





A Green-Mediterranean Diet, Supplemented with Mankai Duckweed, Preserves Iron-Homeostasis in Humans and Is Efficient in Reversal of Anemia in Rats

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ABSTRACT

Background

Decreased dietary meat may deplete iron stores, as plant-derived iron bioavailability is typically limited.

Objectives

We explored the effect of a low-meat Mediterranean (green-MED) diet, supplemented with *Wolffia globosa* duckweed (Mankai: rich in protein and iron) as a food source for humans, on iron status. We further examined the iron bioavailability of Mankai in rats.

Methods

Two hundred and ninety-four abdominally obese/dyslipidemic [mean age=51.1 y; body mass index (kg/m^2)=31.3; 88% men] nonanemic participants were randomly assigned to physical activity (PA), PA+MED diet, or PA+green-MED diet. Both isocaloric MED groups consumed 28 g walnuts/d and the low-meat green-MED group further consumed green tea (800 mL/d) and Mankai (100 g green shake/d). In a complementary animal experiment, after 44 d of an iron deficiency anemia-inducing diet, 50 female rats (age=3 wk; Sprague Dawley strain) were randomly assigned into: iron-deficient diet (vehicle), or vehicle+iso-iron: ferrous gluconate (FG) 14, Mankai 50, and Mankai 80 versions ($1.7 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ elemental iron), or FG9.5 and Mankai 50-C version ($1.15 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ elemental iron). The specific primary aim for both studies was changes in iron homeostasis parameters.

Results

After 6 mo of intervention, iron status trajectory did not differ between the PA and PA+MED groups. Hemoglobin modestly increased in the PA+green-MED group (0.23 g/dL) compared with PA (−0.1 g/dL; $P<0.001$) and PA+MED (−0.1 g/dL; $P<0.001$). Serum iron and serum transferrin saturation increased in the PA+green-MED group compared with the PA group (8.21 $\mu\text{g}/\text{dL}$ compared with −5.23 $\mu\text{g}/\text{dL}$ and 2.39% compared with −1.15%, respectively; $P<0.05$ for both comparisons), as did folic acid ($P=0.011$). In rats, hemoglobin decreased from 15.7 to 9.4 mg/dL after 44 d of diet-induced anemia. After depletion treatment, the vehicle-treated group had a further decrease of 1.3 mg/dL, whereas hemoglobin concentrations in both FG and Mankai iso-iron treatments similarly rebounded (FG14: +10.8 mg/dL, Mankai 50: +6.4 mg/dL, Mankai 80: +7.3 mg/dL; FG9.5: +5.1 mg/dL, Mankai 50-C: +7.1 mg/dL; $P<0.05$ for all vs. the vehicle group).

Conclusions

In humans, a green-MED low-meat diet does not impair iron homeostasis. In rats, iron derived from Mankai (a green-plant protein source) is bioavailable and efficient in reversal of anemia. This trial was registered at clinicaltrials.gov [↗](#) as NCT03020186.



Key Words

iron homeostasis parameters; Mediterranean diet; plant iron sources; vegetarian diet; weight loss

Abbreviations used

DIRECT-PLUS, Dietary Intervention RandomizEd Controlled Trial PoLyphenols-UnproceSsed; FG, ferrous gluconate; MCH, mean cell hemoglobin; MCV, mean corpuscular volume; MED, Mediterranean; MET, metabolic equivalent; PA, physical activity; TIBC, total iron-binding capacity; UIBC, unsaturated iron-binding capacity

Introduction

Vegetarianism is thought to be a healthy lifestyle, providing beneficial effects on various cardiovascular disease risk factors such as hypertension, serum lipid levels, glycemic control, and the incidence of type 2 diabetes (1, 2, 3, 4). However, iron, a mineral that is biologically essential for oxygen transport capacity, and/or its bioavailability may be limited in a vegetarian lifestyle (5, 6). The presence of iron in some plant foods, as well as iron absorption enhancers, on one hand, and iron absorption inhibitors, such as phytates, tannins, and fibers, on the other, constitutes an apparent paradox. Thus, there is a need to define specific dietary interventions which can supply bioavailable iron from vegetarian sources.

Another type of diet, referred to as “flexitarian,” is characterized by replacing the majority (though not all) of red meat consumed with plant food sources (7). The Mediterranean (MED) diet, which is increasingly associated with favorable health outcomes (8, 9, 10), is rich in fruits and vegetables and, therefore, may constitute a good platform for a plant-rich flexitarian diet (11).

