Vitamin D_3 supplementation for 8 weeks leads to improved haematological status following the consumption of an iron-fortified breakfast cereal: a double-blind randomised controlled trial in iron-deficient women. (1-2)

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ABSTRACT

The effect of 38 µg (1500 IU) daily vitamin D_3 supplementation, consumed with an iron-fortified breakfast cereal for 8 weeks, on haematological indicators in iron-deficient female subjects was investigated. Fifty iron-deficient subjects (plasma ferritin concentration < 20 µg/L; mean age ± SD: 27.4 ± 9.4 years) were randomised to consume an iron-fortified breakfast cereal containing 9 mg of iron daily, with either a vitamin D_3 supplement or placebo. Blood samples were collected at baseline, interim (4 weeks) and post-intervention (8 weeks) for measurement of iron and vitamin D status biomarkers. The effect of intervention was analysed using mixed-model repeated measures ANOVA. Significant increases were observed in two main haematological indices: haemoglobin concentration and haematocrit level from baseline to post-intervention in the vitamin D group, but not in the placebo group. The increase from baseline to post-intervention in haemoglobin concentration in the vitamin D group (135 ± 11 to 138 ± 10 g/L) was significantly higher compared to the placebo group (131 ± 15 to 128 ± 13 g/L) (P=0.037). The increase in haematocrit level from baseline to post-intervention was also significantly higher in the vitamin D group (42.0 ± 3.0 to 43.8 ± 3.4%) compared to the placebo group (41.2 ± 4.3 to 40.7 ± 3.6%) (P=0.032). Despite the non-significant changes in plasma ferritin concentration, this study demonstrates that 38 µg supplemental vitamin D, consumed daily, with iron-fortified breakfast cereal led to improvement in haemoglobin concentration and haematocrit levels in women with low iron stores. These findings may have therapeutic implications in the recovery of iron status in iron-deficient populations at a healthcare level.

INTRODUCTION

Combatting anaemia or iron deficiency requires a cohesive approach, as its occurrence is suggested to be multifactorial. Iron supplements have been widely used to correct iron deficiency in at-risk groups, whereas in the general population either modification of the diet or iron fortification in selected food vehicles may be implemented [(1)]. Dietary modification requires long-term objectives, and evidence from recent experimental trials has shown that iron fortification, which is considered a medium-term strategy[(2)], will not completely improve general iron status, whilst the efficacy of oral iron supplementation in improving iron status is often limited by low adherence as a result of adverse events following supplementation [(3)]. The measurement of different iron parameters is fundamental to determining of iron status, due to a broad spectrum which extends between iron deficiency and iron overload, which both occur due to the failure of iron homeostasis and causes flaws at functional and structural levels [(4)]. The presence of anaemia can be confirmed with a single biomarker of haemoglobin concentration, whilst measurement of ferritin concentration

is deemed to be the best measurement to identify iron deficiency [5], on condition that there is no presence of inflammation [6]. Hepcidin has been reported to be a systemic iron regulator [7] and a clearer understanding of the interaction between inflammation, erythropoiesis, and hypoxia, which are regulated by hepcidin, may benefit in designing effective iron interventions that may result in fewer adverse events[2]. Understanding of this interaction may require clinical laboratory-based iron interventions that are focused on measuring hepcidin and designed to be implemented in a specific population, instead of the general population[2].

Vitamin D, a secosteroid hormone, which exists in two major forms (D_3 and D_2)[8] has recently been implicated in the stimulation of erythroid precursors and ultimately rate of erythropoiesis[9]. An *in vitro* study carried out in human cell lines demonstrated that administration of 25(OH)D and 1,25-dihydroxyvitamin D ($1,25(OH)_2D$) for 6 hours led to a 50% reduction in hepcidin mRNA expression[10], supporting the postulated hypothesis that hepcidin suppression may facilitate iron status regulation by increasing iron uptake and storage. Evidence from a pilot human study using 7 healthy subjects, supplemented with single oral dose of vitamin D_2 (2500 µg) supports the *in vitro* findings, and showed that serum hepcidin concentration was significantly reduced by 34% following 24 hours ($P<0.05$) and by 33% after 72 hours ($P<0.01$) of supplementation. However, serum ferritin concentration, which signifies iron stores, was found to have significantly decreased[10]. A small sample size limits interpretation of the findings, however, the significant decline observed in serum hepcidin concentration, in addition to the evidence from the *in vitro* study[11] warrants larger scale investigations.

To the best of our knowledge, limited and inconsistent published evidence exists supporting the link between concurrent incidence of iron and vitamin D deficiencies in the normal population, as previous studies were predominantly carried out in kidney disease, heart failure or diabetic patients[12]. There is a paucity of randomised controlled trials, investigating the effect of the vitamin D supplementation administered routinely, as an iron absorption enhancer on iron status, especially in populations at risk of iron deficiency. The present study integrates the two strategies of supplementation and fortification to treat iron deficiency by using vitamin D_3 supplements in combination with the consumption of iron-fortified foods, in premenopausal UK women with low iron stores. Based on existing evidence from observational, human interventions and *in vitro* studies, anaemia was clearly associated with the incidence of vitamin D deficiency, though no clear mechanism has been identified [13]. The proposed theory, however, revolves around the mechanism of action of vitamin D that affects hepcidin expression, pro-inflammatory cytokine production, and rate of erythropoiesis [13; 14; 15; 16]. We hypothesise that vitamin D supplementation will exert further improvement in haematological indices, and the measurement of plasma hepcidin concentrations,

parathyroid hormone (PTH) and vitamin D binding protein (VDBP) concentrations will enable the investigation of a potential mechanism linking vitamin D and iron deficiencies.

SUBJECTS AND METHODS

Subjects

Fifty premenopausal women aged 19-49 years were recruited to the study using posters, emails, and press releases as a recruitment medium at the University of Chester and the wider city of Chester, UK. The inclusion criteria were: females, healthy, and non-pregnant nor lactating. Exclusion criteria were: history of gastrointestinal and metabolic disorders, blood donation within the past 6 months, and regular consumption of nutritional supplements.

Sample size:

Sample size was estimated using serum ferritin concentrations (µg/L) from a double-blind placebo-controlled study carried out in previous study in women aged 20-40 years with serum ferritin concentrations of < 22 µg/L[17]. The study was designed to determine the effect of 8 weeks iron-fortified milk supplementation on iron stores. The study found no significant difference of mean (±SD) serum ferritin concentration at baseline between the iron-fortified group (13.3 ± 6.9 µg/L) and control group (12.6 ± 6.8 µg/L) (P=0.69). At post-intervention, mean (±SD) serum ferritin concentrations were significantly higher (17.7 ± 11.9 µg/l) in the iron-fortified group, compared to 10.6 ± 8.1 µg/L in the control group (P=0.01). With a Cohen's effect size (d) of 0.7, the total sample size required in the present study was 26 per group (power=0.80, α error probability = 0.05). Allowing for a 20% drop-out rate, the total sample size required was estimated to be 62 (31 subjects/group). The sample size was estimated using of G-Power Software (Version 3.1.7).

