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Randomized Control Trials

Daily oral iron supplementation produced greater improvements in hematological parameters than alternate day doses – A pilot double-blind randomized control trial in iron-deficient young women

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SUMMARY

Background and aims: There is little universal consensus on the optimal regime for oral iron supplementation to treat iron deficiency (ID) and iron-deficiency anemia (IDA) in women of reproductive age (WRA). A few studies in a high-income country have reported higher fractional absorption from oral iron supplements (OIS) given on alternate days than daily doses; however, there were no significant improvements in hematological indices in the women in these studies who received the alternate-day doses. There are also concerns about adverse gastrointestinal effects resulting from daily OIS. Data on these aspects from low and middle-income countries (LMIC), where the burden of IDA is high, are limited.

Methods: We conducted a double-blinded, parallel-arm, non-inferiority, randomized controlled trial in non-pregnant WRA aged 18–45 years with ID (serum ferritin <20 μg/L) (CTRI/2020/03/024144). They were randomized to receive either 60 mg elemental iron daily (n = 30) or 120 mg elemental iron on alternate days (n = 30) for 14 days. The primary outcome was to determine the comparative effectiveness of daily versus alternate-day OIS in improving hematological and iron-related parameters in blood, at the end of the intervention. Secondary outcomes included extent of adherence to intervention, adverse events experienced, and changes in fecal calprotectin concentrations (a marker of gut inflammation) and the gut microbiome profile.

Results: Adherence to the regimes was excellent (≥90 %) in both arms. Both regimes significantly improved hematological and iron-related parameters in blood at the end of 14 days. Daily OIS resulted in greater increases in mean corpuscular volume (fL) [1.25 (0.25, 2.32) vs. 0.50 (−0.35, 1.42); $p = 0.043$], mean corpuscular hemoglobin (pg/cell) [0.52 (0.54) vs. 0.17 (0.56); $p = 0.019$], and reticulocyte counts (%) [0.32 (0.13, 0.75) vs. 0.27 (0.02, 0.45); $p = 0.055$] than alternate-day doses. There were no significant differences between the groups in extent of improvements in iron-related parameters, incidence of adverse effects, and effects on gut inflammation and microbiome profile.

Conclusion: In iron-deficient WRA in an LMIC setting, daily OIS (60 mg) for 14 days was more effective than equivalent amounts on alternate days in improving hematological parameters.

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1. Introduction

Globally, anemia affects approximately 30 % of women of reproductive age (WRA) and 40 % of children aged 6–59 months [1]. In India, the prevalence of anemia in these groups was 57 % and 67.1 %, respectively [2]. Iron deficiency (ID) is the commonest cause of anemia worldwide [3]. Children aged below 5 and WRA bear the brunt of this burden, due to high demands for iron in these groups [3–5].

Oral iron supplements (OIS) (in the form of bioavailable ferrous salts) are effective in treating such deficiency and mild-to-moderate anemia. Doses ranging from 40 mg to 200 mg of elemental iron per day have been used for this purpose [6,7]. Only about 10 % of an oral dose of iron is usually absorbed [8]. High doses are commonly used because of such low absorption rates [6,7]. Thus, OIS often results in the intestinal lumen being exposed to large amounts of unabsorbed iron. Such exposure has been shown to produce deleterious intestinal effects, such as inflammation and increased production of free radicals [9–11].

The microbial flora in the gastrointestinal tract has also been shown to be affected by high concentrations of iron. Gut dysbiosis, with decreases in commensal bacteria and increases in pathogenic strains, has been reported in the presence of increased amounts of iron in the gut of African infants [12,13] and due to iron fortification in African children [14] and infants [12]. A rise in fecal calprotectin has been reported after iron fortification, suggesting the development of gut inflammation [12,14]. Studies examining dysbiosis due to OIS in adults are limited and report variable findings. Premenopausal women with iron-deficiency anemia (IDA), who received OIS were found to have significantly higher numbers of the commensals [15]. Another study on women (aged 40–65 years), showed that high-dose OIS (>100 mg/day) increased pathogenic *Proteobacteria* and reduced beneficial taxa [16]. Overweight and obese pregnant women on OIS (>60 mg/day) had a significantly lower abundance of beneficial bacteria at 16 weeks of gestation [17]. Such reported effects may contribute to adverse gastrointestinal effects (sometimes seen in response to OIS) [18], often resulting in poor patient compliance, thus reducing the therapeutic efficacy of OIS.

Hepcidin, the key regulator of systemic iron homeostasis, is produced by the liver in response to increased iron stores and elevated concentrations of transferrin-bound iron in circulation [19]. It binds to ferroportin, involved in cellular iron efflux, leading to its internalization and subsequent degradation. When this occurs in enterocytes, it inhibits iron absorption from the gut [20]. High doses of OIS have been shown to result in elevated concentrations of hepcidin in blood [14]. Such elevated concentrations were found 6 h after an oral iron dose and returned to baseline values 24 h later [21]. Increases in serum hepcidin have been reported to result in reduced absorption of oral iron, when administered as daily or twice-daily doses [21].

