Relationship between vitamin D deficiency, bone remodelling and iron status in iron-deficient young women consuming an iron fortified food.

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Abstract

Background: Iron and vitamin D deficiencies are two of the most widespread nutritional disorders in the world. Our aim was to know if the consumption of an iron fortified fruit juice modifies bone remodelling and the possible influence of baseline vitamin D status on the recovery of iron status in a group of iron deficient women.

Methods: Iron biomarkers, 25-hydroxyvitamin D levels, and dietary intake were measured in 123 iron deficient menstruating women. A subgroup (n=41) participated in a randomised double-blind placebo-controlled study of 16-weeks during winter. They consumed a placebo fruit juice (P) or iron-fortified fruit juice (F). Dietary intake, 25-hydroxyvitamin D, parathormone (PTH), bone alkaline phosphatase (ALP), aminoterminal telopeptide of collagen I (NTX), and iron biomarkers were determined.

Results: 92% of the iron-deficient women were vitamin D deficient or insufficient. Transferrin saturation and 25-hydroxyvitamin D were positively correlated. Iron status improved in F, 25-hydroxyvitamin D decreased in F and P, and PTH, ALP and NTX levels were within the normal range and did not vary. Women with 25-hydroxyvitamin D≥50nmol/L compared to 25-hydroxyvitamin D<50nmol/L showed a higher increase in transferrin saturation (a marker of iron supply to tissues) during iron recovery.

Conclusion: The prevalence of vitamin D deficiency or insufficiency is very high in iron deficient women. The recovery of iron status by consuming an iron fortified food does not affect 25-hydroxyvitamin D levels, however the increase in iron supply to tissues is lower if the women also present vitamin D deficiency. Although bone health does not seem to be affected in this group of women, correction of iron and vitamin D deficiencies should be promoted in young women to improve present and future health.

Keywords: Iron-deficiency anaemia, 25-hydroxyvitamin D, Bone remodelling, Fortified food, Menstruating women

Introduction

Iron deficiency anaemia and vitamin D deficiency are extremely common and widespread nutritional disorders in the world [1,2]. Some studies have been suggested a relationship between iron deficiency and low 25-hydroxyvitamin D levels [3-5]. Nevertheless, there are no reports regarding the effect that the combined presence of these two deficiencies could have on bone metabolism.

The relationship between these two deficiencies is not clear, but seems to be reciprocal. On one hand, clinical observations suggest a role of 1,25-dihydroxyvitamin D [6] in erythropoiesis, as this hormone directly stimulates erythroid precursors [7]. On the other hand, iron participates in the second activation step of vitamin D, necessary to make this hormone functional. This conversion is done by a renal 25-hydroxyvitamin D 1α-hydroxylase (1α-OHase) enzyme which comprises a cytochrome P-450, a ferredoxin, and a ferredoxin reductase [8]. Therefore, less available iron could compromise production of the active form of vitamin D.

With respect to the effect that these deficiencies could have on bone metabolism, the role of vitamin D in mineralizing the skeleton by increasing serum calcium concentrations is well known [9]. Vitamin D deficiency is associated with rickets in children and osteomalacia in adults, and a higher risk of osteoporosis [1]. Iron is essential in collagen synthesis since it is a required cofactor for the prolyl and lysyl hidroxylases. These enzymes act on the nascent peptide chain prior to triple helix formation and the reaction products serve to stabilize the collagen triple helices under physiological conditions [10]. It is hypothesized that in an iron deficiency situation, there may be less iron available to the prolyl and lysyl hidroxylases which could result in decreased cross-linking activity and, subsequently, weaker collagen fibres. In fact, severe iron deficiency anaemia has been associated with deleterious effects on bone in rats. Previous studies showed that iron-deficient anaemic rats had lower bone mineral content (BMC), bone mineral density (BMD), and mechanical strength than iron-replete animals [11-13].

In humans, few studies are available, and the results are less consistent. A trend for positive association between bone mineral density of the radius and serum ferritin was found in adolescent girls [14]. One study carried out in postmenopausal women showed that osteoporotic women with fractures presented a relative iron deficiency compared with controls [15]. Therefore, women that suffer from iron deficiency during decades may develop osteoporosis later in life. However, there is no information on bone metabolism in iron deficient menstruating women and the possible changes in bone turnover during iron status recovery.