The duckweed (Mankai) is based on a specific strain of *Wolffia globosa*, an aquatic plant which can serve as a plant protein source. In Asian cuisines, *Wolffia globosa* is considered a natural food source or “vegetable meat ball” (12). Nutritionally, Mankai is characterized by high protein content (>45% of the dry matter), the presence of 9 essential and 6 conditional amino acids (13), and a demonstrated protein digestibility-corrected amino acid score (12) of 89%. The Mankai plant is rich in nonsoluble fiber, iron, vitamins, and polyphenols (14, 15) and, therefore, could serve as a food source for humans. However, the phytate content in the duckweed plant (16) is as high as in other leafy vegetables (i.e., kale and spinach) (17), and thus has the potential to inhibit iron absorption.

As vegetarian and flexitarian dietary patterns grow in popularity worldwide, there is an increasing need for safe and feasible alternative dietary iron sources that can replace meat in terms of protein and iron content. We hypothesized that, among nonanemic humans, the

decrease in iron stores observed in meat-restricted diets can be significantly moderated by dietary consumption of *Wolffia globosa*. We further hypothesized that, among anemic rats, the relatively high iron content of *Wolffia globosa* would be highly bioavailable. To test our hypotheses, 2 complementary approaches were taken. In a human intervention study, participants were directed to reduce animal-based protein intake and instead consume a daily *Wolffia globosa* green shake. Our specific aim for the human trial was to assess changes in iron homeostasis parameters within 6 mo of intervention as a secondary analysis in a weight loss trial. In addition, we aimed to specifically examine the effect of *Wolffia globosa* duckweed on recovery from anemia in rats, induced by an iron-deficient diet.

Methods

DIRECT-PLUS human trial

Participants

The study (NCT03020186) was conducted in an isolated workplace with a monitored provided lunch (Nuclear Research Center Negev, Dimona, Israel). Most of the medical measurements, and the lifestyle intervention sessions were conducted in a convenient in-house clinic at the facility. Eligibility criteria included age >30 y and abdominal adiposity (waist circumference: men >102 cm, women >88 cm) or dyslipidemia (TGs >150 mg/dL; HDL cholesterol ≤40 mg/dL for men, ≤50 mg/dL for women). Individuals unlikely to be able to partake in physical activity (PA) in the gym were excluded, as were those with serum creatinine ≥2 mg/dL, disturbed liver function, major illness that might require hospitalization, pregnant women, active cancer patients, those who underwent chemotherapy in the last 3 y, participants in another trial, those taking Coumadin (warfarin), and those with a pacemaker or platinum implant, given the use of MRI for analysis of fat deposition.

The Institutional Review Board of Soroka Medical Center approved the study protocol. All participants provided written informed consent and received no financial compensation for their participation.

Protocol and procedures

The Dietary Intervention RandomizEd Controlled Trial PoLyphenols-UnproceSsed (DIRECT-PLUS) was initiated in May, 2017.

After baseline measurements, the intervention started in 1 phase, after participants' random assignment to 1 of the 3 following groups: PA, PA+MED diet, or PA+green-MED diet (a flowchart of the DIRECT-PLUS trial is provided in [Supplemental Figure 1](#)). Participants were aware of their assigned intervention (open label).

Lifestyle intervention groups

PA

All groups received free gym memberships and education sessions to encourage moderate-intensity PA, ~80% of which had an aerobic component. The full workout program, including a resistance-training regimen that was added to the aerobic training, is provided in [Supplemental Method 1](#). PA group participants were not guided for caloric restriction but received basic health-promoting guidelines for a healthy diet.

PA+MED

In addition to the aforementioned PA intervention, PA+MED participants were guided to follow a calorie-restricted traditional MED diet ([11](#)) low in simple carbohydrates, as described in our previous DIRECT ([9](#)) and CENTRAL ([10](#), [18](#)) trials. This diet was rich in vegetables, with poultry and fish replacing beef and lamb. The diet included 28 g walnuts/d (160 kcal/d, 84% fat, mostly ω -3 α -linolenic acid) provided free of charge.

PA+green-MED

In addition to the PA intervention and 28 g walnuts/d provided, the green-MED diet was lower in processed and red meat than the MED diet. Furthermore, the green-MED diet was richer in plants and polyphenols, as participants were further instructed to consume the following provided items: 3–4 cups/d (800 mL/d) of green tea and 100 g frozen cubes of *Wolffia globosa* (Mankai) plant as a 500-mL green shake (dinner replacement). Both the prescribed MED and green-MED diets were calorie-restricted (1500–1800 kcal/d for men and 1200–1400 kcal/d for women).