Fractional iron absorption (FIA) is the proportion of an oral dose of iron that is absorbed [22]. It is influenced by concentrations of circulating hepcidin. Iron absorption was reported to be increased when serum hepcidin concentration was below 3 nmol/L and serum ferritin was below 51 μg/L [23], highlighting how serum hepcidin and ferritin concentrations affect the extent to which oral iron is absorbed. Studies have shown that fractional iron absorption was higher with OIS given on alternate days, than with daily doses [24,25]. However, these studies have several limitations in that they were short-term studies on European women only, were not blinded, sample sizes were small (ranging from 13 to 40), were done in highly controlled environments in a high-income country and they did not measure meaningful clinical endpoints [21,24–26]. In a subsequent randomized controlled trial (RCT), these investigators reported that increases in serum ferritin were similar in the groups that received daily or alternate day iron, at time points when both groups had received equivalent amounts of iron (day 93 for the daily iron group and day 186 for the alternate-day group) [27]. Hemoglobin concentration on day 93 were significantly higher in the daily iron supplementation group than in the alternate-day group. No significant differences in serum ferritin and hemoglobin concentrations were found between the groups at other time points studied (days 46, 139, and 186). The incidence of gastrointestinal side effects was reported to be lower in the alternate-day group [27].

There is limited data from low and middle-income countries (LMICs), such as India, on the comparative effectiveness of alternate and daily OIS. Such data is important, as these countries bear a significant brunt of the burden of anemia. Indian studies in this area, currently available in the published literature, are quite heterogeneous and possess several methodological limitations [28–32]. Some of these studied males and females [30–32] or pregnant women [28], without considering differing iron requirements and responses in the 2 sexes or during pregnancy. Most of them were non-blinded [28–31]. In one, OIS were given after meals, without controlling for the effect of dietary components on iron absorption [31]. The cut-off values used to define ID varied among the studies (ranging from 20 to 50 μg/L) [29,30,32]. Participants in these studies had varying degrees of anemia, ranging from mild to severe, thus making interpretations of the results and comparisons across studies difficult [28–30]. Furthermore, these studies only estimated hemoglobin, ferritin, and hepcidin in blood; they did not investigate other markers of anemia or iron status or inflammation, all of which are relevant in this context. Gut microbiome profiles and markers of gut inflammation in response to supplementation were also not studied. These factors limit the wider applicability and relevance of the results reported.

Banerjee et al. [33] (2024), in their systematic review, recommended that intermittent iron supplementation (administered once, twice, or three times a week, or on alternate days) was more effective in improving hemoglobin concentrations and iron status of pregnant women than those taking no iron [33]. They, however, showed no comparison with those who took daily doses. Some studies have shown that alternate day dosing is as good as daily supplementation [32,34], while another showed that daily doses of iron were more effective in improving hematological parameters in pregnant women [28,35]. However, many of these studies have major limitations, as has been detailed earlier. Hence, robust evidence on whether daily or alternate-day oral iron supplementation is more effective is still lacking.

Given the above background, the objective of the present study was to determine the comparative effectiveness of daily and alternate days of OIS in improving hematological and iron-related parameters in blood in iron-deficient Indian WRA. Their responses to these supplementation regimens and effects on the gut microbiome profile were studied.

2. Methods

Ethical approval for the study was obtained from the Institutional Review Board of Christian Medical College, Vellore, India (IRB MIN no: 12457 [INTERVEN] dated 25th Jan 2020). The trial was registered with the Clinical Trials Registry- India (CTRI/2020/03/024144). The study was initiated on 27th May 2020 and completed on 24th May 2021.

Women of reproductive age, who presented with minor health complaints at a clinic of the institution where the study was carried out, were screened for eligibility. Inclusion criteria were

women with iron deficiency (with serum ferritin concentrations less than 20 μg/L) aged between 18 and 40 years, who were not pregnant, with a body mass index (BMI) ranging from 18 to 30 kg/m^2. Exclusion criteria included presence of current or recent major illnesses or conditions (e.g., inflammatory illness, diabetes mellitus, endocrine disorders, etc) that could interfere with study outcomes, those with infections or febrile illnesses in the previous two weeks, those already on iron supplements or who had taken them in the last 2 weeks, those taking dietary supplements containing vitamins or minerals, or herbal or other plant-based preparations, or those with serum CRP concentrations >6 mg/L or those who had donated blood recently. Additional exclusion criteria for the study are described in the supplementary file. They were recruited after obtaining written informed consent (n = 30, in each group).

