index (PDI) of the Bayley Scales of Infant Development (BSID) at approximately 12 months of age in the iron-supplemented group compared with the control group (mean difference: 4.21; 95% confidence interval [CI] 2.31 to 6.12). No significant effect of iron supplementation on the Mental Developmental Index (MDI) or behaviour was found. A follow-up study of children aged 9 years

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who participated in a randomised trial during infancy (daily supplementation of iron, zinc, iron and zinc, or placebo at 4 to 6 months of age for 6 months; 92% of subjects available for follow-up evaluation) has been recently published.[15] Cognitive and school performance did not differ significantly between the 4 groups.[17]

Another recently published systematic review aimed to assess effectiveness of oral iron treatment to improve the developmental and hematologic outcomes of preschool children (1-5 years) with non-anaemic ID (normal hemoglobin concentration and depleted iron stores).[18] Two randomized trials were identified (n=69), both of moderate risk of bias due to insufficient information on allocation concealment and open-label intervention in one of them. One of the trials showed a statistically significant difference in the post-treatment MDI score among children who received oral iron therapy compared to no therapy (mean difference : 6.3, 95 % CI 1.5 to 11.0). Authors concluded that the evidence was insufficient to recommend oral iron therapy to school-aged children with non-anaemic ID. Adequately powered, randomized trials are needed in children with non-anaemic iron ID.

Given that ID is a common problem in young children, measures to prevent it are being taken. According to the Committee of Nutrition of the European Society for Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN CoN), this should be achieved by delayed umbilical cord clamping, the use of iron-fortified formulas when formula feeding is needed, the postponement of the introduction of whole cow milk as the main drink until the end of the first year of life, and the promotion of consumption of complementary foods rich in iron.[6] Furthermore, the ESPGHAN CoN concluded that there is no convincing evidence that iron supplements should be provided to normal birth weight, exclusively breastfed infants during the first 6 months of life in populations with a low prevalence of IDA among 6-month-old infants. In contrast, the American Academy of Pediatrics (AAP) recommends iron supplementation (1 mg/kg/day) in exclusively breastfed infants beginning at 4 months of age that should be continued until iron from complementary foods is available.[10] As the level of iron intake is uncertain in partially breastfed infants, the AAP recommends that those who receive more than one half of their daily feedings as human milk also should be supplemented with 1 mg/kg/day of iron beginning at 4 months.[10]

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This considerable difference between European and US guidelines is based on different interpretations of the scarce available evidence. The only study that has previously addressed this issue was performed by, among others, a member of our group (MD). In that study, 101 Swedish and 131 Honduran, non-anaemic, breastfed infants were randomised into 3 groups: placebo from 4 to 9 months, iron supplements (1 mg/kg/day) from 4 to 9 months, or placebo from 4 to 6 months and iron supplements from 6 to 9 months of age.[19] The study showed that iron supplements effectively decreased the risk of IDA at 9 months in Honduran infants, but not in the Swedish infants, who already had a low prevalence of IDA at 9 months (<3%). However, in the Swedish infants who received iron supplements, a negative effect was observed on growth.[20] Unfortunately, these infants were not followed up for longer than to 9 months and neurodevelopmental outcomes were not assessed.

There is, therefore, a clear need for interventional studies of good methodological quality to evaluate the role of iron supplementation in non-anaemic infants on their mental and psychomotor development, as well as on possible adverse effects on growth and infections.[6, 21]

Iron preparations in the form of syrup or drops, which are usually prescribed for the treatment of anaemia in children, have an unpleasant taste, may stain the teeth, and cause adverse effects mostly related to the gastrointestinal tract. Additionally, these preparations need careful measuring to obtain the desired dose.[22] Micronized ferric pyrophosphate is a novel form of microencapsulated iron, which can be packaged in easy-to-use sachets and added to milk or weaning foods. It has been generally recognized as safe (GRAS) as a nutrient supplement in food.[23] Ferric pyrophosphate, marketed as SunActive Fe, has been shown to be effective in infants with anaemia.[24] Its use was less often related to episodes of diarrhoea, vomiting, and teeth staining compared to ferrous glycine sulfate drops.[24] The easy-to-use form of the powder, which can be added to liquids or foods without changing the taste, makes long-term supplementation easier to pursue.[22]

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Trial objectives and hypotheses

The main objective of this trial is to assess the effectiveness of low-dose iron (1 mg/kg/day) supplementation in healthy, term, breastfed infants from 4 to 9 months of age in regard to optimizing their developmental outcomes measured at 12, 24, and 36 months of age. The hypothesis being tested is that psychomotor and mental development will be superior in iron-supplemented children.