Lifestyle interventions were accompanied by 90-min nutritional and PA sessions in the workplace with multidisciplinary guidance (physicians, clinical dietitians, and fitness instructors). Additional information regarding the lifestyle sessions, as well as motivation techniques, is available in [Supplemental Method 2](#).

Wolffia globosa duckweed

The daily Mankai shake contributed 18% of the total protein recommended in the DRIs for men, 5.7% of carbohydrates, and 2.4% of fat (19). The vitamin contribution of Mankai (as percentages of men's DRIs) was 49% of vitamin A, 21% of vitamin B-12, 26% of vitamin E, and 61% of folic acid. The mineral contribution of the Mankai shake was 168% for iron, and 35% for zinc (as percentages of men's DRIs) (20). The *Wolffia globosa* supplied for this study was grown under optimized controlled conditions, thus it was considered free of heavy metal contamination, as confirmed by laboratory analyses.

Outcome measures

Clinical parameters and fasting blood biomarkers

Height was measured to the nearest millimeter using a standard wall-mounted stadiometer. Body weight was measured without shoes to the nearest 0.1 kg. Waist circumference was measured halfway between the last rib and the iliac crest to the nearest millimeter by standard procedures using a 150-cm anthropometric measuring tape. Blood samples were taken at times 0 and 6 mo, after a 12-h fast. The samples were centrifuged at room temperature (3500 RPM for 15 min) and stored at -80°C . Serum iron, ferritin, transferrin, transferrin saturation, and folic acid levels were assayed at the University of Leipzig, Germany. All parameters were measured on the fully automated Cobas 8000 platform with reagents from Roche Diagnostics, according to the manufacturer's protocol. Serum transferrin was measured by immuno-turbidimetry with rabbit-anti-human-transferrin antibodies. Serum ferritin was measured by the Electro-Chemi-Luminescence-ImmunoAssay (ECLIA) sandwich approach. Folic acid was measured by the ECLIA competitive approach. Hemoglobin concentrations in freshly drawn whole blood samples were assessed at the workplace clinic at baseline (290 participants) and at the time of 6-mo follow-up measurements (± 2 mo, 264 participants).

Nutrient intake and PA intensity

Electronic questionnaires were administered at baseline and after 6 mo (21, 22). We followed overall changes in intake of specific food groups, as described previously (23, 24), and further used lifestyle and validated PA questionnaires (symptoms, adverse effects, quality of life, medication usage, and safety questionnaires). PA intensity levels were measured using metabolic equivalent (MET) units, defined as the ratio of work metabolic rate to standard resting metabolic rate (25). MET was calculated using a tracking guide (26) and results from the validated PA questionnaire.

Statistical analysis: human trial

The general primary aim of the 18-mo DIRECT-PLUS study was to evaluate adiposity in terms of MRI-assessed visceral and hepatic fat changes (a sample size calculation for this study is presented in [Supplemental Method 3](#)). In this secondary analysis, the specific primary aim was to evaluate changes in iron homeostasis parameters (serum iron, hemoglobin, transferrin saturation, and ferritin) and serum folic acid concentration after 6 mo of treatment. Continuous variables are presented as means \pm SDs, unless specified otherwise, whereas categorical variables are presented as numbers and percentages. Variables were tested for normal distribution using the Shapiro–Wilk test. In order to calculate changes in nutritional patterns (based on foods or food groups) after 6 mo of intervention, the total change score of consumption for each nutritional pattern was summarized according to the participants' responses to the food change questionnaire, as follows: increased consumption=+1, decreased consumption=-1, no change=0 (per participant). These values were then divided by the number of participants in each intervention group, such that each score represents the percentage of people who changed ([24](#)). Differences between time points are expressed as absolute values, unless specified otherwise. Differences within intervention groups were evaluated using a paired-sample *t* test for normally distributed variables, and using the Wilcoxon Signed Rank test for nonnormally distributed variables. To detect differences between intervention groups, we performed an ANOVA; when the overall ANOVA was significant, a post hoc Tukey's honestly significant difference (HSD) test or a Kruskal–Wallis 1-factor ANOVA with multiple comparisons was applied. Chi-square tests were applied to evaluate differences between categorical variables' distributions. Further analysis was performed via ANCOVA, in order to control for the possible confounding effect of weight loss.