Figure 1 shows the consort flow chart for the study. As this was a pilot study, a sample size of 30 participants per group was used (Fig. 1). At baseline, blood and stool samples were collected from each participant. They were then randomized to receive oral elemental iron, either 60 mg daily or 120 mg on alternate days, for 14 days (placebo with 0 mg iron on alternate days), under fasting conditions and with no food intake for at least 30 min afterwards. Details of randomization, allocation concealment, blinding and administration of intervention are described in the supplementary file. Daily follow-up calls were made to ensure compliance and to monitor adverse effects. On day 15, blood and feces samples were obtained, as done at baseline (Supplementary Fig. 1). The primary outcome was to assess the changes in hematological and iron-related parameters from baseline to the end of the intervention. Hematological parameters included hemoglobin (Hb), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red blood cell (RBC) count, reticulocyte count, and reticulocyte hemoglobin (Retic Hb). Iron-related parameters included serum iron, ferritin, transferrin saturation (TSAT), total iron-binding capacity (TIBC), soluble transferrin receptor (sTfR), hepcidin, erythroferrone, and erythropoietin. Secondary outcomes included extent of adherence to intervention, adverse events experienced, and changes in fecal calprotectin concentrations (a marker of gut inflammation) and composition of gut microbiome at the end of the intervention.

Hematological and iron-related parameters, and serum C-reactive protein were estimated on automated analyzers. Details of the analyzers used are described in the supplementary file. Serum hepcidin and erythroferrone concentrations were estimated by commercially available enzyme-linked immunosorbent assays (Intrinsic LifeSciences, USA). Fecal calprotectin (Epitope Diagnostics, Inc) was estimated. For the stool microbiome analyses, 16S rRNA amplification and library preparation were carried out as described earlier [36]. The samples were sequenced on the MiSeq platform (2 x 300); alpha diversity was assessed with the Shannon index, Faith's phylogenetic diversity, and Evenness, while beta diversity was evaluated using Bray–Curtis distances and Unifrac metrics.

Data analysis was carried out using SPSS version 29. Continuous variables were represented as means ± standard deviations or medians (interquartile ranges). Categorical variables were reported as numbers and percentages. Data in the 2 groups were compared using the paired t-test or Wilcoxon's signed rank t-test (for comparisons between baseline and post-intervention data) and independent t-test or Mann–Whitney U test (for changes seen from baseline post-intervention), as appropriate. Differences in proportions were compared using the Chi-square test or Fisher's exact test. A p value of less than 0.05 was taken to indicate statistical significance in all cases.

3. Results

Three participants (one in the daily group, two in the alternate-day group) experienced minor adverse effects and were not willing to continue with the oral iron supplementation. However, all 3 consented to remain in the study till the end of the intervention period. They completed all post-intervention assessments. Their data were included in the final analyses, as per the intention-to-treat approach (Fig. 1).

There were no significant differences between the two groups at baseline, with regard to clinical characteristics, and hematological and iron-related parameters (Tables 1–3). The extents of adherence to OIS over 14 days were similar in the two groups (95 % and 98 % in the alternate-day and daily groups, respectively).

The interventions resulted in improvements in hematological parameters in both groups (Table 2). Significant improvements were seen in values for MCV, MCH and reticulocyte counts in response to daily iron doses, while alternate-day dosing significantly increased only reticulocyte counts (Table 2). Post-intervention, values of hematological parameters in the 2 groups were similar, except for reticulocyte counts, which were higher in those who had received daily iron (p = 0.062) (Table 2). The proportion of anemic participants (at the time of enrollment) reduced from 50 % to 56.6 % in the daily and alternate-day groups, respectively, to 40 % (post-intervention) in both groups (Table 2).

Both interventions produced improvements in iron status (from baseline) as indicated by significant changes observed in values for serum ferritin, TIBC, sTfR, sTfR index, and total body iron (Table 3). Serum hepcidin concentrations were found to be significantly elevated only in those who received daily doses of iron (Table 3). Post-intervention, values of iron-related and inflammatory markers were similar in the 2 groups (Table 3).

Values for MCV and MCH showed significantly greater increases (from baseline values) in those who received iron daily than those who received it on alternate days (Table 4). The mean value for reticulocyte counts was also higher in those who received iron daily (p = 0.055). Changes seen in iron-related parameters were similar in the 2 groups, except for a greater increase in serum hepcidin concentrations in the daily-iron group (p = 0.062) (Table 5). Serum CRP concentrations decreased in those in the daily iron group, while they increased in those in the alternate day group (p = 0.053). Changes in fecal calprotectin concentrations were similar in the 2 groups (Table 5).

Table 6 summarizes the adverse effects reported by participants in each group. Based on the number of adverse effects reported, participants were assigned a cumulative score from 0 to 6 (1 point for each adverse effect reported and 0 for each adverse effect not reported). The scores were similar in in the 2 groups.

No significant differences in alpha diversity of the gut microbiome were observed in response to OIS in the 2 interventional arms. Beta diversity also showed no significant clustering in the 2 groups (Supplementary Figs. 2 and 3).