Secondary objectives are to assess the effects of iron supplementation on infant growth (weight, length, head circumference) and laboratory parameters of iron status (haemoglobin [Hb], mean corpuscular volume [MCV], haematocrit ([HCT], serum ferritin [s-Ferritin], reticulocyte Hb, hepcidin, and soluble transferrin receptor concentration [sTfR]). Behaviour at 36 months will also be assessed.

METHODS AND ANALYSIS

The trial is registered at www.clinicaltrials.gov (NCT02242188). Any important modifications in the protocol will be entered there.

Study design

Pragmatic, placebo-controlled, blinded, parallel-group, superiority, randomised, placebo-controlled trial.

Setting and participants

Healthy singleton infants will be considered for inclusion before completion of 4 months of age. Parents will be approached either shortly after birth at the obstetrics department (a tertiary care clinical hospital for women: Department of Obstetrics and Gyneacology, The Medical University of Warsaw, Poland) or during well-baby visits at general practitioners' offices located within the community of Warsaw. Those who are eligible will be invited to participate in the study. Parents considering participation will receive oral and written information on the study. A researcher will contact the caregivers by telephone at approximately 3.5 months of age to check for eligibility criteria again. Those exclusively breastfed or predominantly breastfed, i.e., receiving breast milk for over 50% of feedings at the age of 4 months, will be considered for inclusion and invited to the study site. After caregivers provide

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informed consent, an infant will be included and blood will be obtained. Those without anaemia, defined as Hb <105 g/L (< 10.5 g/dL)[5], will be randomised to receive either the iron supplement or placebo from the age of 4 completed months until 9 months of age. Apart from recruitment, all study procedures will be pursued in the Department of Paediatrics, Medical University of Warsaw, Poland.

Inclusion criteria

To be included in the study, a child must be a healthy singleton infant aged 1 day to 4 months old, born at term (37 to 42 weeks of gestation), with a normal birth weight of >2500 g. If approached shortly after delivery, the mother of a child must express the intention to breastfeed. If recruited at an older age, an infant must be breastfed either exclusively or predominantly (>50% feedings). A caregiver must provide written informed consent. This will be obtained by one of two physicians involved in the study.

Exclusion criteria

The following exclusion criteria will be applied: preterm delivery (<37 weeks of gestation), birth weight < 2500 g, multiple pregnancy, major illness or congenital anomaly, being <50% breastfed at the time of inclusion, food allergy, anaemia (Hb <105 g/L [10.5 g/dL]) at inclusion, lack of informed consent, and difficult communication with caregivers.

Randomisation criteria

The participants will be randomised at the age of 4 completed months, after re-checking the inclusion and exclusion criteria, under the condition of being exclusively breastfed or receiving breast milk for at least 50% of their daily feeds at the time of randomisation.

Interventions

Infants in the experimental group will receive the powder preparation of iron pyrophosphate and maltodextrin (Actiferol, SunActive Fe, Sequoia, Poland) in a single daily dose of approximately 1 mg per kilogram of body weight from 4 months to 9 months of age (dose in line with the AAP recommendations). Three doses will be

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used: 7 mg for infants up to 7 kg of body weight, 10 mg for infants from 7 to 10 kg of body weight, and 15 mg for those exceeding the weight of 10 kg. Caregivers will be instructed to administer the daily dose at the same time of a day, after mixing the content of the sachet with a little amount of breastmilk or milk formula. Infants in the placebo group will receive maltodextrin alone. Maltodextrin is almost flavourless, easily digestable polysaccharide commonly used as food additive.