Recruitment and screening:

A total of 186 women attended the initial screening clinics and 62 were eligible based on the plasma ferritin concentration threshold of < 20 µg/L to define marginal iron deficiency and plasma 25(OH)D concentration of < 250 nmol/L. However, 12 of the eligible subjects withdrew from participating in the study. The reasons were; sickness (n=1), did not respond to the invitation email (n=6), and declined to participate following the screening session (n=5). After screening, 50 subjects were included and randomised.

Study design

A placebo controlled, double-blind randomised controlled trial (RCT) was carried out for a period of 8 weeks. The data collection phase was between September 2015 and April 2016. The study design consisted of 2 phases: **Phase 1** was the recruitment and screening phase when potential

subjects were screened for iron deficiency. **Phase 2** was the intervention phase where all subjects consumed iron-fortified breakfast cereal, together with either vitamin D_3 supplements (vitamin D group) or placebo (placebo group) daily, for 8 weeks. This study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects were approved by the Faculty of Life Sciences Research Ethics Committee (FREC reference: 1078/15/SF/CSN). Written informed consent was obtained from all subjects. The present study was registered at ClinicalTrials.gov (Trial registry number: NCT02714361 - www.clinicaltrials.gov).

Phase 1 (Screening):

All subjects attended a screening clinic to ascertain plasma ferritin concentration, which was used to indicate physiological iron stores in the present study. During the screening clinic, 4 mL of blood was collected in a lithium heparin blood collection tube at the clinical laboratory within the Department of Clinical Sciences & Nutrition, at the University of Chester by the researcher, a trained phlebotomist. The plasma sample collected was used to determine the concentration of ferritin and 25(OH)D. Eligible subjects were then invited to continue to Phase 2 of the study. Subjects who had abnormally high plasma ferritin concentrations from the screening (n=2) were excluded from study and were advised to arrange a consultation with their general practitioner.

Phase 2 (Intervention):

A total of 50 subjects who were eligible to participate in the study were randomised to receive either 38 µg of vitamin D_3 or placebo. Both groups were instructed to consume an iron-fortified breakfast cereals with either vitamin D_3 or a placebo, daily for 8 weeks. The subjects were also provided with UHT semi-skimmed milk and were required to consume 60 g of pre-weighed iron-fortified cereals provided in sealed plastic containers with 200 mL of milk in the morning every day for the duration of the study. Subjects were instructed to consume the vitamin D_3 capsules or placebo capsules with 200 mL of water daily, in the evening, for the duration of the study. All subjects were reminded not to modify their dietary habits and physical activity, in addition, to abstaining from donating blood during the course of the study.

Study clinics:

Subjects were all required to attend clinics after overnight fasts of approximately 8 hours and were expected to attend 3 clinics in total. Subjects were asked to consume only water during the overnight fast. Each clinic lasted approximately 30 minutes and took place between 8-10 am. Details of each clinic are as follows:

Clinic 1 (Week 0, baseline): Height (cm) was measured using a digital stadiometer and body weight (kg) measured using weighing scales, followed by collection of a 30 mL fasted venous blood sample collection. Subjects were given a 3-day food diary to be completed within the first week and returned at the next clinic. A 4-week supply of both iron-fortified breakfast cereals in individually pre-weighed sealed plastic containers and UHT semi-skimmed milk were provided to the subjects, together with a supplement bottle, containing 8 weeks of supplements with assigned subject ID. The protocol was explained to subjects and an email reminder was sent to the subjects one day before the following clinic.

Clinic 2 (Week 4, interim): Body weight was measured and a 30 mL fasted venous blood was drawn. Subjects were given a further 3-day food diary to be completed during the week before the final clinic and the previous food diary was collected. A further 4-week supply of iron-fortified breakfast cereals and UHT semi-skimmed milk was provided to the subjects.

Clinic 3 (Week 8, post-intervention): Body weight was measured and a 30 mL fasted venous blood was drawn. The final 3-day food diary and supplement bottle with any remaining capsules, for assessment of the compliance, were collected.

Randomisation and blinding of subjects

The randomisation process was carried out using computer-generated software (www.randomization.com) by a third party, independent to the study. Subjects (n=50) were randomised to 2 groups: vitamin D_3 (vitamin D group) or placebo (placebo group). The third party allocated 62 capsules of vitamin D_3 and placebo into each identical supplement bottle according to the generated plan. An excess of 6 capsules in each bottle enabled the researcher to estimate compliance once the study was completed. Each tamper proof bottle was then sealed and numbered, ready to be provided to subjects.

The subjects and researcher were blinded to which groups subjects were assigned to. The researcher administered the numbered supplement bottle (i.e; 001) to the subjects based on the sequence that subjects attended their baseline clinic (week 0). The blinding was maintained throughout the study period of 8 weeks and allocation was not unlocked until the end of the data analysis.

Iron-fortified breakfast cereals

The iron-fortified breakfast cereal used in the study was a commercially available whole grain rice and wheat flakes cereal (Weight Watchers UK Ltd, Berkshire, UK). Both breakfast cereal and UHT semi-skimmed milk were provided to the subjects at Clinic 1 and 2. Subjects were instructed to consume one pre-weighed tub of cereal (60 g) containing approximately 9 mg of iron with 200 mL of UHT semi-skimmed milk (Tesco PLC, Hertfordshire, UK), daily. Each subject was also given a measuring cup to measure approximately 200 mL of UHT semi-skimmed milk to consume with the cereal, to ensure standardisation of meal consumed by each subject. It was emphasised during the first clinic that the subjects needed to consume the cereal daily. However, in the event of missing a tub of the provided cereal, it was advised that the cereal was consumed immediately, or double the amount the following day.

Subjects were asked to record and report any problems regarding consumption of the provided cereal during the interim clinic (week 4) and were requested to notify researcher of any adverse events during the course of study. The specific cereal was selected as it is fortified with iron, and previous iron-fortified cereal studies have reported the use of between 7-18 mg of total iron. A total of 9 mg iron in the present study provides approximately 60% of the Reference Nutrient Intake (RNI) per day for women aged 19-50 years[18]. The nutritional content of the breakfast cereal as stated on the product label is shown in **Table 1**.

Vitamin D supplement and placebo

The supplement used was vitamin D_3 cholecalciferol (38 µg, 1500 IU, Pharma Nord ApS, Vejle, Denmark), liquefied in cold-pressed olive oil and encapsulated in a clear soft gel 7 mm diameter capsule. Each capsule was made of a combination of olive oil, gelatine, glycerol, and purified water. The supplements were packaged in blister packs, and manufactured specific to the research requirement and according to pharmaceutical standards (EC and Scandinavia).

The matching placebo was custom-produced to the requirement of the study by Pharma Nord. Placebo was identical to the gel capsule in appearance, size, colour and taste but without the active ingredients. A certificate of analysis provided by the manufacturer confirmed the vitamin D content of supplements (36.6 mcg, with limits 30.4-57 mcg).

Assessment of Compliance

Compliance to the supplementation (%) was estimated as follows: (62 – remaining capsules in the bottle)/56 x 100. For the total duration of 8 weeks, subjects were required to consume 56 capsules of vitamin D_3 or placebo.