Mankai iron status in rats

Experimental animals

The rat experiment (HNM-001-IRON) was performed at Pharmaseed (Nes Ziona, Israel) using standard operation procedure (SOP) and according to Organisation for Economic Co-operation and Development principles of Good Laboratory Practice ENV/MC/CHEM (98)17 ([27](#)). Fifty Sprague Dawley strain female 3-wk-old rats (Envigo RMS) were randomly allocated to cages (2 rats/cage) according to Pharmaseed SOP 027 and the study was performed in compliance with The Israel Animal Welfare Act and following The Israel Board for Animal Experiments guidelines (approval IL-16-07-221). The rats were kept in a climate-controlled environment with a temperature range of 18–24°C, relative humidity of 30–70%, and a 12-h light/12-h dark cycle (0600/1800).

Feeding protocol and experimental design

In the anemia induction phase, the rats were rendered iron-deficient and anemic by feeding them a Teklad iron-deficient diet (TD.99397+vitamins, detailed in **Supplemental Table 1**) for 44 d.

The rats were randomly assigned in 2 independent cycles ($n=25$ each; a flowchart of the rat experiment is provided in **Supplemental Figure 2**).

The iron-deficient diet was maintained for the duration of the study to preserve iron deficiency anemia achieved during the first 44 d. In the 3 subsequent weeks (after the depletion treatment period), test meals were administered by oral gavage (4.5 mL, a mixture of Mankai powder with water as fluid slurry), once daily (in the afternoon). To allow maximal iron absorption from the test item, food was removed daily from the animals' cages in the morning, and reintroduced immediately after the gavage feeding.

After the anemia induction phase, the rats were divided into 6 experimental groups. The routes of administration were formulated to attain delivery of equal amounts of elemental iron as follows:

Vehicle: an iron deficient-diet [protein, 17.7%; carbohydrates, 69.8%; fat, 5.2%; all percentage by weight. Casein (low Cu and Fe), 200 g/kg; DL-methionine, 3.0 g/kg; sucrose, 545.19 g/kg; corn starch, 150 g/kg; corn oil, 50 g/kg; mineral mix (iron deficient), 35 g/kg; vitamin mix (AIN-76A), 14 g/kg; choline bitartrate, 2.8 g/kg; ethoxyquin, 0.001 g/kg].

Vehicle +ferrous gluconate (FG): 2 doses of FG: 9.5 mg/kg or 14 mg/kg.

Vehicle +Mankai: 3 doses of Mankai: Mankai 80, Mankai 50-C, and Mankai 50, with the 3 versions of Mankai referring to different methods of plant drying.

The interventions provided the following amounts of elemental iron: $1.7 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ for the iso-iron groups FG 14, Mankai 50, and Mankai 80 versions, and $1.15 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ for the iso-iron groups FG 9.5 and Mankai 50-C version (calculation of formulations of the equivalent amounts of elemental iron needed for the study is provided in **Supplemental Table 2**). The nutrient content, as well as the amount of Mankai per group, are provided in **Supplemental Table 3**. Overall, the reported nutritional composition of Mankai was high in protein (45%) and carbohydrate (25%) and low in fat (7%) and heavy metals, when measured by inductively coupled plasma analysis. Quality testing for heavy metals, microbiological content, pesticides, and aflatoxin content revealed compatibility with common safety

requirements for vegetables in the United States. The trial was completed with 47 rats (1 rat each was excluded from the Mankai 50, Mankai 50-C, and Mankai 80 groups) owing to sporadic and non-treatment-related events.

Outcome measures

Body weight was recorded twice weekly during the first weeks and weekly thereafter. Blood was collected from the retro-orbital sinus. Blood hemoglobin concentrations were monitored before anemia induction, at the end of weeks 2 and 3, after anemia induction (before beginning of treatment), at week 2 after treatment initiation, and once a week thereafter until study termination. Iron, total iron-binding capacity (TIBC), unsaturated iron-binding capacity (UIBC), RBCs, hematocrit, mean cell hemoglobin (MCH), mean corpuscular volume (MCV), and mean corpuscular hemoglobin concentration were monitored at the beginning of treatment and at the end of the treatment period.

Statistical analysis: animal experiment

The primary aim of the HNM-001-IRON trial was to assess the influence of Mankai duckweed on iron status in iron-deficient anemic rats, by measuring blood hemoglobin concentrations. ANOVA was used to examine 22-d changes (as absolute values) between the groups followed by post hoc Bonferroni correction, whereas a paired-sample *t* test was used to examine changes within each group. Sensitivity analysis was performed via the comparison of hemoglobin concentration between the vehicle and the treatment groups at day 22 using ANOVA with Bonferroni correction. Differences between time points are expressed as absolute values, unless specified otherwise. Significance in both studies was set at $P < 0.05$ (2-sided). Statistical analysis was performed using SPSS software, version 22.0 (IBM).