Allocation concealment and blinding

A computer-generated randomisation list prepared by a person unrelated to the trial will be used to allocate subjects to the study groups (A or B) in blocks of ten. Stratification by infant gender will be applied. Consecutive randomisation numbers (each number assigned to intervention or placebo in a blinded way, as described below) will be given to participants at enrolment. This procedure will be performed by one of two physicians involved. The study products will be delivered to the centre in boxes labelled with the letters A and B (meaning of A and B blinded, information deposited in a sealed envelope in a safe at the administrative part of the department). The boxes will also carry the information on the specific dose (7, 10 or 15mg). Subsequently, the letters A and B will be removed from the boxes by an independent person unrelated to the study planning and conduct, and replaced with numeric codes corresponding to the randomisation numbers, e.g. 001 – 7mg, 001 – 10mg, 001 – 15mg, 002 – 7mg, 002 – 10mg, 002 – 15mg etc. Sachets containing the study product will be packed in small packages of 30 pieces each. Neither the collective packages, nor the sachets will carry any labelling and all the unused sachets will always be returned by parents before switching to higher dose. The active product and placebo will be packed in identical sachets and the content will look and taste the same. Researchers, caregivers, outcome assessors, and a person responsible for the statistical analysis will be blinded to the intervention until a statistical report for the 12-months' developmental assessment is available. After that, both the caregivers and main outcome assessor will remain blinded until the completion of the study. The information on intervention assignment will be stored in a sealed envelope in a safe in the administrative part of the department.

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The personal information about potential and enrolled participants will be stored in a locker within the study site, accessible for the involved researchers only.

Compliance

The caregivers will be asked to bring the remaining study product to the study site each time they bring the infant for a check-up visit during the intervention, i.e. at 6 and 9 months of age. Compliance with the study protocol will be checked by counting the number of sachets left unused. Those subjects receiving less than 75% of the recommended doses will be considered noncompliant.

Primary outcome

Psychomotor development at the age of 12 months will be the primary outcome measure. Additional assessments will be performed at 24 and 36 months. The Bayley Scales of Infant and Toddler Development (Bayley-III or BSID-III) will be used.[25] Fine and gross motor, cognitive, language, and social-emotional development scores will be measured with the Bayley-III (in the previous version of this tool [BSID–II] these components of assessment were presented as psychomotor development index [PDI] and mental development index [MDI]). A psychologist qualified and experienced in assessment with use of the Bayley-III will perform the test within one month of the moment a child reaches 12, 24, and 36 months of age. Psychomotor development as the main outcome of the study will be adjusted for gestational age, parental education, and socioeconomic status.

Secondary outcomes

Behaviour

At 3 years, additional screening for behavioural and emotional problems will be performed with use of Child Behaviour Checklist (CBCL), a version for preschool children.[26] This will be applied by the psychologist performing psychomotor development assessment.

Laboratory tests

Haematological status will be assessed at 4, 12, and 24 months. Samples will be analysed for Hb concentration, MCV, reticulocyte Hb concentration, s-Ferritin

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concentration, C-reactive protein (CRP). Novel parameters of iron status such as hepcidin concentration, and soluble transferrin receptor concentration (sTfR) will also be measured. Hepcidin is a recently characterized oligopeptide that has emerged as the master regulator of iron metabolism. [27,28] It has been shown to be closely associated with iron status and iron intakes in infants. [29] Further evaluation of hepcidin and sTfR in children has been listed among the future research directions by the ESPGHAN Committee on Nutrition. [6] Hepcidin will be analysed by ELISA (BioTek ELx800) and sTfR with use of immunoturbidimetric method (COBAS c501, Roche). Blood samples will be taken by paediatric nurses and analysed at the study site.

Iron deficiency anaemia will be defined as Hb concentration <105 g/L (i.e. <10.5 g/dL) and s-Ferritin concentration of <45 pml/L (i.e. <20 mcg/L) at 4 months or <27 pml/L (i.e. <12 mcg/L) for 12 and 24 months.[6] Non-anaemic iron deficiency will be defined as Hb concentration >105 g/L and s-Ferritin concentration <45 pml/L at 4 months or <27 pml/L for subsequent measurements.[6] Children diagnosed with anaemia will be offered clinical evaluation and treatment within the department or referred to a paediatrician.

Growth

Growth parameters (weight, length/height, and head circumference) will be recorded during the follow-up visits at 4, 6, 9, 12, 24, and 36 months of age. Measurements will be performed by paediatric nurses in a standardized way with use of periodically calibrated scales and measuring boards, according to the World Health Organization (WHO) recommendations.[30]

Dietary iron intake

Dietary assessment in terms of iron intake will be performed at 9 months by means of a 3–day, prospective food diary, which will be prepared on a standardized form by the caregiver(s) and delivered to a health provider at the 9-month follow-up visit. Iron intake will be calculated with use of a DIETA 5.D software (version 2015)[31].