Anthropometric measurements

Height (cm) and weight (kg) were measured using electronic scales (Model 875 SECA, Hamburg, Germany) and a wall mounted digital stadiometer (Model 264 SECA, Hamburg, Germany) at baseline and repeated at subsequent clinics as previously described. The instruments used were calibrated before every measurement. Body mass index (BMI) was then calculated using the equation: BMI = weight (kg)/height (m^2) and categorised using threshold values from the WHO.

Blood handling

Both whole blood and plasma obtained from the venepuncture were used for analysis. The blood sample was collected in lithium heparin and EDTA blood collection tubes (BD Company, New Jersey, USA) for blood biomarker analysis. Whole blood was used immediately after each clinic to measure full blood counts (FBC). Venous blood samples collected were centrifuged for 10 minutes (1600 g) at 4°C to obtain plasma samples required for iron and vitamin D biomarker assays, which were then aliquoted into microcentrifuge tubes and stored at -80 °C before being used for analysis. Plasma samples were used to analyse iron status biomarkers (C-reactive protein, ferritin, soluble transferrin receptor (sTfR) and hepcidin) and vitamin D metabolism biomarkers (25(OH)D, PTH, and VDBP) concentrations.

Measurement of iron and vitamin D biomarkers

Whole blood samples were used to measure full blood counts indices using an automated Ac.T diff Haematology Analyser (Beckman Coulter, Inc., Brea, USA). The intra-assay CV for this measurement was 1.9%.

Plasma samples were used for the analysis of ferritin and 25(OH)D concentrations using a miniVIDAS automated immunoanalyser (Biomerieux, Marcy-I'Etoile, France), with the detectable range of > 1.5 µg/l and 20.3 nmol/l respectively. The analytical reliability of 25(OH)D assay in miniVIDAS automated analyser used in the present study achieved the set performance target by Vitamin D External Quality Assessment Scheme (DEQAS, London, UK), with intra-assay CV of 4.7% and 2.0% for ferritin and 25(OH)D, respectively. A number of plasma samples were re-analysed using 25(OH)D ELISA kit (Calbiotech Inc, Spring Valley, USA) (n=32 samples from 19 participants) and human ferritin ELISA kit (Elabscience Biotechnology Co. Ltd, Houston, USA) (n=3 samples from 2 participants), as the concentrations were below the detectable range of the miniVIDAS immunoanalyser. Plasma CRP, hepcidin, sTfR and VDBP concentrations were each measured using commercially available human ELISA kits specific to each biomarker (Quantikine

Human Immunoassay ELISA kit, R&D Systems Inc., Minneapolis, USA) and plasma PTH concentrations were measured using commercially available human ELISA kits (Calbiotech Inc, Spring Valley, USA). The intra-assay CVs were 11.3% (25OHD), 12.9% (Ferritin), 2.7% (CRP), 11.5% (Hepcidin), 5.0% (sTfR), 11.3% (VDBP) and 7.0% (PTH).

Assessment of dietary intake

Subjects were required to complete a 3-day food diary during the first and last weeks of the intervention (to include 2 weekdays and 1 weekend day) to estimate their habitual dietary intake. The diary comprised instructions on how to appropriately record dietary intakes, including a guide to portion sizes, how to describe the foods/drinks in detail, together with a sample diary. In each section of the diary, there were columns for time of consumption, location, description of food/drink consumed, brand and amount/quantity for the subjects to complete. Subjects were also requested to specify any physical activity performed during that particular day. If the foods/drinks consumed were homemade, there were also recipe sections provided for the subjects to specifically note in the diary.

Dietary records were analysed for nutritional content using Nutritics Professional Nutrition Analysis Software (Nutritics Ltd, Dublin, Ireland). The food items used for the analysis were derived from McCance and Widdowson's 'The Composition of Foods'[19]. The DRVs from the Committee on Medical Aspects of Food Policy (COMA) 1991[18] in combination with the Scientific Advisory Committee on Nutrition (2015)[20] were used as a reference to compare the intake of energy, macro, and micronutrients of the subjects.

Statistical Analysis

All statistical analyses were performed using IBM SPSS Statistic Data Editor Software (Version 21) (IBM Corporation, New York, USA). Shapiro-Wilks or Kolmogorov-Smirnov tests were used to determine the normal distribution of data as appropriate. Descriptive statistics were used to describe frequencies, means and standard deviations. Baseline comparisons between the groups (vitamin D and placebo groups) were carried out using independent t-test for normally distributed data or the Mann-Whitney test for non-normally distributed data. Mixed model repeated measures ANOVA was performed to determine the effect of intervention and the interaction with time points for all iron status and vitamin D status blood biomarkers. Post-hoc analyses were carried out when intervention × time point interactions were observed, to identify the differences within or between the groups. Changes in all iron status and vitamin D status blood biomarkers from baseline (week 0) to post-intervention (week 8) between the two groups were compared and analysed using independent t-test for normally distributed data or Mann-Whitney test for non-normally distributed

data. Sub-group analyses (n=32) were carried out in subjects with a plasma ferritin thresholds of <15 µg/L to ascertain whether the severity of iron deficiency influenced subjects' iron status response. Pearson's or Spearman's correlation coefficient tests were performed as appropriate, to investigate the associations between (i) baseline concentration of haemoglobin and 25(OH)D with changes in iron status biomarkers and vitamin D biomarkers and (ii) iron and vitamin D biomarkers. Differences and associations were considered significant with a p-value ≤ 0.05 (two-sided).

RESULTS

Baseline characteristics of subjects

Of a total of 186 women screened, 62 were eligible and 50 women commenced the study. Four subjects dropped out after the first clinic due to feeling unwell (n=2) or without specific reason and did not respond to emails (n=2). A further two subjects dropped out after the interim clinics due to ill health therefore 44 subjects completed the intervention. The baseline characteristics are reported based on the data available for the total of 50 subjects (including drop-outs). **Figure 1** shows a flow diagram of study phases.

The baseline characteristics of subjects are summarised in **Table 2.** The subjects recruited were aged between 19-49 years, with a mean (±SD) age of 27.4 ± 9.4 years. The majority of subjects were white (80%), followed by Asian (12%) and others (8%). The mean (±SD) plasma ferritin and 25(OH)D concentrations were 11.5 ± 5.6 µg/L and 38.3 ± 21.4 nmol/L, respectively. This indicates that the subjects were largely iron deficient (plasma ferritin < 15 µg/L, n=32)[21] and vitamin D deficient (IOM deficient threshold of plasma 25(OH)D <30 nmol/L, n=31)[22] at screening and eligible for the study. The mean (±SD) plasma 25(OH)D concentration did not change from screening to when eligible subjects commenced the study at baseline (P=0.205), which ranged between 5 and 10 days. On the contrary, a higher mean (±SD) plasma ferritin concentration was observed at baseline (13.2 ± 7.8 µg/L) compared to screening (11.5 ± 5.6 µg/L) (P=0.012). The concentrations of both plasma ferritin and 25(OH)D were not corrected for inflammation, as no subjects had elevated plasma CRP concentrations (<10 mg/L) at all time points, indicated by mean of < 4 mg/L in both groups.