Consumption of iron supplemented foods can be useful to prevent iron deficiency anaemia. In this sense, iron enriched fruit juices have recently been found to be very efficacious at improving iron status in young iron deficient women [16] which was attributed to the amount of iron in the juice, the presence of iron absorption enhancers, such as ascorbic acid, and absence of inhibitors.

The present study was carried out in iron deficient menstruating women in order to know: 1) if there is a relationship between 25-hydroxyvitamin D levels and iron status biomarkers; 2) if consumption of an iron fortified fruit juice that improves iron status modifies bone remodelling; and 3) if the recovery of iron status by consuming this iron fortified food varies depending on baseline vitamin D status.

This trial was registered at clinicaltrials.gov as NCT01135576.

Subjects and methods

Subjects

A group of 123 women was recruited during winter season. The inclusion criteria were: women aged 18-35 years, non-smoker, non-pregnant, non-breastfeeding, with low iron stores, defined as serum ferritin <40ng/mL and haemoglobin ≥11g/dL. Subjects were excluded from the study if they had amenorrhea, menopause or any known health problems likely to influence iron status including iron-metabolism-related diseases (iron deficiency anaemia, thalassaemia, haemochromatosis), chronic gastric diseases (inflammatory bowel disease, Crohn disease, gastric ulcers, celiac disease, hemorrhagic diseases), renal disease or allergy to any of the components of the assay juices. Other exclusion criteria were: blood donors or to have regularly consumed iron supplements within the four months prior to participating in the intervention.

The participants signed a written informed consent to a protocol approved by the Clinical Research Ethics Committee of Hospital Puerta de Hierro, Madrid and the Spanish National Research Council Committee, Madrid, Spain.

Nutritional intervention

A subgroup of the aforementioned volunteers selected at random (n=41) participated in a randomised double-blind placebo-controlled study of 16-weeks of duration, from November to March. Two groups: P group (n=18) or F group (n=23) consumed 500 mL/day of placebo fruit juice or iron-fortified fruit juice, respectively. The fortified juice supplied 18 mg of iron per 500 mL carton, in the form of microencapsulated iron pyrophosphate coated with lecithin, equivalent to 100% of the recommended

dietary allowance (RDA) per day [17], and all juices were fortified with vitamin C. Details of the nutritional composition of the juices and conditions of the study were reported previously [16]. At baseline, 8 and 16 weeks blood samples and 24-h urine samples were collected and stored at -80°C for analytical determination.

Each subject's dietary intake was evaluated at baseline and every 8 weeks to control any possible changes in energy and nutrient intake. They completed a 72-h detailed dietary intake report, previously validated and proved valuable to assess nutrient intake [18,19], specifying the types of food consumed and serving weights. Daily food, energy intake, nutrient intake and energy provided by macronutrients were calculated with a computer application using the Spanish Food Composition Database [20].

Blood sampling and biochemical assays

Blood samples were collected by venipuncture after a 12-h fasting period, between 08:00 h and 09:00 h. Serum and plasma were obtained after centrifugation at 1000g for 15 minutes and stored at -80ºC.

Total red blood cells, haematocrit, mean corpuscular volume (MCV) and haemoglobin were determined following standard laboratory techniques using the Symex NE 9100 automated haematology analyser (Symex, Kobe, Japan). Serum iron, serum ferritin and serum transferrin were determined by the Modular Analytics Serum Work Area analyser (Roche, Basel, Switzerland). Transferrin saturation (%) was calculated as follows: serum iron (µmol/L)/ TIBC (µmol/L) x 100, where TIBC is total iron binding capacity, calculated as 25.1 x transferrin (g/L).