Adverse events

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Parents will be asked to fill out a form of possible adverse events of the intervention daily. The symptoms listed in the form will include diarrhoea, vomiting, constipation, discolouration of the stool, fever, and respiratory tract infections. The forms will be collected at each check-up visit during the intervention.

Data collected during the study will be stored safely in a locker accessible only for the researchers involved in the study. Data will be transferred from the paper case report forms to the electronic database. Double data entry will be applied. The final dataset will be available for the authors of this protocol only.

Timeframes of activities during the study

Recruitment for the trial has started in August 2015 and the whole study, including the 36-month follow-up, will last until mid-2018. The time points of all participant-related actions to be taken during the study period are presented in **Table 1.**

Table 1. Timetable of activities planned during the course of the study directly related to participants.

Age (months) / Activity	1	4	5	6	7	8	9	10	11	12	24	36
Enrollment	+											
Randomisation		+										
Intervention			+	+	+	+	+					
Dietary assessment							+					
Anthropometry		+		+			+			+	+	+
Blood withdrawals		+								+	+	
Neurodevelopmental assessment										+	+	+
Behaviour												+

Retention of participants in the study

Retaining participants in a clinical trial of long-term duration represents a substantial difficulty for research teams. To make the obtained results the most credible, a high percentage of subjects should complete the study and their data should ideally be

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available for the assessment of main outcomes.[32] The current study is a trial with a relatively long intervention period and a long follow-up. To avoid high attrition rate, actions recommended in the literature are planned.[33] The caregivers of participating children will be free to contact a researcher (paediatrician) or a nurse by telephone or email at any time. The researchers and study nurse will contact parents by telephone during the intervention period to ensure that the infant receives the supplement. Visits will be scheduled by telephone 2 weeks in advance, and the parents will receive a reminder text message on the day before the scheduled visit. Transportation cost refund will be offered.

Data monitoring

Data monitoring committee has not been established since the intervention within the trial (iron 1mg/kg per day) does not differ from standard of care in infants from risk groups.[6]. The profile of potential side effects is also known.

Power calculation

The sample size was calculated for the main outcome of fine and gross motor, cognitive, language, and social-emotional development scores to be measured using the Bayley-III at the age of 12 months.[25] In the previous editions of the test, these elements of the assessment were combined into the PDI and the MDI. To detect a difference of 5 points in the PDI between the study groups with a power of 80% and α =0.05, a sample of 91 infants is needed in each study group. This sample size is based on the assumption that the standard deviation would be 12 points for the PDI in each study group. To account for 20% of loss to follow up, we aim to recruit a total of 220 infants for this study.

Achieving the target sample size might be challenging within the planned period. If the recruitment pace will be considered too slow, all efforts will be made to increase the number of parents approached mainly by involving additional paediatric practices.

Statistical analysis

The SAS System (SAS System 9.4, SAS Institute Inc., Cary, NC, USA, 2013) will be used for calculations: Power, NPAR1WAY, TTEST and MIXED Procedure. Results

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will be analysed on an intention-to-treat (ITT) basis. Per-protocol (PP) analysis will be applied to compliers only (>75% of doses taken). The experimental and control groups will be compared in terms of developmental scores by means of the Student t test or the Mann–Whitney test. Repeated–measures analysis of variance will be performed for developmental scores at 12, 24, and 36 months. For categorical variables, Fisher's exact test will be used. In exploratory analysis, 2 factors will be taken into account: gender and birth weight (2500-3000 g vs. >3000 g). Psychomotor development as the main outcome of the study will be adjusted for gestational age, parental education, and socioeconomic status. A two–tailed p–value of the test statistics < 0.05 will be considered significant.

Practical importance of the project

Iron supplementation in infants at risk of iron depletion is an established practice and one of the main objectives of this project is to optimise neurodevelopment by means of preventing non-anaemic iron deficiency and anaemia. Exclusive breastfeeding increases the risk of iron deficiency, but controversies exist about whether iron supplementation in this population should be recommended. The results of our project may shed light on these uncertainties and will contribute to optimising child healthcare. If repeated by other research teams, these results may substantially influence early feeding recommendations and practices.