4. Discussion

4.1. The need for the present study

Studies that have reported higher fractional and total iron absorption with alternate-day oral supplementation in iron-deficient women in high income countries did not show significantly higher serum ferritin and hemoglobin concentrations (than in those who received daily doses), at the end of the interventions [24,25]. Increases in iron absorption, thus, do not necessarily translate into improved hematological outcomes. Limitations of the above studies, which have been detailed in the introduction section,

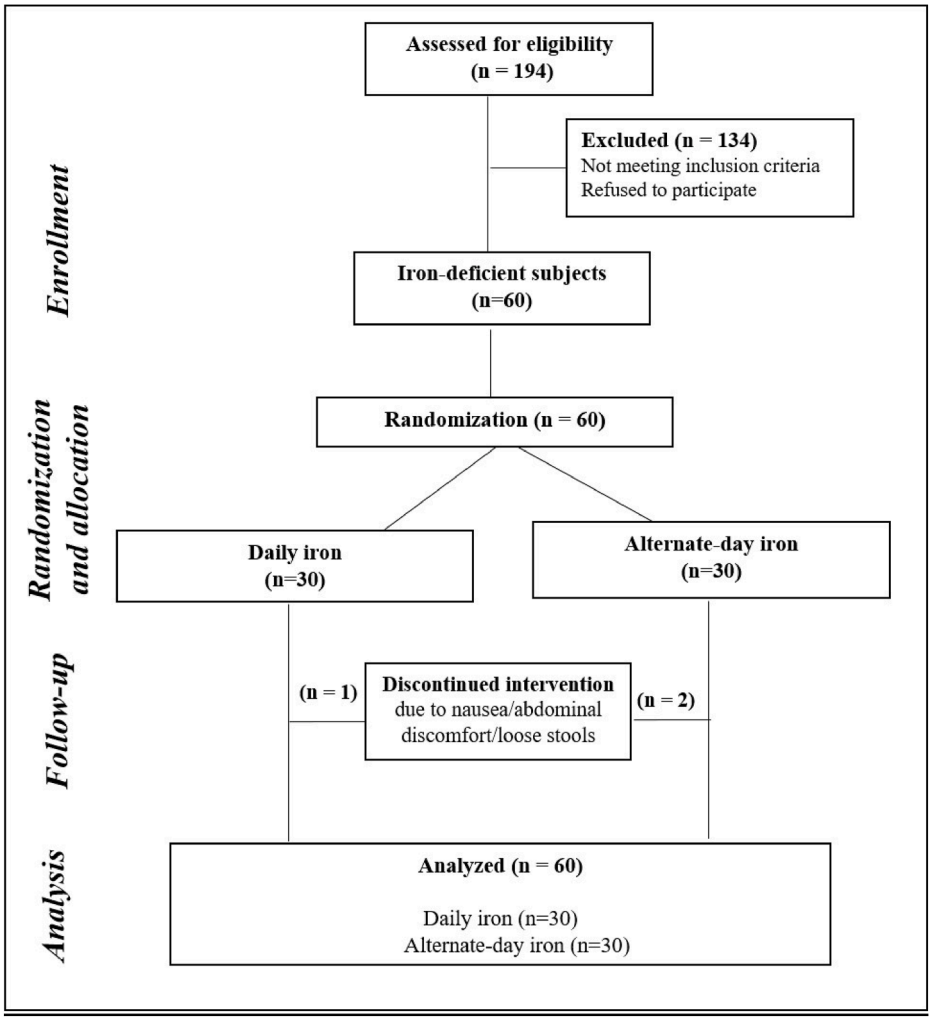

Fig. 1. CONSORT flow diagram depicting participant screening, enrollment, randomization and follow up. Three participants discontinued treatment due to experiencing minor adverse effects and being unwilling to continue taking the supplements but consented to be followed up and completed all post-intervention assessments. They were included in post-intervention analyses, as per an intention-to-treat approach.

Table 1
Clinical characteristics of the participants at the time of recruitment.

	Daily iron (n = 30)	Alternate day iron (n = 30)
Age (years)	25.5 (23, 31)	29 (24, 34.5)
Height (cm)	157 (155, 162.25)	160 (155, 163.25)
Weight (kg)	60.73 ± 10.57	61.32 ± 10.79
BMI (kg/m^2)	24.03 ± 3.61	24.33 ± 4.03

Data are shown as mean (SD) or as median (IQR). Data from the two groups were compared, using either independent t test or Mann–Whitney U test (as appropriate).

constrain the applicability of these findings in LMICs, which have high burdens of ID and IDA.

In their RCT, von Siebenthal et al. [27] also reported similar hemoglobin and MCV values and serum ferritin concentrations in the daily and alternate day groups, at the end of the intervention periods (90 days for the daily group and 180 days for the alternate-day group) and a significantly lower prevalence of ID in the alternate-day group than in the daily group, when assessed at the 6-month mark. However, the validity of this observation to suggest the superiority of alternate-day iron supplementation over daily supplementation is questionable, as the alternate-day group continued receiving supplements for 90 days prior to the point of assessment, while the daily group received no iron during that period. More robust evidence would be required to show that increased FIA (that may occur with alternate-day doses) results in greater improvements in hematological responses and better functional outcomes than with daily doses. The present study was an attempt to address this gap.