Serum 25-hydroxyvitamin D (25-hydroxyvitamin D_2 plus 25-hydroxyvitamin D_3), was determined using an ELISA technique (25-hydroxyvitamin D EIA, IDS, UK). The ELISA was performed in an automated microplates analyser (Personal Lab, Adaltis, Italy). Intra- and inter-assay coefficients of variation of the method were 5.6% and 6.4%, respectively. Sensitivity of the method is 2 ng/ml. Deficient vitamin D status was defined as a circulating 25-hydroxyvitamin D concentration <50nmo/L (<20ng/mL), insufficient status was considered 51-74nmol/L (21-29ng/mL), and sufficient >75nmol/L (>30ng/mL) [1]. Also, biochemical markers of bone turnover and PTH were only determined during the nutritional intervention. As a marker of bone formation, serum isoenzyme of bone alkaline phosphatase (ALP) was quantified with an ELISA technique (Ostase®Bap, IDS, UK) using two monoclonal antibodies specific for bAP over liver AP. The ELISA was performed in an automated microplates analyser (Personal Lab, Adaltis, Italy). Sensitivity of the method is 7 µg/L. Intra- and inter-assay coefficients of variation are 4.5% and 6.1%, respectively. Normal range: 3-15 µg/L. The biochemical marker of bone resorption,

aminoterminal telopeptide of collagen I (NTX) was determined in 24h-urine pool by an ELISA test (Osteomark®NTx Urine, Wampole Laboratories, USA). The ELISA was performed in an automated microplates analyser (Personal Lab, Adaltis, Italy). Sensitivity of the method is 1 nM of bone collagen equivalents (BCE). Intra- and inter-assay coefficients of variation are 5% and 5.5% respectively. Normal range: 15-80 nM BCE/mM creatinine. PTH was determined by chimioluminiscence assay of second generation PTH (Elecsys, Roche Diagnostics, USA). Intra- and inter- assay coefficients are 6.5% and 2.7% respectively and normal range is 15-65 pg/mL. Normal ranges of ALP, NTX and PTH for healthy women (30-45 years old) were estimated at the Fundación Jiménez Díaz Institute for Medical Research (IIS-FJD), Madrid, Spain.

Statistical analysis

Data are presented as means with their standard deviations. A normal distribution of variables was determined by the Kolmogorov-Smirnov test. Serum ferritin values were log transformed for statistical testing. Pearson's linear correlation tests between serum 25-hydroxyvitamin D levels and iron biomarkers at baseline were performed.

In the nutritional intervention data, repeated-measures ANOVA and post hoc Bonferroni test were used to study time effect within groups. Comparisons were also made between the F group and the P group using ANOVA. To know whether the recovery of iron status after consuming this iron fortified food varies depended on baseline vitamin D status, a two-way repeated-measures ANOVA with 25-hydroxyvitamin D levels (<50 nmol/L and ≥ 50 nmol/L) as the between-subjects factor were applied for each parameter in P and F group. Comparisons between volunteers with 25-hydroxyvitamin D levels <50 nmol/L and volunteers with 25-hydroxyvitamin D levels ≥ 50 nmol/L at each point were measured using ANOVA. $P<0.05$ was considered significant. The SPSS statistical package for Windows (version 19.0) was used to analyze the data.

Results

Table 1 shows the baseline characteristics of the subjects. No significant differences between the total group and the subgroup that participated in the nutritional intervention were found. In this group of iron deficient women (n=123), 42.3% presented vitamin D deficiency, 49.6% vitamin D insufficiency and 8.1% vitamin D sufficient. There was a significant positive correlation between serum 25-hydroxyvitamin

D and transferrin saturation (p=0.007), but the correlations between serum 25-hydroxyvitamin D and haemoglobin, serum ferritin and serum transferrin were not significant.

Dietary characteristics of the total group are presented in Table 2. No significant associations between nutrient intake and 25-hydroxyvitamin D levels and iron parameters were found.

Table 3 shows energy and nutrient intakes at baseline and week 16 of iron deficient women consuming placebo or iron-fortified fruit juices. Although energy intake did not show significant differences between baseline and week 16 in either groups, a decrease in the energy percentage from proteins (p=0.02 in P group) and a trend toward increase in that from carbohydrates were observed during the study. At baseline, there was no difference in iron intake between both groups, but for the duration of the intervention, due to the consumption of the iron-fortified juice, iron intake was approximately double in F group compared to P group. Vitamin C intake increased in both groups from baseline, due to juice composition, without significant differences between P and F groups. There were no changes in the other nutrient intakes determined.