Results

DIRECT-PLUS human trial

Baseline characteristics

Of 378 volunteers, 294 participants met the inclusion criteria. Baseline characteristics of the 294 study participants are presented in **Table 1**. At baseline, 59% of the participants had $\text{BMI} \geq 30 \text{ kg/m}^2$ (60% in the PA group, 59% in the PA+MED group, and 57% in the PA+green-MED group; $P=0.708$). Waist circumference was $111 \pm 9.1 \text{ cm}$ for men ($n=259$) and $103 \pm 9.6 \text{ cm}$ for women ($n=35$). Hemoglobin concentrations were $15.1 \pm 1.0 \text{ g/dL}$ for men ($n=257$) and

13.4±0.9 g/dL for women ($n=33$). Serum iron and folic acid concentrations were within the normal range at baseline [reference values for healthy adults for iron: 80–180 µg/dL for men and 60–160 µg/dL for women; for folic acid: 5–25 ng/mL (28)]. All baseline parameters were distributed similarly across the 3 randomly assigned groups. No significant differences were observed between the groups in terms of folate supplement (chi-square=2.82; $P=0.24$) or multivitamin use (PA: 2.1%; PA+MED: 4.2%; PA+green-MED: 7.2%; chi-square=3.04; $P=0.22$). One participant from group PA reported using iron supplementation (chi-square=2.01; $P=0.367$). Supplements containing inhibitors or promoters of iron absorption did not differ in usage between the intervention groups (magnesium supplement, $P=0.432$; calcium supplement, $P=0.601$; vitamin C supplement, $P=0.762$).

TABLE 1. Baseline characteristics and iron status of the entire study population and of each assigned intervention group, DIRECT-PLUS, human study¹

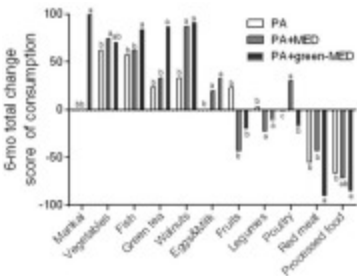
	Entire study ($n=294$)	PA ($n=98$)	PA+MED ($n=98$)	PA+green-MED ($n=98$)	<i>P</i> value between groups
Age, y	51.1±10.5	51.1±10.6	51.7±10.4	50.6±10.8	0.758
Men, n (%)	259 (88%)	86 (88%)	86 (88%)	87 (89%)	0.968
Smoking, n (%)	48 (16%)	19 (19%)	13 (13%)	16 (16%)	0.511
BMI, kg/m ²	31.3±3.99	31.2±3.8	31.3±3.97	31.3±4.2	0.986
Waist circumference, cm	109.7±9.5	109.9±10.3	110.0±9.5	109.3±8.7	0.588
Serum iron, µg/dL	106±31.8	110±30.9	108±30.2	101±33.7	0.103
Serum ferritin, ng/mL	168±113	163±114	179±119	163±108	0.551
Serum transferrin, mg/dL	268±35.0	270±37.7	271±36.0	263±30.2	0.198
Serum transferrin saturation, %	28.7±9.1	29.4±8.8	29.1±9.3	27.5±9.3	0.301
Serum folic acid, ng/dL	8.0±3.6	7.8±3.5	8.4±3.7	7.9±3.5	0.427
Blood hemoglobin, g/dL	14.9±1.1	14.9±1.1	14.8±1.0	14.9±1.2	0.659

Values are presented as means±SDs for continuous variables and as *n* (%) for categorical variables.
MED, Mediterranean; PA, physical activity.

Adherence to the dietary intervention

After 6 mo of intervention, study adherence was 98.3% (based on the DIRECT-PLUS trial co-primary outcome, the adherence was calculated according to the attendance at mid-term measurements). Apart from the Mankai shake content, intake of iron, as calculated from the FFQs (21, 22), decreased in all weight loss intervention groups ($P=0.55$ between groups). Dietary folic acid showed a similar trend ($P=0.17$ between groups). For the PA+green-MED group, the additional intake from 100 g of frozen Mankai cubes provided 13.5 mg Fe/d and 0.24 mg folate/d.

Changes in nutritional patterns after 6 mo of intervention are presented in **Figure 1**. The green-MED diet was distinguished by decreased red meat and poultry intake and increased fish, green tea, and Mankai intake, compared with the PA+MED diet ($P<0.05$ for all, compared with other groups).