4.2. Rationale for the period chosen for the intervention

An intervention period of 14 days was chosen for the present study, based on evidence that early hemoglobin changes can predict longer-term responses to oral iron therapy [37]. The short duration of the intervention facilitated effective monitoring of compliance through daily phone calls, thus ensuring very high levels of adherence to the protocol, as evidenced by the fact that both daily and alternate-day supplementation regimens significantly improved iron-related and hematological parameters in blood (Tables 2 and 3).

4.3. Effect of the interventions on hematological parameters

In the present study, improvements in hemoglobin concentrations at the end of the intervention period were similar in the

Table 2
Hematological parameters in the daily and alternate-day iron groups at baseline and post-intervention.

	Baseline		Post-intervention		
	Daily iron (n = 30)	Alternate day iron (n = 30)	Daily iron (n = 30)	Alternate day iron (n = 30)	p value (comparisons between the 2 groups, post-intervention)
Presence of anemia (Hb < 12 gm/dL) (%)	50 %	56.6 %	40 %	40 %	1.00
Hemoglobin (g/dL)	12 (11.32, 12.5)	11.8 (11.42, 12.82)	12.15 (11.35, 12.9)	12.1 (11.5, 12.67)	0.75
Hematocrit (%)	36.75 (34.6, 38.2)	36.6 (35.5, 38.6)	37.2 (35.7, 38.32)	37.65 (35.92, 38.40)	0.5
Mean corpuscular volume (MCV) (fL)	82.00 (79.35, 86.52)	81.2 (77.75, 85.90)	83.00[a] (80.95, 86.92)	81.7 (79.32, 85.42)	0.55
Mean corpuscular hemoglobin (MCH) (pg)	26.95 (25.27, 28.3)	26.3 (24.27, 28.52)	27.7[a] (25.72, 28.40)	27 (24.80, 28.22)	0.59
Mean corpuscular hemoglobin concentration (MCHC) (g/dL)	32.41 (1.59)	32.40 (1.30)	32.55 (1.56)	32.43 (0.43)	0.76
Red blood cells count (10^6 cells/μL)	4.45 (0.26)	4.52 (0.34)	4.44 (0.29)	4.54 (0.31)	0.22
Reticulocyte count (%)	1.23 (0.99, 1.55)	1.27 (0.93, 1.59)	1.64[a] (1.34, 2.15)	1.52[b] (1.21, 1.79)	0.06
Reticulocyte hemoglobin content (pg)	29.55(27.25, 32.22)	28.2 (25.90, 31.30)	30.85 (29.05, 33.00)	30.8 (28.30, 32.55)	0.55

Data are shown as percentages, and as mean (SD) or median (IQR), depending on their distribution. Data were analyzed, using either independent t test or Mann–Whitney U test (as appropriate). Difference in proportions were compared using Chi-square test. A p value less than 0.05 was taken to indicate statistical significance.
[a] **p < 0.05** when compared to baseline values in the daily group.
[b] **p < 0.05** when compared to baseline values in the alternate-day group.

Table 3
Iron-related and inflammatory markers in the daily and alternate-day iron groups at baseline and post-intervention.

	Baseline		Post intervention		
	Daily iron (n = 30)	Alternate day iron (n = 30)	Daily iron (n = 30)	Alternate day iron (n = 30)	p value (comparisons between the 2 groups, post-intervention)
Presence of iron deficiency, post intervention (%)	100	100	60	56.6	0.79
Serum iron (μg/dL)	45.50 (28.00, 69.25)	46.10 (29.00, 78.50)	45.50 (35.75, 67.25)	52.50 (42.75, 89.25)	0.16
Serum ferritin (ng/ml)	8.45 (5.42, 12.80)	9.85 (5.67, 17.40)	18.40[a] (15.67, 22.57)	19.05[b] (14.62, 27.30)	0.66
Total iron-binding capacity [TIBC] (μg/dL)	389.72 (50.03)	394.47 (44.22)	362.83 (39.46)[a]	371.74 (35.72)[b]	0.36
Transferrin saturation (%)	13.00 (6.87, 18.12)	12.10 (8.05, 20.47)	12.55 (9.40, 19.32)	14.15 (10.97, 23.95)	0.23
Soluble transferrin receptor (mg/L)	4.54 (3.53, 6.67)	4.87 (3.75, 7.03)	4.10[a] (3.54, 5.74)	4.44[b] (3.46, 6.28)	0.98
sTfR index	5.14 (3.33, 8.98)	4.87 (3.21, 9.42)	3.18[a] (2.67, 4.75)	3.16[b] (2.62, 4.70)	0.83
Total body iron (calculated) (mg/kg)	24.96 (23.14, 27.97)	26.19 (22.55, 28.42)	28.78[a] (26.72, 29.89)	29.19[b] (26.92, 30.62)	0.99
Hepcidin (ng/mL)	10.21 (3.20)	11.50 (4.45)	14.88 (6.25)[a]	13.40 (7.21)	0.32
Erythroferrone (ng/mL)	0.99 (0.29, 2.74)	0.87 (0.07, 1.79)	0.65 (0.26, 1.26)	0.66 (0.10, 1.86)	0.97
Erythropoietin (mIU/mL)	13.80 (8.41, 22.60)	11.80 (8.00, 16.25)	11.70[a] (8.25, 15.37)	9.41[b] (7.45, 14.55)	0.35
Serum CRP (mg/L)	0.23 (0.04, 0.52)	0.19 (0.07, 0.54)	0.23 (0.06, 0.47)	0.26 (0.06, 0.67)	0.68
Fecal calprotectin (μg/g)	10.67 (8.05, 18.04)	10.59 (8.56, 19.39)	12.29 (10.43, 22.08)	12.34 (10.77, 26.18)	0.75