Table 4 shows the results of iron and bone biomarkers of iron deficient women consuming placebo and iron-fortified fruit juices during 16 weeks. In F group, significant increases in haemoglobin, serum ferritin and transferrin saturation levels and decreases in serum transferrin levels, show recovery from iron deficiency ($p=0.03$, $p<0.001$, $p=0.008$, $p<0.001$ respectively), and compared to P group haemoglobin and serum ferritin levels were significantly higher at week 16 ($p<0.001$ and $p=0.05$). Serum 25-hydroxyvitamin D significantly decreased from baseline to the end of the study in P and F ($p<0.001$), without differences between groups. No changes were observed in PTH, ALP and NTX in P or F group.

Transferrin saturation did not change during the experimental period in the P group. Figure 1 shows transferrin saturation increase in volunteers of F group depending on baseline 25-hydroxyvitamin D levels (time influence, p=0.008, time x 25-hydroxyvitamin D level, NS). Transferrin saturation was significantly higher in the F subgroup with 25-hydroxyvitamin D levels $\geq$ 50 nmol/L compared to 25-hydroxyvitamin D levels < 50 nmol/L at 8 and 16 weeks (p=0.05 and p=0.04 respectively).

Discussion

Present results show for the first time a relationship between iron deficiency anaemia and vitamin D deficiency in menstruating Spanish women. A positive association between 25-hydroxyvitamin D and transferrin saturation was observed in these women. The best marker of vitamin D status is 25-

hydroxyvitamin D while transferrin saturation is the marker of the supply of iron to tissues. Recovery of transferrin saturation, due to the consumption of an iron fortified fruit juice, was found to be lower if the iron deficient women had also vitamin D deficiency.

It is remarkable that the majority (92%) of the women that participated in the study were vitamin D deficient or insufficient, and this percentage is higher than data of hypovitaminosis D described for Spanish adolescent women (63%) [21] and Spanish young adults (61%) [22]. Therefore, this suggests that there is an association between the deficiencies of both micronutrients in our population.

There are many different factors that could independently influence the development of these two deficiencies in this population group, such as nutritional, genetic, physiological and environmental factors. Concerning dietary intake at baseline (Table 2), iron and vitamin D intakes were below the Recommended Dietary Allowance (RDA) for this population group (18 mg/day and 5 µg/day respectively) [23,24]. Regarding iron intake, the RDA is not easily reached by menstruating women, as previously reported by our research group [25,26]. Although the influence of dietary components on iron absorption is well known [27], in the present study no association between nutrient intake and iron biomarkers was found. However, the increase in iron intake due to the consumption of the iron fortified fruit juice clearly improved iron status, showed by the increasing in haemoglobin, ferritin and transferrin saturation. The influence of this iron pyrophosphate-fortified fruit juice on other iron parameters was detailed in a recent article [16]. Few foods naturally contain vitamin D, so a deficient intake is generally observed in the general population [28]. In agreement with this, vitamin D intake in the present study only reached 60% of the RDA. The lack of association between dietary vitamin D and 25-hydroxyvitamin D levels in our volunteers could be explained since the production of vitamin D in the skin through sunlight exposure is the main contributor to vitamin D status [29]. With respect to other nutrients related to bone health, dietary intakes reached the RDA. We observed adequate intakes of calcium, magnesium, phosphorus, vitamin C and vitamin K, which are essential nutrients to maximise bone formation and minimise bone loss [30].

Very scarce data are available on simultaneous vitamin D and iron deficiency in humans. Two observational studies in children and adolescent immigrants from Africa and Asia to northern regions detected high prevalence of both deficiencies, which were associated with several factors such as skin pigmentation, covering clothes in females or malnutrition [3,4]. But to the best of our knowledge, there

are no data from controlled humans studies in which the sample is selected as being iron deficient. Furthermore, no data are available on the possible influence of vitamin D status in iron recovery.