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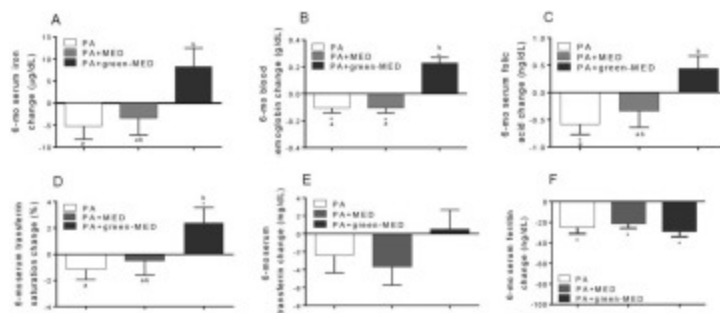
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FIGURE 1. Changes in nutritional patterns in obese, dyslipidemic adults who engaged in PA with or without consuming a MED diet or who engaged in PA and consumed a green-MED diet for 6 mo (DIRECT-PLUS human study). Data are presented as a percentage change after 6 mo for each group. Groups without a common letter differ ($P<0.05$). PA: $n=82$; PA+MED: $n=85$; PA+green-MED: $n=75$. DIRECT-PLUS, Dietary Intervention RandomizEd Controlled Trial Polyphenols-UnproceSsed; MED, Mediterranean; PA, physical activity.

All 3 intervention groups similarly increased their PA level ($P=0.303$ between groups), measured in MET units, compared with baseline (PA: 13.4 ± 46.6 MET/wk, $P=0.014$; PA+MED: 24.1 ± 44.6 MET/wk, $P<0.001$; PA+green-MED: 16.5 ± 37.4 MET/wk, $P<0.001$).

Changes in iron homeostasis parameters

After 6 mo of lifestyle intervention, significant weight reduction in all intervention groups was observed (PA: -1.5 ± 3.9 kg; PA+MED: -5.4 ± 5.6 kg; PA+green-MED: -6.2 ± 5.9 kg; $P < 0.001$ compared with baseline for each intervention group). Mean changes in biochemical parameters associated with iron metabolism, as well as changes in folic acid concentrations, are presented in **Figure 2A–F**. The change in serum iron was significantly greater for the PA+green-MED than for the PA group ($P = 0.031$) and tended to be greater than for the PA+MED ($P = 0.075$) group. Blood hemoglobin was modestly, although significantly, increased in the PA+green-MED group ($P < 0.001$ compared with baseline), and mildly but significantly decreased in the PA ($P = 0.009$ compared with baseline) and PA+MED groups ($P = 0.007$ compared with baseline). These changes were significantly greater for the PA+green-MED than for both the PA and PA+MED groups ($P < 0.001$ for all).



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FIGURE 2. Changes in serum iron (A), blood hemoglobin (B), serum folic acid (C), serum transferrin saturation (D), serum transferrin (E), and serum ferritin (F) in obese, dyslipidemic adults who engaged in PA with or without consuming a MED diet or who engaged in PA and consumed a green-MED diet for 6 mo (DIRECT-PLUS human study). For 6-mo serum iron, folic acid, transferrin saturation, transferrin, and ferritin changes: $n = 96$ for each group; for 6-mo blood hemoglobin changes: PA: $n = 87$; PA+MED: $n = 84$; PA+green-MED: $n = 88$. Data are presented as means \pm SEMs. *Significant within a group ($P < 0.05$). Groups without a common letter differ ($P < 0.05$). DIRECT-PLUS, Dietary Intervention RandomizEd Controlled Trial PoLyphenols-UnproceSsed; MED, Mediterranean; PA, physical activity.

Serum folic acid concentration decreased significantly in the PA group ($P = 0.003$ compared with baseline) and was significantly lower than in the PA+green-MED group ($P = 0.011$). Whereas serum transferrin saturation nonsignificantly decreased in the PA and PA+MED

groups, it significantly increased in the PA+green-MED group ($P=0.042$ compared with baseline, $P=0.041$ compared with changes in the PA group).

Serum ferritin protein concentrations decreased across all intervention groups ($P<0.001$ compared with baseline for all), with no significant difference between the groups. There were no significant changes in serum transferrin concentrations within or across the intervention groups.