Data are shown as percentages, and as mean (SD) or median (IQR), depending on their distribution. Data were analyzed, using either independent t test or Mann–Whitney U test (as appropriate). Difference in proportions were compared using Chi-square test. A p value less than 0.05 was taken to indicate statistical significance.
[a] **p < 0.05** when compared to baseline values in the daily group.
[b] **p < 0.05** when compared to baseline values in the alternate-day group.

two groups, in keeping with results from previous studies [24,25,30,32]. In contrast, Dhanush et al. [29] and Mehta et al. [31] have reported greater increases in hemoglobin concentrations with alternate-day iron supplementation. Dhanush et al. [29] observed this on days 14 and 28, with 120 mg of elemental iron given on alternate days (compared to those who received 60 mg iron daily), while Mehta et al. [31] noted this effect by day 21 (with 60 mg iron on alternate days, compared to those who received 60 mg daily). However, numerous limitations of these studies (differences in baseline characteristics of the participants, lack of blinding of the interventions, unequal dosing regimens, small sample sizes and high dropout rates) limit the validity of these observations.

The present study showed that daily iron supplementation resulted in significantly greater increases in MCV, MCH, and reticulocyte counts than the alternate-day regimen (Table 4), indicating that daily oral iron supplementation may be superior to alternate-day dosing in improving these parameters. However, increases in hemoglobin concentrations were similar in the 2 groups. These results suggest that hemoglobin concentration may not be a sensitive enough indicator to evaluate early responses to oral iron therapy. Thus, the absence of significant increases in hemoglobin should not be misinterpreted as a lack of therapeutic response or as evidence of the ineffectiveness of oral iron therapy. Assessments of other hematological indices, as carried out in the present study, may serve as more reliable markers of response to

Table 4
Changes observed in hematological parameters (from baseline), in response to the interventions.

	Daily iron (n = 30)	Alternate day iron (n = 30)	P value
Hemoglobin (g/dL)	0.20 (0.73)	0.11 (0.47)	0.58
Hematocrit (%)	0.47 (2.35)	0.34 (1.40)	0.80
Mean corpuscular volume (fL)	1.25 (0.25, 2.32)	0.50 (−0.35, 1.42)	***0.04***
Mean corpuscular hemoglobin (pg)	0.52 (0.54)	0.17 (0.56)	***0.02***
Mean corpuscular hemoglobin concentration (g/dL)	0.14 (0.57)	0.03 (0.59)	0.45
Red blood cells (10^6 cells/μL)	−0.01 (0.28)	0.01 (0.19)	0.72
Reticulocytes count (%)	0.32 (0.13, 0.75)	0.27 (0.02, 0.45)	***0.05***
Reticulocyte hemoglobin content (pg)	1.55 (0.10, 2.67)	1.25 (0.35, 3.30)	0.84

Data are shown as means (SD) or as medians (IQR). Data from the two groups were compared, using either independent t test or Mann–Whitney U test (as appropriate). A p value less than 0.05 was taken to indicate statistical significance.

Table 5
Changes observed in iron-related and inflammatory parameters (from baseline), in response to the interventions.

	Daily iron (n = 30)	Alternate day iron (n = 30)	P value
Serum iron (μg/dL)	1.00 (−13.57, 12.75)	2.5 (−4.75, 27.32)	0.71
Serum ferritin (ng/ml)	10.2 (5.93)	9.36 (7.97)	0.65
Total iron-binding capacity [TIBC] (μg/dL)	−26.88 (33.73)	−22.72 (32.79)	0.63
Transferrin saturation (%)	1.53 (−2.45, 4.69)	1.91 (−0.60, 7.24)	0.72
Soluble transferrin receptor (mg/L)	−0.52 (−1.01, −0.01)	0.006 (−0.02, 0.12)	0.67
sTfR index	−1.87 (−3.59, −0.52)	−1.48 (−4.69, −0.68)	0.32
Total body iron (calculated) (mg/kg)	3.60 (2.01, 4.56)	2.92 (1.86, 4.42)	0.33
Hepcidin (ng/mL)	3.89 (−0.51, 7.62)	−0.32 (−2.07, 5.72)	0.06
Erythroferrone (ng/mL)	−0.36 (−1.22, 0.10)	0.00 (−0.86, 0.45)	0.72
Erythropoietin (mIU/mL)	−3.13 (−6.77, 1.13)	−2.26 (−5.39, 1.13)	0.50
CRP (mg/L)	−0.14 (0.76)	0.21 (0.66)	0.05
Fecal calprotectin (μg/g)	2.57 (−8.47, 4.81)	4.10 (−2.67, 7.83)	0.54

Data are shown as means (SD) or as medians (IQR). Data from the two groups were compared, using either independent t test or Mann–Whitney U test (as appropriate). A p value less than 0.05 was taken to indicate statistical significance.