It is noteworthy that these young women with iron deficiency exhibited very low 25-hydroxyvitamin D levels. However, the improvement of iron status after the supplementation with the iron fortified food did not increase 25-hydroxyvitamin D. Moreover, 25-hydroxyvitamin D levels after 16 weeks of treatment were lower than before supplementation (Table 4), most likely due to the reduction of UV light exposure produced in winter season at latitudes higher than 40º [31], since the study was performed from November to March in Madrid (Spain) and there were no differences in vitamin D intake. But, on the other hand, we found that, even though the volunteers who consumed the iron fortified fruit juice clearly improved their iron status, the recovery of transferrin saturation was lower if they had also vitamin D deficiency (Fig 1). Therefore, the obtained results support the hypothesis that there is an association between vitamin D and iron status, although the nature of this relationship and the underliying mechanism remains uncertain. Different studies attributed this connection to the role of iron in vitamin D activation [11,15], and to vitamin D modulating the risk of anaemia by decreasing inflammation [5] and stimulating erythropoiesis [7]. More recent studies found a metabolic association between iron status and 25-hydroxyvitamin D levels through fibroblast growth factor 23 (FGF23) [32]. This hormone controls 1α-OHase enzyme expression, and its levels increase with low serum 25-hydroxyvitamin D [33]. One study in subjects with rickets showed that low serum iron and low haemoglobin were associated with elevated FGF23 and low 1,25-dihydroxyvitamin D [34]. However, there is a lack of evidence regarding aetiological mechanism, especially in healthy adults such as our volunteers.

Given the low levels of 25-hydroxyvitamin D found in these women, we expected the PTH levels would be higher than normal. When low 25-hydroxyvitamin D levels lead to reduced calcium levels, PTH is secreted causing an increase in bone resorption to correct hypocalcemia. This can have a negative influence on bone mass, preventing the maximum peak bone mass from being reached, which is very important in order to avoid the development of osteoporosis after menopause [35]. Nevertheless, levels of PTH and bone remodelling markers are considered to be in the normal range in these volunteers. Our research group observed that postmenopausal osteoporotic women with low 25-hydroxyvitamin D levels presented a higher decrease in bone mass than postmenopausal osteporotic women with normal levels of 25-hydroxyvitamin D [36]. This fact has been described by other authors [37]. In a similar way, in a recent work, we found that levels of 25-hydroxyvitamin D lower than 18 ng/ml produced

hyperparathyroidism (PTH>65 pg/ml) in a high percentage of postmenopausal women [38]. However, in the present study, although approximately 50% of the women at baseline and 75% at the end of the study presented 25-hydroxyvitamin D <45 nmol/L (<18 ng/ml), their PTH levels were not higher than the upper limit of normality. In agreement, another study performed in young people living in a sunny place did not observe an increased in PTH levels associated with low 25-hydroxyvitamin D levels [39]. Some studies suggested that different factors could have an influence in the PTH and 25-hydroxyvitamin D relationship. For the same levels of 25-hydroxyvitamin D, higher age and BMI have been related with higher levels of PTH [40,41]. Therefore, this lack of correlation between low levels of 25-hydroxyvitamin D and high levels of PTH observed in our volunteers could be explained as they were young and normal-weight women.

With respect to bone turnover markers, studies that established their reference ranges for menstruating women excluded those under 30 years [42]. Given that the mean age of the women that participated in the study was 25 years, it was difficult to classify them in a range of normality. Compared to other data of the bibliography, bone turnover markers, were higher than data observed in premenopausal women with higher or similar age [43,44]. It is known that bone turnover in women is elevated until skeletal maturity is reached, usually in the 3rd decade of life, thus the women participating in the present study were expected to have a high rate of bone remodelling, which explains the present results.

We found no relationship between iron levels and bone biomarkers, in contrast to data obtained in animal models about deleterious effects on bone in anaemic rats [11-13]. The biochemical markers of bone formation and bone resorption remained stable independently of iron status. The obtained results could be explained by the fact that our volunteers presented iron deficiency without anaemia and were young, so bone formation may prevail over bone resorption. However, it could be hypothesised that continued iron deficiency may result in substantial bone loss and higher risk of osteoporosis. In this sense, one study in postmenopausal women found that osteoporotic patients that have suffered from fragility fracture presented lower iron status that other osteoporotic women with no fractures and control women [15]. Therefore, it would be very interesting to investigate whether iron deficiency, maintained during years, could predispose to osteoporosis.