There were no significant differences in the subjects' baseline physical characteristics (height, weight and BMI) between the vitamin D group and the placebo group. No significant differences in subjects' iron and vitamin D status (haemoglobin, plasma ferritin, plasma hepcidin and plasma 25(OH)D concentrations) between the vitamin D group and the placebo group at baseline were observed. With reference to dietary intake, no significant differences in energy, protein, carbohydrate, fat, iron, vitamin D, calcium and vitamin C intake between the vitamin D group and the placebo group, except for carbohydrate (as % of energy) were detected. When

excluding drop-outs (n=6) from the analysis, no significant differences in physical characteristics, iron and vitamin D status, and dietary intakes at baseline between the two groups were found.

Different thresholds were used to classify anaemia (haemoglobin < 110 g/L), iron deficiency (plasma ferritin < 15 μg/L) and vitamin D deficiency (plasma 25(OH)D < 30 nmol/L). Prevalence of anaemia, iron deficiency and vitamin D deficiency at baseline were 12% (n=6), 64% (n=32) and 62% (n=31) respectively. It was observed that 13% of the vitamin D deficient (VDD) participants were also anaemic and a higher proportion were iron deficient (ID) (61%). This indicates the possible association between iron status and vitamin D status, which has been shown in previous observational studies.

Compliance and adverse events

Overall mean (±SD) compliance of supplementation was 92.9 ± 8.0%, indicating good compliance. Compliance was similar in both groups with 93.2% in the vitamin D group compared to 92.6% in the placebo group. No subjects reported any adverse events associated with the consumption of supplements.

The consumption of iron-fortified breakfast cereal was examined using the diary recorded by the subjects during the first and last weeks of the intervention. All subjects recorded consumption of the provided iron-fortified cereal at breakfast every day in both diaries indicating good compliance. There were also no adverse events reported by the subjects connected to the cereal consumption.

Effect of vitamin D supplementation on iron and vitamin D status biomarkers

No increase in the concentrations of the two main iron status biomarkers: haemoglobin and haematocrit level from baseline (week 0) to post-intervention (week 8) in the vitamin D group, compared to the placebo group were observed **(Table 3). Figure 2** shows the improvement in haemoglobin concentration (4 g/L) in the vitamin D group was significantly higher compared to the placebo group (-3 g/L) (P=0.037). The improvement in haematocrit level was also significantly higher in the vitamin D group (1.8%) compared to the placebo group (-0.5%) (P=0.032). In the vitamin D group, consumption of vitamin D_3 supplements with the iron-fortified cereals, however, did not impact upon the other iron status biomarkers including red blood cell (RBC), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), plasma ferritin, plasma hepcidin and plasma sTfR, compared to the placebo group.

Effect of vitamin D supplementation on vitamin D status biomarkers

As expected, plasma 25(OH)D concentration was significantly higher in the vitamin D group, compared to the placebo group at 4-weeks and post-intervention, relative to baseline. The increase from baseline to post-intervention in plasma 25(OH)D concentration in the vitamin D group (28.0 nmol/L) was significantly higher than the change in the placebo group (-5.7 nmol/L) (P=0.0001) **(Table 3)**. From the total number of 44 subjects who completed the study, the increase from baseline to 4-weeks in plasma 25(OH)D concentration in the vitamin D group (23.1 nmol/L) was also significantly higher than the change in the placebo group (0.2 nmol/L) (P=0.0001). In addition, the increase at post-intervention from 4-weeks in the vitamin D group (5.0 nmol/L) was also significantly higher than the change in the placebo group (-5.9 nmol/L) (P=0.003) (Mean ±SD not shown in Table 3 and Supplemental Table). However, no significant difference between the vitamin D group and the placebo group in plasma PTH and plasma VDBP concentrations were observed**.**

Time effect of the intervention within each group on iron and vitamin D status biomarkers

In the vitamin D group, mean (±SD) haemoglobin concentration was significantly higher at post-intervention (138 ± 10 g/L) compared to baseline (136 ± 12 g/L) (P=0.035) **(Supplemental Table)**. No significant difference in haemoglobin concentration was observed within the placebo group, between each time point. In the vitamin D group, mean (±SD) haematocrit level was significantly higher at post-intervention (43.8 ± 3.4%) compared to both; baseline (42.5 ± 3.2%, P=0.017) and 4-weeks (42.7 ± 3.2%, P=0.044). No significant difference in haematocrit level was observed within the placebo group, between each time point.

In the vitamin D group, mean (±SD) RBC count was significantly higher at post-intervention (4.8 ± 0.3 $x10^{12}$/L) compared to baseline (4.6 ± 0.3 $x10^{12}$/L) (P=0.007). No difference in RBC count was observed within the placebo group, between each time point. In the vitamin D group, mean (±SD) MCHC was significantly lower at post-intervention (316 ± 10 g/L) compared to both baseline (320 ± 8 g/L, P=0.028) and 4-weeks (320 ± 8 g/L, P=0.032). No difference in MCHC was observed within the placebo group, between each time point.

Difference within groups in MCH was observed in both groups. In the vitamin D group, mean (±SD) MCH was significantly lower at post-intervention (29.1 ± 1.9 pg) compared to 4-weeks (29.3 ± 1.8 pg) (P=0.017). In the placebo group, mean (±SD) MCH was significantly lower at post-intervention (28.1 ± 3.0 pg) compared to both baseline (28.7 ± 3.0 pg, P=0.008) and 4-weeks (28.7 ± 3.0 pg, P=0.0001).

No significant difference was observed within both groups in MCV, plasma ferritin, plasma hepcidin and plasma sTfR concentrations, between each time point. Based on a WHO ferritin normal threshold of >15 µg/L, there were 10 subjects in the vitamin D group who had an increase in their plasma ferritin concentrations from baseline to 4-weeks. The number of subjects who had increased plasma ferritin concentrations was slightly higher (n=13) from the 4-week time point to post-intervention, to an overall mean (±SD) plasma ferritin concentration of 16.0 ± 10.8 µg/L which is within the normal range. A similar trend was observed in the placebo group, however, the majority of the subjects in the placebo group (n=16) remained iron deficient at post-intervention, indicated by an overall mean (±SD) plasma ferritin concentration of 13.8 ± 13.3 µg/L.

A sub-group analysis carried out in subjects who were iron deficient based on a plasma ferritin threshold of <15 µg/L at baseline showed no significant effect of vitamin D_3 supplementation on all biomarkers of iron status was observed.