Table 6
Incidence of adverse effects reported by participants receiving daily and alternate-day iron.

	Daily iron (n = 30)	Alternate day iron (n = 30)	P value (Chi square test or Fisher's exact test)
Nausea	56.6 % (17/30)	73.3 % (22/30)	0.17
Abdominal discomfort	33.3 % (10/30)	26.6 % (8/30)	0.57
Loss of appetite	10 % (3/30)	3.3 % (1/30)	0.61
Loose stools	13.3 % (4/30)	6.6 % (2/30)	0.67
Constipation	16.6 % (5/30)	16.6 % (5/30)	1.0
Headache	6.6 % (2/30)	13.3 % (4/30)	0.67
Overall incidence of adverse effects based on cumulative scoring	1.3 (0.89)	1.4 (1)	0.96

Data are shown as percentages and numbers. Difference in proportions were compared using Chi-square test while differences in the overall incidence of adverse effects (based on cumulative scoring) were compared, using either independent t test or Mann–Whitney U test (as appropriate).

OIS. In this context, reticulocyte indices (reticulocyte counts and reticulocyte hemoglobin content) have been reported to be sensitive markers for monitoring the response to oral iron therapy in children and adults with IDA [38–40]. Kaundal et al. [30] showed that individuals receiving daily iron had significantly higher MCV and MCH values at 3 and 6 weeks after starting therapy than those who received alternate-day supplementation. They also showed that reticulocyte counts increased more markedly in the daily iron group by the end of the first week (1.2 ± 1.0 % vs. 0.38 ± 1.3 %; p = 0.09). This trend aligns with the one observed in the present study, suggesting that daily dosing probably results in a more rapid hematological response than alternate-day dosing.

In the present study, no significant differences were observed in the increases in reticulocyte hemoglobin content (Ret-He) in the daily and alternate-day iron groups. However, some studies have reported early increases in Ret-He in response to OIS. For example, Kaundal et al. [30] observed significant increases in Ret-He within a week of initiating daily OIS, compared to alternate-day supplementation. Similarly, Mehta et al. [31] reported significant increases in Ret-He from baseline concentrations on days 2 and 3 in the alternate-day group, but not in the daily group. However, these studies were conducted in adult populations of both sexes and were not randomized or blinded, which limits the ability to draw definitive and valid conclusions from these findings and to make direct comparisons with the present study.

4.4. Effect of the interventions on the gut microbiome

Excess unabsorbed iron in the gastrointestinal tract has been associated with an abundance of iron-dependent entero-pathogens and the presence of intestinal mucosal inflammation [41]. Some studies in children have reported that the gut microbial balance was disrupted by oral iron [12,14], while others have not [42,43]. The present study found no significant differences in fecal calprotectin concentrations or gut microbiota profiles in the two groups, showing that the iron doses taken by WRA for 14 days did

not have significant effects on gut inflammation and microbiome diversity (Supplementary Figs. 2 and 3).

4.5. Adverse effects reported in the 2 groups

We found no significant differences in adverse effects reported in the daily and alternate-day iron groups. Stoffel et al. [24] reported a higher incidence of nausea and abdominal discomfort with daily dosing; however, their study was not blinded and the difference reported was not statistically significant. Pasupathy et al. [32] noted no significant differences between the two regimens, except for more nausea in the alternate-day group at 4 weeks. von Siebenthal et al. [27] found fewer gastrointestinal side effects with alternate-day dosing in their double-blinded randomized control trial. They used a real-time mobile application to document this. Other studies [28,30] have also reported fewer side effects with the alternate day regimens, but suffer from the limitation that their studies were not blinded. In contrast, Mehta et al. [31] observed no significant differences in side effects between the two groups, consistent with the findings of the present study. These varying observations highlight that not all studies have found clear differences in tolerability between daily and alternate-day iron supplementation. These differences in reports may be attributed to various factors such as the variations in study design, absence of blinding, retrospective reporting of side effects, differences in population demographics, differing durations of interventions, and dosing regimens, all of which could influence the reporting.

4.6. The importance of adequate adherence to OIS for effective treatment of iron deficiency and iron-deficiency anemia

It has been shown that when adherence to therapy is good, OIS are effective in treating IDA and ID in women of reproductive age (WRA) [44]. This is borne out by the results of the present study as well. These observations emphasize the importance of measures to ensure adherence to OIS to mitigate ID/IDA effectively. It should also be borne in mind that in the case of medication prescribed on alternate days, there is a very real risk of forgetting to take the medication on the prescribed days.