Another important issue is that the maintenance of an adequate iron and vitamin D status is essential for integral health. Iron deficiency may affect physical and cognitive performance, immunity and temperature regulation [27]. Vitamin D deficiency has been related to an increased risk of many chronic illnesses,

including common cancers, autoimmune diseases, infectious diseases, and cardiovascular disease [45]. These actions not only depend on renal 1α-OHase activity, but on extra-renal 1α-OHase mediated by vitamin D receptors distributed in many tissues [46]. Iron acts as a cofactor of all 1α-OHases and the activity of the extra-renal hydroxylases is regulated by the concentration of the substrate, 25-hydroxyvitamin D [47]. Therefore, low iron and 25-hydroxyvitamin D levels could lead to different health disorders derived from the low activity of body extra-renal 1α-OHases.

The prevalence of iron deficiency in young menstruating women is highly frequent. According to our results, this situation could be accompanied by low levels of 25-hydroxyvitamin D, which can obstruct the recovery of iron status. Although bone health does not seem to be affected in this group of women, correction of iron deficiency and low 25-hydroxyvitamin D levels is an issue of great importance in the context of public health. Therefore, clinical and/or nutritional interventions should be considered in order to maintain adequate levels of vitamin D and iron.

Further studies should be done in anaemic and non-anaemic subjects to clarify the possible interactions between iron and vitamin D metabolism, and the possible health effects that these two deficiencies could have, taking into account also the modulating effects of diet and genetic polymorphisms. Present results add new information regarding the influence of 25-hydroxyvitamin D levels on iron status recovery but other studies should be carried out to confirm if the improvement of 25-hydroxyvitamin D leads to better recovery of iron status in response to iron fortified food or supplements. It is also important to study the effect of iron deficiency on bone turnover after the skeletal maturity is reached; and to perform prospective studies in pre- and postmenopausal women, with and without osteoporosis. Finally, the possible effect that iron and vitamin D deficiencies can have on extra-renal 1α-OHase functionality and their repercussions on health should be studied.

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Fig 1. Transferrin saturation at baseline, 8 and 16 weeks in volunteers that consumed the iron-fortified fruit juice depending on 25-hydroxyvitamin D levels: < 50 nmol/L (open circle) and > 50 nmol/L (filled circle). Values are means with 95% confident intervals expressed by vertical bars. Comparisons between volunteers with 25-hydroxyvitamin D levels < 50 nmol/L and volunteers with 25-hydroxyvitamin D levels > 50 nmol/L at each point were measured using one-sided tests (*$p \leq 0.05$).

Table

Table 1. Iron biomarkers and 25OH vitamin D of the total group and the subgroup of volunteers.

	Total group (n=123)	Subgroup (n=41)
Age (years)	24.3±4.8	25.5±5.9
Body mass index (kg/m^2)	21.7±2.2	21.8±2.3
Haemoglobin (g/dL)	13.2±0.9	13.2±0.8
Serum ferritin (ng/mL)	26.4±17.5	22.6±12.2
Serum transferrin (mg/dL)	314.5±60.5	318.6±53.8
Transferrin saturation (%)	19.3±9.2	17.1±7.7
25-hydroxyvitamin D (nmol/L)	54.2±18.0	50.2±14.0
% Deficient vitamin D status (< 50nmol/L)	42.3	53.6
% Insufficient vitamin D status (51-74 nmol/L)	49.6	41.5
% Sufficient vitamin D status (> 50nmol/L)	8.1	4.9

Differences between groups were not significant. Data are mean±SD

Table 2. Energy and macronutrient intakes of iron deficient women

Energy (kJ/d)	9091±2512
Protein (% energy/d)	14.6±4.2
Carbohydrate (% energy/d)	41.8±13.0
Lipids (% energy/d)	40.2±13.4
Iron (mg/d)	14.1±4.7
Calcium (mg/d)	977.5±355.8
Magnesium (mg/d)	283.8±87.9
Phosphorus (mg/d)	1423±363
Vitamin D (µg/d)	3.0±2.4
Vitamin C (mg/d)	131.2±60.8
Vitamin K (µg/d)	134.0±89.6

Data are mean±SD (n=123)

Table 3. Energy and nutrient intakes of iron deficient women consuming placebo and iron-fortified fruit juices during 16 weeks