Association between different variables of iron and vitamin D biomarkers

Baseline haemoglobin concentrations were found to have a significant impact on the subjects' response to iron-fortified breakfast cereal consumption **(Figure 3)**. The more anaemic the subjects were at baseline, the greater the improvement in RBC counts, haemoglobin concentrations and haematocrit levels. Strong and moderate inverse associations between baseline haemoglobin concentration and change in RBC, haemoglobin concentration and haematocrit levels were noted. No significant association was observed between baseline plasma ferritin concentrations with any of the changes in iron status biomarkers . However, we acknowledge that if vitamin D stimulates erythropoiesis, it will draw on both endogenous reserves and stimulate dietary iron and this is indistinguishable in the present study. However, a strong and positive association was observed between plasma ferritin and plasma hepcidin concentrations (r=0.605, P=0.0001, data not shown), indicating the role of hepcidin in suppressing iron uptake once the subjects' iron stores are replete. Positive associations were observed between baseline plasma 25(OH)D concentrations with RBC count, haemoglobin concentration and haematocrit level, as well as a significant inverse association with plasma PTH concentration, as expected **(Table 4)**. Interestingly, improvement in plasma VDBP concentrations were found to be strongly associated with the recovery of several key full blood count indices (Table 4).

Dietary iron intake following vitamin D supplementation and iron-fortified cereal consumption.

Increases in dietary iron intake were expected in both intervention groups, as both received iron-fortified cereals. However, changes in both groups were non-significant. In the vitamin D

group, mean (±SD) dietary iron intake was 16.5 ± 1.8 mg/day at baseline and 16.8 ± 2.3 mg/day at post-intervention (+0.3 mg/day, P=0.6), and in the placebo group, dietary iron intake was 16.6 ± 2.7 mg/day at baseline, and 17.5 ± 2.9 mg/day post-intervention (+1.0 mg/day, P=0.134).

DISCUSSION

We investigated the effect of vitamin D_3 supplementation with a dose of 1500 IU (38 µg), consumed with an iron-fortified breakfast cereal (9 mg) on iron status. To the best of our knowledge, this is the first RCT reporting the increase of two key haematological indices; haemoglobin concentrations and haematocrit levels following consumption of vitamin D with iron-fortified foods, indicated by increased haemoglobin concentrations and haematocrit levels.

Haemoglobin concentrations reflect the presence or absence of anaemia and oxygen-carrying capacity required for normal cell functions, whilst haematocrit levels indicate the proportion of circulating RBCs relative to whole blood total volume. The shifts (increase or decrease) in these two key indices of iron status are typically interrelated[23]. The change in haemoglobin concentration (4 g/L vs -3 g/L, P=0.037) and haematocrit levels (1.8% vs -0.5%, P=0.032) were significantly higher in the vitamin D group as opposed to the placebo group. It was observed that these two biomarkers were improved in the vitamin D group, whilst diminishing in the placebo group, suggesting that daily consumption of iron-fortified cereal with vitamin D supplements, compared to only iron-fortified cereal, may exert an additional beneficial effect on iron status.

Studies carried out in clinical settings among IDA and hypertensive patients [16; 24] or healthy adults [15] observed no improvement in haemoglobin, or serum ferritin concentrations. However, haemoglobin concentration and haematocrit levels were found to be significantly higher in the intervention group at 8 weeks in a iron/vitamin D fortification study by Toxqui *et al.* [25], but no significant impact on any iron status biomarkers was observed in a calcium/vitamin D fortification study by Hennigar *et al.* [26]. Mean (±SD) baseline concentration of haemoglobin was 134 ± 14 g/L, and 7 of the subjects (n=50, vitamin D=3, placebo=4) were anaemic based on the WHO threshold of <120 g/L[21].

A greater improvement in these biomarkers may have been observed with a higher proportion of anaemic subjects at baseline, however, it was evident in the present study that the baseline concentration of haemoglobin dictates the subjects' response to the intervention. It was previously demonstrated in a meta-analyses by Casgrain *et al.* [27] that participants' response to iron intervention vastly depends on their initial iron status, and that the improvement is greater in anaemic participants, as the iron requirements are higher due to physiological demands. The mean baseline concentration of plasma 25(OH)D was also found to influence the response to intervention. Greater improvement was observed in the RBC counts, haematocrit levels, and haemoglobin concentrations, if the subjects were vitamin D deficient at baseline. Factors such as (i) amounts/form of iron and vitamin D used in the study (low/high/none or

fortification/supplementation); (ii) duration of interventions; or (iii) baseline characteristic that defines anaemia or vitamin D deficiency by using different thresholds, contributes to inconsistencies in published findings.

The active metabolite of vitamin D, calcitriol has recently been demonstrated in pilot human studies to play a role in suppression of the hepcidin expression, which is dictated by a gene known as antimicrobial peptide (HAMP) [10; 14]. Vitamin D has been demonstrated in previous studies to be capable of increasing proliferation of erythroid precursors in the bone marrow to support erythropoiesis by decreasing the expression of pro-inflammatory cytokines which cause the suppression of hepcidin. Decreased cytokines and suppressed hepcidin leads to higher iron bioavailability for RBC production and haemoglobin synthesis [14].

The mechanism of action of vitamin D in exerting additional effects on the recovery on iron status was proposed to revolve around suppression of hepcidin expression, pro-inflammatory cytokine production, and rate of erythropoiesis. Under normal circumstances, iron homeostasis involves the circulation of transferrin-iron complexes that move to the bone marrow to produce red blood cells in erythropoiesis. The senescent erythrocytes will degenerate and will be engulfed by macrophages. Iron is then reutilised and released back into the circulation to repeat the same erythropoiesis process [14]. Depending upon physiological demands, haem and non-haem dietary iron will enter the labile iron pool from intestinal iron uptake and when increased concentrations of pro-inflammatory cytokines are present, production of the RBCs in the bone marrow is suppressed. This will then lower half-life of RBCs as a result of elevated macrophages and phagocytic activity activation. IL-6 and IL-1β are among the cytokines that are capable of stimulating the liver into increasing production of the *HAMP* gene, which leads to increased or decreased iron uptake [14].

Observational studies in different populations previously reported the concurrent incidence of both vitamin D deficiency and anaemia[12; 28]. Whilst it is evident that hepcidin is the principal iron regulator, the underpinning mechanism of the action of vitamin D on iron status is unclear as there is very limited evidence in humans, and is largely from *in vitro* and *in vivo* studies.

We hypothesise that the action of vitamin D on the recovery of iron status occurs via a mechanism by which vitamin D suppresses plasma hepcidin expression, leading to an increase in ferroportin availability for iron uptake, and ultimately increases plasma ferritin concentration in subjects with low iron stores. A recent *in vivo* study observed that a single bolus oral ingestion of 2500 µg vitamin D_2 led to a significant decrease in serum hepcidin concentrations at 24 hours (34%, $P<0.05$) and 72 hours (33%, $P<0.01$)[10]. Vitamin D also potently decreased transcription of the *HAMP* gene that regulates hepcidin expression, however, no significant improvement in iron stores was observed following supplementation, which is likely due to the inclusion of healthy iron replete

subjects at baseline in the study. A further *in vitro* study reported at least a 15-fold suppression of the *HAMP* gene in a monocyte cell line (THP-1) after 6 hours of treatment with $1,25(OH)_2D$[11], indicating the direct action of vitamin D on *HAMP* gene transcription. The *in vivo* part of the study in which 38 kidney patients were supplemented with vitamin D_3 (1250 µg) found a moderate, significant, negative association between the serum hepcidin and serum 25(OH)D concentrations (r=-0.38, P=0.02)[11].