ETHICS AND DISSEMINATION

The Bioethical Committee of The Medical University of Warsaw issued the study approval before recruitment commenced. Any important modifications in the protocol will be communicated to the Committee. The full protocol will be available freely due open access publication. The findings of this RCT will be submitted to a peer-reviewed journal (paediatric, nutrition, or gastroenterology). Abstracts will be submitted to relevant national and international conferences.

AUTHORS' CONTRIBUTIONS: AC and HS conceptualised the study. MD and GC contributed to the study design. ZL and AC planned the statistical analysis. AC wrote the first draft of the protocol. All authors read and approved the final version.

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COMPETING INTERESTS STATEMENT: No, there are no competing interests.

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SPIRIT 2013 Checklist: Recommended items to address in a clinical trial protocol and related documents*

Section/item	Item No	Description	Page
Administrative information			
Title	1	Descriptive title identifying the study design, population, interventions, and, if applicable, trial acronym	1
Trial registration	2a	Trial identifier and registry name. If not yet registered, name of intended registry	2
	2b	All items from the World Health Organization Trial Registration Data Set	included
Protocol version	3	Date and version identifier	1
Funding	4	Sources and types of financial, material, and other support	13
Roles and responsibilities	5a	Names, affiliations, and roles of protocol contributors	1
	5b	Name and contact information for the trial sponsor	13
	5c	Role of study sponsor and funders, if any, in study design; collection, management, analysis, and interpretation of data; writing of the report; and the decision to submit the report for publication, including whether they will have ultimate authority over any of these activities	13
	5d	Composition, roles, and responsibilities of the coordinating centre, steering committee, endpoint adjudication committee, data management team, and other individuals or groups overseeing the trial, if applicable (see Item 21a for data monitoring committee)	NA
Introduction			
Background and rationale	6a	Description of research question and justification for undertaking the trial, including summary of relevant studies (published and unpublished) examining benefits and harms for each intervention	3 - 5
	6b	Explanation for choice of comparators	8
Objectives	7	Specific objectives or hypotheses	5 - 6
Trial design	8	Description of trial design including type of trial (eg, parallel group, crossover, factorial, single group), allocation ratio, and framework (eg, superiority, equivalence, noninferiority, exploratory)	6

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Methods: Participants, interventions, and outcomes			
Study setting	9	Description of study settings (eg, community clinic, academic hospital) and list of countries where data will be collected. Reference to where list of study sites can be obtained	6
Eligibility criteria	10	Inclusion and exclusion criteria for participants. If applicable, eligibility criteria for study centres and individuals who will perform the interventions (eg, surgeons, psychotherapists)	7
Interventions	11a	Interventions for each group with sufficient detail to allow replication, including how and when they will be administered	7
	11b	Criteria for discontinuing or modifying allocated interventions for a given trial participant (eg, drug dose change in response to harms, participant request, or improving/worsening disease)	n/a
	11c	Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return, laboratory tests)	8 -9
	11d	Relevant concomitant care and interventions that are permitted or prohibited during the trial	n/a
Outcomes	12	Primary, secondary, and other outcomes, including the specific measurement variable (eg, systolic blood pressure), analysis metric (eg, change from baseline, final value, time to event), method of aggregation (eg, median, proportion), and time point for each outcome. Explanation of the clinical relevance of chosen efficacy and harm outcomes is strongly recommended	9 - 10
Participant timeline	13	Time schedule of enrolment, interventions (including any run-ins and washouts), assessments, and visits for participants. A schematic diagram is highly recommended (see Figure)	11, Table1
Sample size	14	Estimated number of participants needed to achieve study objectives and how it was determined, including clinical and statistical assumptions supporting any sample size calculations	12
Recruitment	15	Strategies for achieving adequate participant enrolment to reach target sample size	12
Methods: Assignment of interventions (for controlled trials)			
Allocation:			
Sequence generation	16a	Method of generating the allocation sequence (eg, computer-generated random numbers), and list of any factors for stratification. To reduce predictability of a random sequence, details of any planned restriction (eg, blocking) should be provided in a separate document that is unavailable to those who enrol participants or assign interventions	8