4.7. What should determine the choice of effective strategies for oral iron supplementation?

The present study did not assess fractional iron absorption in response to the oral iron supplements, but observed a greater increase in serum hepcidin concentrations (post-intervention) in the daily iron group ($p = 0.062$) than in the alternate iron group (on day 15), consistent with results reported by Stoffel et al. [24,25] and von Siebenthal et al. [27], who found significant increases in hepcidin concentrations in those who received daily iron. These earlier studies have linked the increased hepcidin concentrations observed to lower iron absorption in the group that received daily iron than in the alternate-day group. Moretti et al. [21] demonstrated that serum hepcidin concentrations rose significantly (from baseline) in iron-depleted women, 24 h after taking 60 mg of oral iron, and returned to baseline 48 h later. They also found that fractional iron absorption was higher when doses were spaced 48 h apart, rather than when administered 24 h apart. However, it is important to emphasize here that transient increases seen in serum hepcidin concentrations or fractional iron absorption over short periods may not be adequate evidence to determine optimal effectiveness of OIS. While these measurements offer valuable insights into the biology of iron metabolism, they are influenced by multiple factors such as the dietary components, diurnal variation, etc, and may not correlate directly with and/or translate into functional outcomes. Hence, the primary determinant of effective treatment strategies should rely on measurable and clinically relevant outcomes, such as improvements in hematological indices and iron status, and functionality in participants.

Given the widely differing results of various studies in this area, it appears that many factors should be considered to decide on an effective regime for OIS. Examples of these include individual patients' needs, the severity of ID/IDA, the desired speed of recovery and incidence of adverse effects. Further randomized controlled trials of longer duration, which are adequately powered and assess clinically relevant endpoints, may be required to identify more clearly the most effective supplementation strategy for different demographic groups.

4.8. Strengths and limitations of the present study

Reports of increased fractional absorption of iron from alternate day OIS from studies done in a high-income European country have garnered much attention, but the fact that such alternate day doses did not produce significant improvements in hematological indices is often missed. The results of the present study in fact indicate that daily doses may be more effective in improving hematological indices. This is particularly relevant information for LMICs where iron deficiency and anemia are highly prevalent, and where the complexity of real-world situations in such countries may make it more complicated to follow alternate day dosing for OIS; daily dosing may be a simpler regime to follow. The present study provides valuable evidence that can guide clinical decision-making and help refine oral iron supplementation strategies.

Another major strength of the present study is its design, which minimized bias and ensured the validity of findings. It also assessed several clinically relevant outcomes, including hematological, iron-related, and inflammatory parameters, and those linked to the gut microbiome. All these data are important in the context of the present study and provide a comprehensive picture of the effects of OIS. Additionally, both groups received equivalent total doses of iron, addressing limitations in previous studies where unequal dosing and time periods hindered direct comparisons and accurate interpretations [21,24,25,27]. However, the relatively short duration of the intervention (14 days) limits its ability to capture long-term outcomes and may have affected the ability to detect differences in hemoglobin concentrations. Future adequately powered studies with longer durations of intervention, in diverse populations, would be desirable to better determine optimal dosing strategies for oral iron supplementation.

5. Conclusion

This double-blind randomized control trial (on iron-deficient Indian WRA), which ensured excellent adherence, showed that daily OIS produced significantly greater improvements in MCV, MCH and reticulocyte counts (but not in hemoglobin), and were associated with lower levels of systemic inflammation than equivalent doses given on alternate days. Incidence of adverse effects were similar in the two groups and there were no significant effects on the gut microbiome. It would be beneficial for future efforts in this area to focus on improving adherence to OIS, to achieve clinically relevant improvements in iron and hematological status of WRA.

Authors' contributions

NMJ was involved in participant recruitment, study execution, data analysis, and interpretation of results, and co-wrote the

manuscript. BA, OJ, and KV assisted with participant recruitment and acquisition of data. DA carried out the microbiome analysis, interpreted the findings, and contributed to manuscript writing. PS was involved in data analysis and interpretation. YS was involved in performing some of the laboratory assays. SKSS contributed to study concept and design and obtained funding. MJ conceptualized and designed the study, obtained funding, supervised the work done and was involved in data analysis and interpretation of results and co-wrote the manuscript. All authors approved the final version of the manuscript.

Availability of data and materials

The datasets generated and/or analysed during the current study will be available from the corresponding author on reasonable request.

Ethical approval, study registration, and permissions

This study was approved by the Institutional Review Board and Ethics Committee of Christian Medical College, Vellore, India (IRB MIN no: 12457 [INTERVEN] dated 25th Jan 2020). The trial was registered with the Clinical Trials Registry- India (CTRI/2020/03/024144).). Written informed consent was obtained from all participants prior to enrolment. All procedures involving human participants were conducted in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki Declaration and its later amendments.

Declaration of Generative AI and AI-assisted technologies in the writing process

No generative AI and AI-assisted technologies were used in the writing process.

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Declaration of interests

The authors report that there are no competing interests to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.clnu.2025.11.005.

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