The findings from the present study showed that vitamin D supplementation led to improvement in two key indices of iron status: haemoglobin and haematocrit, and are consistent with limited published evidence[25].

Hepcidin expression was suppressed in a vitamin D supplementation study (carried out without any iron intervention)[14] and in an earlier study[10] in an attempt to investigate the underlying mechanism behind the action of vitamin D on iron regulation. Both studies did not observe a significant effect of the vitamin D intervention on the improvement of iron stores, indicated by plasma ferritin concentrations, which is consistent with the present study. On the contrary, the present study did not observe any significant influence of vitamin D intervention on hepcidin expression, as reported in previous studies[10; 14]. However, 5 subjects had increased haemoglobin concentrations, haematocrit levels and simultaneously decreased hepcidin concentrations. In this group of subjects, mean (±SD) haemoglobin concentration and haematocrit level were significantly higher at post-intervention (137 ± 15 g/L; 44.1 ± 5.0%) compared to baseline (127 ± 13 g/L; 39.5 ± 3.2%) (P=0.042; 0.043), simultaneously with significant reduction in hepcidin concentration from 8.1 ± 8.0 ng/mL at baseline to 3.1 ± 3.3 ng/mL at post-intervention (P=0.043). However, 5 subjects who had increases in both haemoglobin concentrations and haematocrit levels did not show a reduction in hepcidin concentrations.

The present study observed a strong association between plasma hepcidin and plasma ferritin concentrations, however, both biomarkers were not affected by vitamin D supplementation. The vitamin D_3 dose used in the present study may have been a limitation and was not sufficient to have substantially affected hepcidin expression, thus, no improvement was observed in the recovery of iron stores, indicated by plasma ferritin concentrations. Also, the vitamin D used in the present study may have not been sufficient to act on both biomarkers in a relatively short study duration of 8-weeks, to allow for a substantial effect to be observed.

An intriguing finding in the present study was the strong link between VDBP concentration and iron status improvement, which has not been identified previously. Improvement in plasma VDBP concentrations were strongly associated with the recovery of the RBC counts (r=0.653, P=0.002), MCV (r=0.612, P=0.004), haematocrit levels (r=0.751, P=0.0001), haemoglobin

(r=0.638, P=0.002) and MCH concentrations (r=-0.592, P=0.006). On the basis of this observation, further substantial restoration may be observed in other iron status biomarkers, especially plasma hepcidin and plasma ferritin concentrations, if a higher dose of vitamin D is used. Similar studies by Bacchetta et al.[10] and Smith et al. [14] used much higher doses of vitamin D in single boluses of 2500 ug vitamin D2 and 6250 ug vitamin D3, respectively. VDBP is known for its role as a 25(OH)D transporter, and , and Dastani et al.[29] previously previously demonstrated an association between 25(OH)D and VDBP. This observation is sufficient to connect the action of vitamin D with improvement of iron status, and warrants further investigation.

The subjects' energy, macronutrients, iron, and vitamin D intake were unchanged from baseline to post-intervention. Interestingly, mean daily iron intake of subjects, in particular, was 1.7-fold higher at baseline, relative to typical iron intake of adult women reported in the UK NDNS[30]. This increased intake remained higher at post-intervention. This may be due to the fact that participants may have increased their intake of iron-containing foods after being informed of their iron-deficient status following screening but before they commenced the study. Despite reduction in the placebo group, the present study also demonstrated the effectiveness of iron-fortified breakfast cereal (irrespective of vitamin D intervention) on raising principal iron biomarkers which is in agreement with recent findings[31; 32; 33; 34].

The present study shows that concurrent incidence of both vitamin D and iron deficiency exists in women with low iron stores, and the clinical aspects of the findings may potentially be applied to the recovery of iron status in iron deficient population at a healthcare level through the therapeutic use of vitamin D supplementation as a novel iron absorption enhancer. Iron-fortified breakfast cereals were found to be efficient as a food fortification vehicle, which could be a suitable adjunct in management of iron deficiency. One limitation of the study was the failure to attain required sample size, with a shortfall of 8 participants due to 12 eligible subjects who decided not to partake in the study following screening. A major strength of present study is the double-blind randomised controlled trial study design, carried out in a specific population of marginally low and low iron stores, which allows interpretation of findings to be extrapolated in clinical settings and included specific measurement of vitamin D biomarkers which was not measured in previous studies.

In conclusion, vitamin D, consumed daily in supplement form, at a dose of 38 µg (1500 IU), was shown to lead to the recovery of haemoglobin concentrations and haematocrit levels in female subjects with marginal and low iron stores. The finding in the present study that shows baseline concentration of haemoglobin plays a role in dictating subjects' response to intervention adds to and strengthens the existing published literature. The study also demonstrated concurrent incidence of

both iron and vitamin D deficiencies in subjects, supporting the findings from observational studies carried out in various settings, and was the basis of the postulated hypothesis related to vitamin D mechanism of action on iron status regulation. A strong link between VDBP upregulation and iron status improvement, which has not been recognised previously, was also identified and merits further investigation.

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686 Figure legends

687 Figure 1. Flow diagram of the screening and intervention phase.

688

689 Figure 2. Effect of intervention on mean (±SD) haemoglobin concentration and haematocrit level
690 from baseline (week 0) to post-intervention (week 8). Error bars represent SD. Mean change in
691 haemoglobin concentration (+4 g/dL) and haematocrit level (+1.8%) are significantly higher in the
692 vitamin D group compared to placebo group. *Represents significant difference from placebo group
693 ($P < 0.05$).

694

695 Figure 3. Association between baseline haemoglobin concentrations, and change from baseline
696 (week 0) to post-intervention (week 8) in (a) RBC count (b) haemoglobin concentration (c)
697 haematocrit level (n=44)

698 Table 1. Nutritional composition of iron-fortified breakfast cereal

Nutrient	Per 100 g	Per recommended serving (60 g)
Energy (kJ/kcal)	1566/370	940/222
Fat (g)	2.1	1.2
Saturated	0.4	0.2
Carbohydrates (g)	70	42
Sugars	16	9.6
Fibre (g)	7.5	4.6
Protein (g)	14	8.4
Sodium (mg)	240	160 mg
Iron (mg)	15.0	9

699 *Nutritional composition information sourced from the product label

Table 2. Baseline physical characteristics, iron and vitamin D status and dietary intake of subjects (n=50)

Variables	**All (n=50)**		**Vitamin D group (n=25)**		**Placebo group (n=25)**		**P-value**
	Mean	SD	Mean	SD	Mean	SD	
Physical characteristics							
Age (years)	27.4	9.4	28.0	9.0	26.9	9.9	0.280
BMI (kg/m^2)	24.4	4.8	24.9	5.7	24.0	3.7	0.930
Iron & vitamin D status							
Haemoglobin (g/L)	134	14	136	12	132	15	0.210
Plasma ferritin (µg/L)	13.2	7.8	14.1	7.7	12.4	8.0	0.467
Plasma hepcidin (x10^{3}ng/L)	3.5	4.3	4.2	5.7	2.9	2.2	0.968
Plasma 25(OH)D (nmol/L)	36.8	23.6	35.0	19.8	38.6	27.2	0.992
Mean daily dietary intake							
Energy (MJ)	6.9	1.6	7.2	1.3	6.8	1.8	0.404
Protein (g)	70.0	16.4	74.7	16.4	66.1	15.6	0.155
Carbohydrate (%)	51.8	6.3	49.5	6.3	53.6	5.7	*0.025
Fats (%)	30.6	7.0	31.1	6.8	30.1	7.2	0.626
Iron (mg)	16.4	2.3	16.5	1.8	16.4	2.6	0.108
Vitamin D (µg)	1.7	1.6	1.4	1.0	1.8	2.0	0.813
Calcium (mg)	813.3	246.8	817.8	170.1	858.4	300.8	0.816
Vitamin C (mg)	83.5	43.6	81.7	42.6	85.1	39.9	0.482

P-values in **bold** represent a significant difference between the vitamin D and the placebo group at baseline. Independent t-test or the Mann-Whitney test was used depending on normal distribution of data.