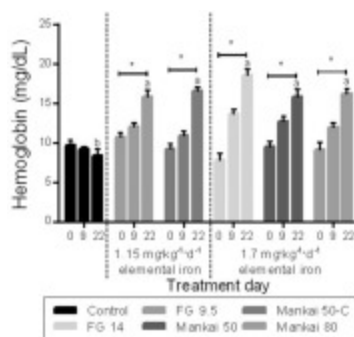
When controlling for weight change after 6 mo, differences between the intervention groups remained significant for blood hemoglobin ($P<0.001$ for all).

Individual responses of selected iron homeostasis parameters are available in **Supplemental Figure 3**.

Mankai iron status in rats

The anemia-inducing protocol adopted was highly efficient; hemoglobin concentrations of the entire cohort dropped from 15.7 ± 2.5 to 9.4 ± 2.3 mg/dL within 44 d ($P<0.001$).

The restoration of hemoglobin concentrations is demonstrated in **Figure 3**. After 22 d of treatment, hemoglobin concentrations decreased further in the vehicle group and increased significantly in the FG 14 ($1.7 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ elemental iron) group ($P<0.001$), in the FG 9.5 ($1.15 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ elemental iron) group ($P=0.013$), in the Mankai 50 ($1.7 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ elemental iron) group ($P=0.003$), in the Mankai 50-C ($1.15 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ elemental iron) group ($P<0.001$), and in the Mankai 80 ($1.7 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ elemental iron) group ($P<0.001$). The 22-d hemoglobin change was greater in both FG groups and all 3 Mankai groups than for the vehicle group ($P<0.05$ for all compared with vehicle). Sensitivity analysis for the differences of hemoglobin concentration at day 22 shows greater hemoglobin concentrations for all the FG and Mankai groups than for the vehicle group ($P<0.001$ for all compared with vehicle).



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FIGURE 3. Iron status of rats fed iron-deficient diet without or with either FG or Mankai (rat experiment). Data are presented as means \pm SEMs. Absolute hemoglobin concentrations on days 0, 9, and 22. The dose level and study design are based on 1.15 mg \cdot kg⁻¹ \cdot d⁻¹ elemental iron for the FG 9.5 and Mankai 50-C groups and 1.7 mg \cdot kg⁻¹ \cdot d⁻¹ elemental iron for the FG 14, Mankai 50, and Mankai 80 groups. * $P < 0.05$ compared with vehicle for 22-d change. Groups without a common letter differ ($P < 0.05$). Vehicle group (iron-deficient diet): $n = 10$; vehicle+FG groups: $n = 5$ each; vehicle+Mankai groups: $n = 9$ each. FG, ferrous gluconate.

Changes in TIBC, UIBC, hematocrit, MCV, serum iron, RBC, MCH, and mean corpuscular hemoglobin concentration after 22 d are presented in **Supplemental Table 4**. TIBC, UIBC, haematocrit, and MCV changes were significantly different between the vehicle and each of the other treatment groups ($P < 0.01$ for all). Changes in iron concentrations after 22 d were significantly different between the vehicle group and FG 14, as well as all 3 Mankai groups ($P < 0.05$ for all). Changes in RBC concentrations differed significantly between the vehicle group and each of the 3 Mankai groups ($P < 0.05$ for all). The change in MCH concentration in the vehicle group at 22 d was significantly different from the change in groups FG 14 and Mankai 50-C over the same period ($P < 0.05$ for both). Overall, the sets of iron homeostasis parameters were similar in both FG and all Mankai groups but significantly different from those in the vehicle group.

During the recovery phase, all rats gained weight, although the vehicle group showed a significantly lower body weight gain than the treatment groups (**Supplemental Figure 4**).

Discussion

In the secondary analysis of 294 nonanemic abdominally obese human participants, our aim was to examine systemic changes in iron status indicators concentrations. The findings suggest that the green-MED diet, lower in red meat and poultry intake and richer in green plants, did not impair but rather appeared to maintain iron status and folic acid concentrations among participants with normal iron status at baseline, who underwent weight loss interventions. We further aimed to evaluate the iron bioavailability of Mankai duckweed, the plant iron source and a component of the green-MED diet, in rats as an iron status study. The results suggest that the iron in Mankai is well absorbed, and restores iron status in anemic rats similarly to ferrous gluconate, a popularly used supplement for treating iron deficiency anemia. Those results likely explain the iron status achieved in the green-MED diet.