	Group	Baseline	Week 16	p Time
Energy (kJ/d)	Placebo	9087±2282	10420±2846	NS
	Fortified	8477±1816	9571±2489	NS
Protein (%energy/d)	Placebo	14.9±2.6	13.3±1.8	p=0.02
	Fortified	14.1±2.4	13.5±2.3	NS
Carbohydrate (%energy/d)	Placebo	38.7±7.7	42.7±8.3	NS
	Fortified	42.5±5.8	43.9±6.6	NS
Lipids (%energy/d)	Placebo	42.5±7.7	39.3±7.0	NS
	Fortified	39.9±605	39.0±6.2	NS
Iron (mg/d)	Placebo	15.6±5.5	14.0±5.5	NS
	Fortified	13.1±3.5	32.1±9.1***	p<0.001
Calcium (mg/d)	Placebo	1044±380	1003±412	NS
	Fortified	905±262	834±310	NS
Magnesium (mg/d)	Placebo	266.8±109.6	275.1±92.1	NS
	Fortified	267.2±49.4	262.2±95.3	NS
Phosphorus (mg/d)	Placebo	1361.6±348.9	1472.0±509.6	NS
	Fortified	1271.2±273.9	1321.6±470.2	NS
Vitamin D (µg/d)	Placebo	2.7±2.2	3.7±2.7	NS
	Fortified	3.1±2.5	3.9±3.3	NS
Vitamin C (mg/d)	Placebo	136.3±62.0	200.6±60.4	p<0.001
	Fortified	122.5±49.1	206.2±81.7	p<0.001
Vitamin K (µg/d)	Placebo	159.6±148.4	151.0±84.0	NS
	Fortified	117.8±63.9	147.1±149.3	NS

Values are presented as means with their standard deviations (n=41). Time-point differences were analysed by repeated-measures ANOVA. Comparisons between Placebo group and Fortified group at each point were measured using one-sided tests (*** $p \leq 0.001$).

Table 4. Iron and bone biomarkers of iron deficient women consuming placebo and iron-fortified fruit juices during 16 weeks

	Group	Baseline	Week 8	Week 16	*p* time
Haemoglobin (g/dL)	Placebo	13.0±0.8[a]	13.4±0.8[b]	13.1±0.7[a]	p=0.02
	Fortified	13.3±0.9[a]	13.7±0.9[ab]	13.7±0.7[b]**	p=0.03
Serum ferritin (ng/mL)	Placebo	22.5±13.7	26.4±18.8	20.2±14.6	NS
	Fortified	22.2±11.6[a]	34.1±15.9[b]	33.5±16.9[b]*	p<0.001
Serum transferrin (mg/dL)	Placebo	315.7±54.8	335.5±59.5	307.8±53.1	NS
	Fortified	318.7±55.8[a]	301.9±57.0[b]	282.3±45.15[c]	p<0.001
Transferrin saturation (%)	Placebo	16.1±7.5	20.3±13.3	18.6±15.8	NS
	Fortified	16.2±7.1[a]	21.1±9.5[ab]	21.7±7.8[b]	p=0.008
25 hydroxyvitamin D (nmol/L)	Placebo	53.3±14.6[a]	42.1±11.0[b]	38.6±10.4[b]	p<0.001
	Fortified	48.2±13.4[a]	39.2±11.5[b]	36.5±13.1[b]	p<0.001
PTH (pg/mL)	Placebo	37.6±11.0	-	35.2±14.5	NS
	Fortified	38.5±18.6	-	33.3±5.7	NS
Alkaline phosphatase (µg/L)	Placebo	10.3±3.1	10.6±2.2	10.7±2.8	NS
	Fortified	12.0±3.6	12.0±2.9	12.5±3.5	NS
NTX (nmol BCE/mmol creatinin)	Placebo	82.5±33.4	86.7±29.4	79.9±23.9	NS
	Fortified	72.8±31.3	82.5±29.6	78.7±30.1	NS

Values are presented as means with their standard deviations (n=41).Within the same row, different letters indicate significant differences (ANOVA of repeated measures followed by Bonferroni test). Comparisons between Placebo group and Fortified group at each point were measured using one-sided tests (*p<0.05; **p≤0.01).

Figure