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Allocation concealment mechanism	16b	Mechanism of implementing the allocation sequence (eg, central telephone; sequentially numbered, opaque, sealed envelopes), describing any steps to conceal the sequence until interventions are assigned	8
Implementation	16c	Who will generate the allocation sequence, who will enrol participants, and who will assign participants to interventions	8
Blinding (masking)	17a	Who will be blinded after assignment to interventions (eg, trial participants, care providers, outcome assessors, data analysts), and how	8
	17b	If blinded, circumstances under which unblinding is permissible, and procedure for revealing a participant's allocated intervention during the trial	8
Methods: Data collection, management, and analysis			
Data collection methods	18a	Plans for assessment and collection of outcome, baseline, and other trial data, including any related processes to promote data quality (eg, duplicate measurements, training of assessors) and a description of study instruments (eg, questionnaires, laboratory tests) along with their reliability and validity, if known. Reference to where data collection forms can be found, if not in the protocol	8
	18b	Plans to promote participant retention and complete follow-up, including list of any outcome data to be collected for participants who discontinue or deviate from intervention protocols	11
Data management	19	Plans for data entry, coding, security, and storage, including any related processes to promote data quality (eg, double data entry; range checks for data values). Reference to where details of data management procedures can be found, if not in the protocol	10
Statistical methods	20a	Statistical methods for analysing primary and secondary outcomes. Reference to where other details of the statistical analysis plan can be found, if not in the protocol	12
	20b	Methods for any additional analyses (eg, subgroup and adjusted analyses)	12
	20c	Definition of analysis population relating to protocol non-adherence (eg, as randomised analysis), and any statistical methods to handle missing data (eg, multiple imputation)	12
Methods: Monitoring			
Data monitoring	21a	Composition of data monitoring committee (DMC); summary of its role and reporting structure; statement of whether it is independent from the sponsor and competing interests; and reference to where further details about its charter can be found, if not in the protocol. Alternatively, an explanation of why a DMC is not needed	12

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	21b	Description of any interim analyses and stopping guidelines, including who will have access to these interim results and make the final decision to terminate the trial	n/a
Harms	22	Plans for collecting, assessing, reporting, and managing solicited and spontaneously reported adverse events and other unintended effects of trial interventions or trial conduct	10
Auditing	23	Frequency and procedures for auditing trial conduct, if any, and whether the process will be independent from investigators and the sponsor	n/a
Ethics and dissemination			
Research ethics approval	24	Plans for seeking research ethics committee/institutional review board (REC/IRB) approval	2, 13
Protocol amendments	25	Plans for communicating important protocol modifications (eg, changes to eligibility criteria, outcomes, analyses) to relevant parties (eg, investigators, REC/IRBs, trial participants, trial registries, journals, regulators)	6,13
Consent or assent	26a	Who will obtain informed consent or assent from potential trial participants or authorised surrogates, and how (see Item 32)	7
	26b	Additional consent provisions for collection and use of participant data and biological specimens in ancillary studies, if applicable	n/a
Confidentiality	27	How personal information about potential and enrolled participants will be collected, shared, and maintained in order to protect confidentiality before, during, and after the trial	8
Declaration of interests	28	Financial and other competing interests for principal investigators for the overall trial and each study site	13
Access to data	29	Statement of who will have access to the final trial dataset, and disclosure of contractual agreements that limit such access for investigators	10
Ancillary and post-trial care	30	Provisions, if any, for ancillary and post-trial care, and for compensation to those who suffer harm from trial participation	n/a
Dissemination policy	31a	Plans for investigators and sponsor to communicate trial results to participants, healthcare professionals, the public, and other relevant groups (eg, via publication, reporting in results databases, or other data sharing arrangements), including any publication restrictions	13
	31b	Authorship eligibility guidelines and any intended use of professional writers	n/a
	31c	Plans, if any, for granting public access to the full protocol, participant-level dataset, and statistical code	13

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Appendices

Informed consent materials	32	Model consent form and other related documentation given to participants and authorised surrogates	ICD and Informa-tion set in Polish
Biological specimens	33	Plans for collection, laboratory evaluation, and storage of biological specimens for genetic or molecular analysis in the current trial and for future use in ancillary studies, if applicable	

*It is strongly recommended that this checklist be read in conjunction with the SPIRIT 2013 Explanation & Elaboration for important clarification on the items. Amendments to the protocol should be tracked and dated. The SPIRIT checklist is copyrighted by the SPIRIT Group under the Creative Commons "Attribution-NonCommercial-NoDerivs 3.0 Unported" license.

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