Several limitations should, however, be considered. First, our human trial included participants with iron status within the normal range. Thus, we cannot conclude that the

green-MED diet will restore extremely depleted iron stores in anemic individuals, although in our complementary animal study, we demonstrated reversal of iron depletion in anemic Mankai fed rats. Second, reflecting the study's workplace profile, only 12% of participants were female. Third, dietary patterns, as well as PA monitoring, were evaluated using self-reported but validated (29) questionnaires, a known limitation in all large-scale dietary intervention studies. For logistical reasons, not all hematological parameters in the human study were available. Finally, we were not able to isolate the unique effect of duckweed because there are several differences between the MED and green-MED diets. At the same time, study strengths include its large sample size, and its 1-phase randomized-controlled design, in which all participants started the intervention on the same day. We performed a complementary animal model-based study to address both iron status and *Wolffia globosa* as a potential dietary source for preventing iron store depletion. The rat experiments were conducted in 2 independent cycles, which yielded similar results.

Intervention trials examining the effects of combining weight reduction and a change in eating patterns to a plant-based diet are limited. The impact of vegetarian diets on iron status in adults was examined in a recent systematic review and meta-analysis (30) of cross-sectional and intervention trials. The findings suggested that vegetarians were more likely to have lower iron stores than nonvegetarians. Our participants in the green-MED diet were guided to avoid red meat, to reduce poultry consumption, and to partially replace these animal foods with a Mankai duckweed green shake. In addition, as part of the green-MED diet, participants were instructed to consume high amounts of green tea (800 mL/d), known to contain polyphenols that are likely to impair iron bioavailability (31). Nonetheless, the green-MED group demonstrated favorable changes in systemic iron status indicators. Specifically, a slight increase in serum iron, blood hemoglobin, and serum transferrin saturation was seen in the PA+green-MED group, as compared to the decreases in all of these biomarkers in the PA and PA+MED groups. Another factor that may influence iron status is intensive PA, because iron can be lost via sweat (32). In our study, PA intensity change was similar for the groups, because all participants were instructed to follow the same PA regime. Our finding of an increase in folic acid in the PA+green-MED group was expected, because vegetarian diets are rich in vitamin B family folate and folic acid (a folate analog), found in green and leafy vegetables (33). This vitamin is associated with clinical benefits (preventing neural tube defects, reducing risk of cardiovascular diseases, etc.) (34).

In the rat study, we found that hemoglobin was increased in both FG groups and in all of the Mankai groups after a 22-d treatment period. The additional biochemical and hematological parameters (i.e., TIBC, UIBC, and iron) were similarly increased. The rat experiment suggests that Mankai could act as a bioavailable iron replacement source, one as good as FG, for

recovery from anemia. Considering all of the results obtained from both the human and rat studies, we speculate that despite containing high dietary fiber and polyphenol levels, the Mankai plant might include more iron enhancers than inhibitors and thus promote iron absorption. Additional explanations for the effect of Mankai on iron concentrations could be a unique ratio of ferrous:ferric iron in the plant or some unknown component that maintains iron in the reduced state, thus allowing for better iron absorption. The possible heavy metal accumulations of arsenic, as well as cadmium and chromium, in duckweed grown in open ponds reported by others (35) were not a concern in this study because the *Wolffia globosa* duckweed supplied was grown indoors under complete control of the plant's growth and environment and its composition was heavily monitored.

In our study, we found a significant decrease in the serum concentrations of ferritin across all 3 intervention groups. Although ferritin serves as an indicator for iron store amounts (36), it is also linked with obesity, with ferritin concentrations being reduced in parallel to weight loss (37). Because ferritin highly reflects the systemic inflammatory state (38) and obesity is a condition known to be associated with chronic low-grade inflammation (39), weight reduction may decrease serum ferritin concentrations.

In conclusion, we found that in an obese but otherwise healthy population, the PA+green-MED regimen was able to maintain iron and folic acid status, despite lower consumption of meat and poultry. Moreover, we found that Mankai, a plant-based protein source, although rich in dietary fibers and polyphenols, can also serve as a dietary iron source with high bioavailability.

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take responsibility for the integrity of the data and accuracy of the data analysis, and read and approved the final manuscript.

Author disclosures

I Shai advises to the Hinoman, Ltd. nutritional committee. AYM, GT, HZ, ER, AK, IY, AR, I Shelef, AT, DB, EP, BS, MB, MS, JT, UC, and MJS, no conflicts of interest.

Supplementary Data



Supplemental Figures 1–4, Supplemental Methods 1–3, and Supplemental Tables 1–4 are available from the “Supplementary data” link in the online posting of the article and from the same link in the online table of contents at <https://academic.oup.com/jn/> ↗.

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