Table 3. Effect of intervention on iron and vitamin D status biomarkers from baseline to post-intervention (n=44)

Biomarkers	**Vitamin D group**						**Placebo group**						**P-value**
	Baseline		**Post -intervention**		**Change**		**Baseline**		**Post-intervention**		**Change**		
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	
Hb (g/L)	135	11	138	10	4	7	131	15	128	13	-3	12	***0.037**
Hct (%)	42.0	3.0	43.8	3.4	1.8	3.1	41.2	4.3	40.7	3.6	-0.5	3.7	***0.032**
RBC (x10^{12}/L)	4.6	0.3	4.8	0.3	0.2	0.2	4.6	0.4	4.6	0.3	-0.0	0.4	0.055
MCV (fL)	91.3	5.1	91.9	5.7	0.7	3.3	89.7	7.4	89.0	7.8	-0.6	3.1	0.117
MCH (pg)	29.3	2.0	29.1	1.9	-0.2	0.6	28.6	3.0	28.1	3.0	-0.4	0.7	0.328
MCHC (g/L)	321	7	316	10	-5	10	318	11	315	11	-3	9	0.425
Fer (µg/L)	14.1	8.2	16.0	10.8	1.9	11.1	12.3	8.1	13.8	13.3	1.5	9.1	0.540
Hep (x10^{3}ng/L)	3.7	4.9	4.1	4.1	0.5	5.8	2.9	2.2	3.4	5.6	0.6	4.5	0.451
sTfR (mg/L)	1.6	0.7	1.8	1.0	0.2	0.3	1.9	1.2	2.0	1.4	0.1	0.3	0.724
CRP (mg/L)	2.1	2.1	2.8	3.2	0.6	2.9	3.1	3.3	3.2	3.8	0.1	4.1	0.642
25(OH)D (nmol/L)	35.2	18.4	62.2	16.1	28.0	28.0	39.3	27.6	34.2	23.6	-5.7	9.7	***0.0001**
PTH (pmol/L)	8.3	8.9	7.2	7.8	-1.1	2.0	7.8	4.4	7.6	3.7	-0.2	2.6	0.198
VDBP (x10^{3}µg/L)	294.9	131.2	289.1	118.7	-5.89	52.2	374.0	144.0	382.7	138.1	8.6	62.2	0.413

Hb, haemoglobin; Hct, haematocrit; RBC, red blood cells; MCV, mean corpuscular volume; MCH, mean corpuscular haemoglobin; MCHC, mean corpuscular haemoglobin concentration; Fer, ferritin; Hep, hepcidin; sTfR, soluble transferrin receptor; 25(OH)D, 25-hydroxy vitamin D; PTH, parathyroid hormone; VDBP, vitamin D binding protein.

P-values in **bold** represent a significant difference in changes from baseline (week 0) to post-intervention (week 8) observed between the vitamin D and placebo groups. Independent t-test or the Mann-Whitney test was used depending on normal distribution of data.

Table 4. Association between iron and vitamin D biomarkers (n=44)

	Baseline 25(OH)D (nmol/L)		VDBP (µg/mL)	
	r	**p**	**r**	**p**
RBC (x10^{12}/L)	0.339	0.0001	0.653	0.002
Hb (g/L)	0.208	0.014	0.638	0.002
Hct (%)	0.199	0.018	0.751	0.0001
PTH (pmol/L)	-0.229	0.006	-	
MCV (fL)	-		0.612	0.004
MCH (pg)	-		-0.592	0.006

Hb, haemoglobin; Hct, haematocrit; RBC, red blood cells; MCV, mean corpuscular volume; MCH, mean corpuscular haemoglobin; 25(OH)D, 25-hydroxy vitamin D; PTH, parathyroid hormone; VDBP, vitamin D binding protein.

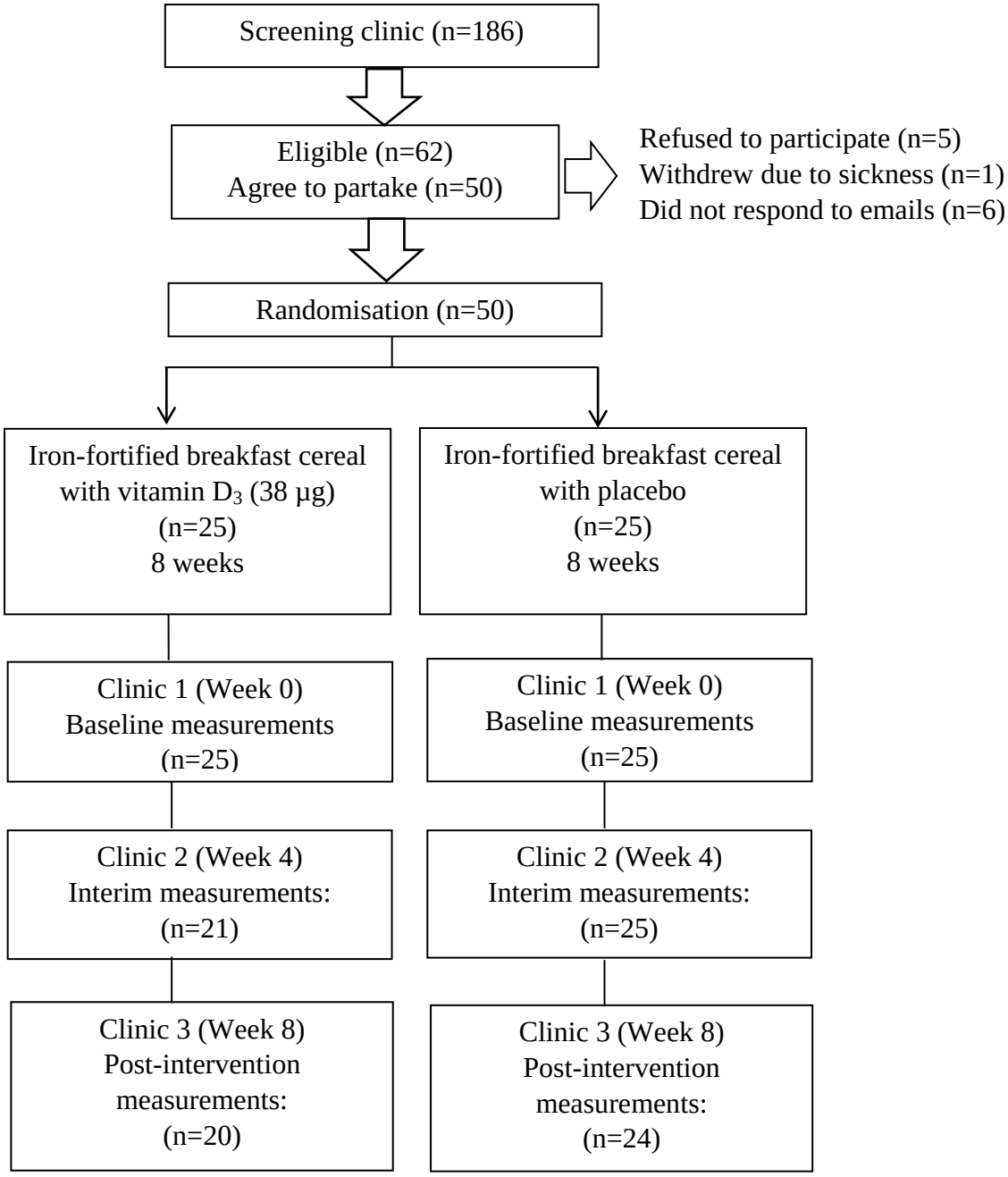

Figure 1. Flow diagram of subjects in the screening and intervention phase

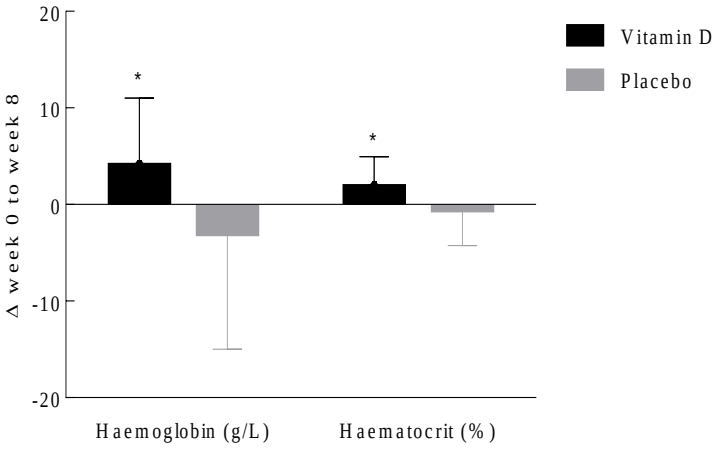

Figure 2. Effect of intervention on mean (±SD) haemoglobin concentration and haematocrit level from baseline (week 0) to post-intervention (week 8). Error bars represent SD. Mean change in haemoglobin concentration (+4 g/L) and haematocrit level (+1.8%) are significantly higher in the vitamin D group compared to placebo group. *Represents significant difference from placebo group.

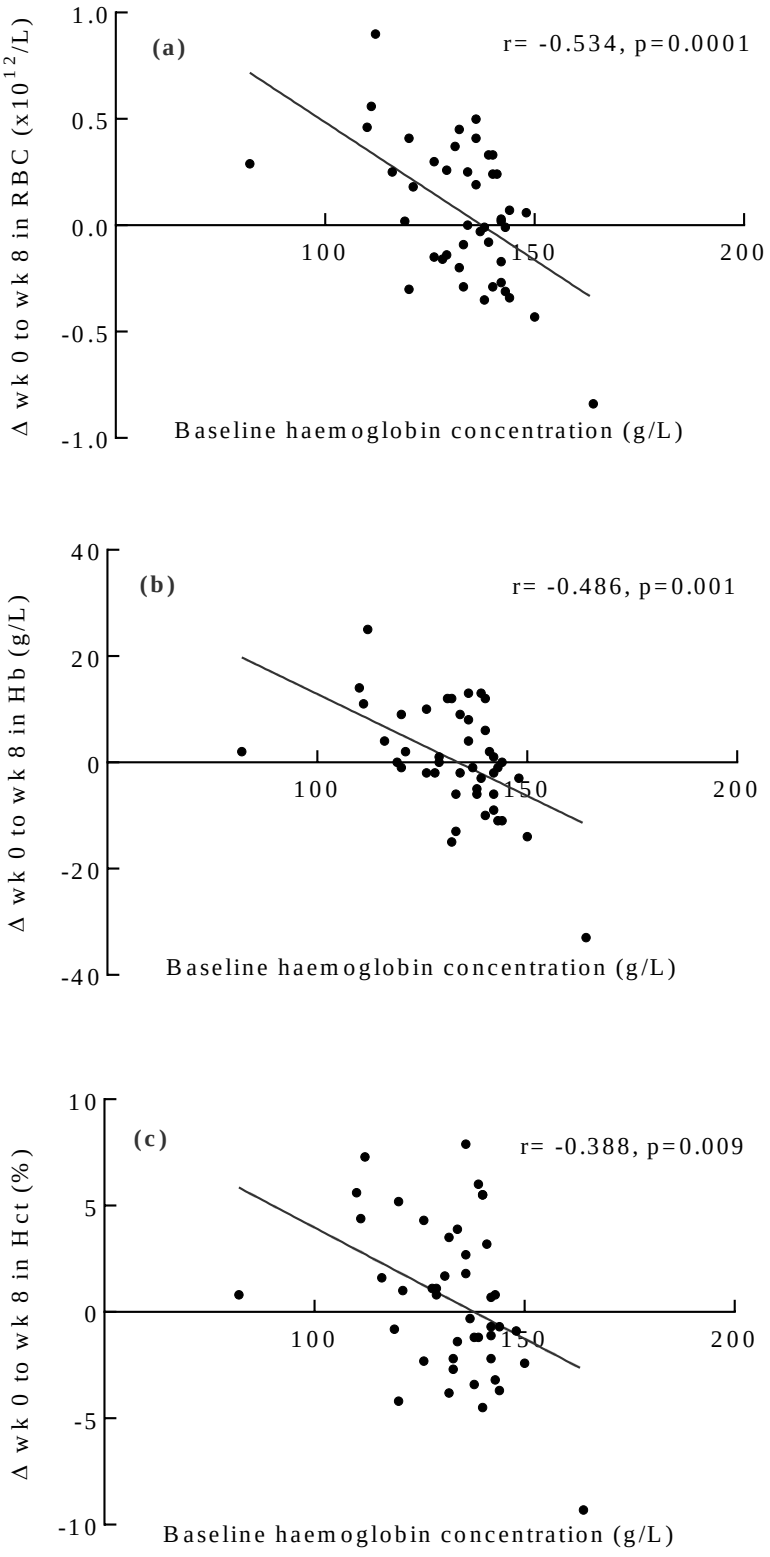

Figure 3. Association between baseline haemoglobin concentrations, and change from baseline (week 0) to post-intervention (week 8) in (a) RBC count (b) haemoglobin concentration (c) haematocrit level (n=44)