

ORIGINAL
ARTICLE

Effects of dietary supplementation with creatine on homocysteinemia and systemic microvascular endothelial function in individuals adhering to vegan diets

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capillary recruitment, intravital video-microscopy, laser speckle contrast imaging, postocclusive reactive hyperemia

ABSTRACT

The incidence of cardiovascular diseases in vegetarian individuals is lower than that in the general population. Nevertheless, individuals who adhere to vegan diets have a higher prevalence of hyperhomocysteinemia with eventual adverse effects on vascular reactivity. Creatine supplementation (CrS) reduces plasma homocysteine levels and enhances vascular reactivity in the microcirculation. Thus, we investigated the effects of CrS on systemic microcirculation and homocysteine blood levels in strict vegan subjects. Forty-nine strict vegan subjects were allocated to the oral CrS (5 g micronized creatine monohydrate daily for three weeks; $n = 31$) and placebo ($n = 18$) groups. Laser speckle contrast imaging coupled with acetylcholine skin iontophoresis was used to evaluate cutaneous microvascular reactivity, and intravital video-microscopy was used to evaluate skin capillary density and reactivity before and after CrS. We demonstrated that CrS reduces the plasma levels of homocysteine and increases those of folic acid. After the CrS period, the homocysteine levels of all of the vegan subjects normalized. CrS also induced increases in baseline skin functional capillary density and endothelium-dependent capillary recruitment in both normo- (N-Hcy) and hyperhomocysteinemic (H-Hcy) individuals. CrS increased endothelium-dependent skin microvascular vasodilation in the H-Hcy vegan subjects but not in the N-Hcy vegan subjects. In conclusion, three weeks of oral CrS was sufficient to increase skin capillary density and recruitment and endothelium-dependent microvascular reactivity. CrS also resulted in plasma increases in folic acid levels and reductions in homocysteine levels among only the H-Hcy individuals.

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INTRODUCTION

Vegan diets, which are defined as a dietary profile characterized by abstention from consuming any animal products, may have positive long-term health impacts, such as reductions in the prevalence of obesity and of cardiovascular disease in the general population [1,2]. Compared with that for omnivores, the incidence of cardiovascular disease and type 2 diabetes is

significantly lower in vegetarians and especially in vegans, who generally have lower body mass index and total and LDL cholesterol levels and moderately lower blood pressure [1].

For ethical and moral reasons, strict vegans abstain from consuming animal diets. Nevertheless, even though vegans adhere to a diet characterized by a large intake of fruits and vegetables, they present several micronutrient deficiencies [2]. In fact, eliminating



all animal products from the diet increases the risk for vitamin B-12 and vitamin D deficiencies, as well as that for marginal iron and zinc blood levels [1,3]. In this context, vitamin B-12 deficiency is known to lead to elevated plasma homocysteine concentrations (hyperhomocysteinemia), a risk factor for neurological and cardiovascular diseases [4]. In fact, lowering plasma homocysteine concentrations can result in a reduction of the risk of ischemic heart disease by about 16% and stroke by 24% [5].

Compared with lacto-ovo vegetarians and omnivores, vegans typically have lower plasma vitamin B-12 concentrations, a higher prevalence of vitamin B-12 deficiency, and higher concentrations of plasma homocysteine, which increase the risk for cardiovascular dysfunction [6–9]. The potential mechanisms by which elevated plasma homocysteine level leads to vascular endothelial dysfunction are the reductions in nitric oxide bioavailability and increased vascular oxidative stress [10,11]. In this context, evidence suggests a significantly higher risk of both ischemic heart disease and deep vein thrombosis in people with the enzyme methylenetetrahydrofolate reductase mutation, which increases homocysteine plasma levels [12]. In this context, creatine supplementation has been suggested to be capable of reducing homocysteine blood levels, exerting positive influences on vascular endothelial function [13,14]. In contradiction, some studies in humans suggest that creatine supplementation does not alter vascular reactivity but instead causes significant elevation of serum homocysteine in normohomocysteinemic subjects, despite that it causes significant reductions in hyperhomocysteinemic individuals [15,16].

Notwithstanding, recent data show that creatine supplementation may be a practical strategy for decreasing plasma homocysteine levels and reducing vascular oxidative stress [9,13,14]. We recently demonstrated that oral supplementation with creatine in healthy, moderately physically active nonvegetarian young adults do not change their plasma homocysteine levels but improves their systemic endothelial-dependent microvascular reactivity and increases their skin capillary density and recruitment [17]. In this study, creatine supplementation was shown to reduce total cholesterol and LDL cholesterol plasma levels and significantly increase T4 levels [17].

The present study intended to investigate the effects of creatine supplementation on homocysteine plasma levels and on the reactivity of the systemic microcirculation in strict vegan subjects.

METHODS

Study design

This study was performed in accordance with the Declaration of Helsinki (revised in 2000) and was registered on clinicaltrials.gov with the number NCT0-2961972 and approved by the Institutional Review Board (IRB) of the National Institute of Cardiology at the Ministry of Health in Brazil (protocol number: CAEE 02471512.4.0000.5272).

Initially, 152 vegetarian volunteers were selected through contact with local vegetarian associations and screened for participation in the study (*Figure 1*). Vegetarian volunteers who were not strict vegans or who supplemented their diets with vitamin B-12 or creatine were excluded from the study. Inclusion criteria included the previous abstention from the intake of any animal product for more than one year, age between 20 and 45 years, and being moderately physically active. After selection, 56 strict vegan subjects were considered eligible for this single-blinded study and signed the informed consent form approved by the IRB. Three volunteers decided to abandon the study before beginning supplementation. Afterward, volunteers were randomly allocated to the creatine supplementation ($n = 31$) and placebo ($n = 22$) groups at an approximately 2:1 allocation basis. There was a loss of follow-up of four volunteers during the three weeks of supplementation in the placebo group, yielding the final distribution of creatine supplementation ($n = 31$) and placebo ($n = 18$). In the end of the study and after unblinding for supplementation, the volunteers were also analyzed according to their homocysteine plasma levels (see Results section). We included individuals who were vegan for more than one year, who did not supplement their diets with creatine or vitamin B-12, and who did not use drugs that alter vascular reactivity. Moreover, considering that creatine is found mostly in meat, fish, and other animal products, the levels of muscle creatine are known to be lower in vegetarians [18]. The volunteers were evaluated regarding their habitual dietary intake on a seven-day basis by a dietitian. The average energy intake of the volunteers was of 2 800 calories per day, containing approximately 65% of carbohydrates, 20% fat, and 15% proteins.

The prospective power analysis was based on intravital microscopy data from previous studies of our group. This analysis indicated that a sample size of 16 subjects per group would have 80% power at the 5% significance level to detect a difference of a 7 capillaries/mm²

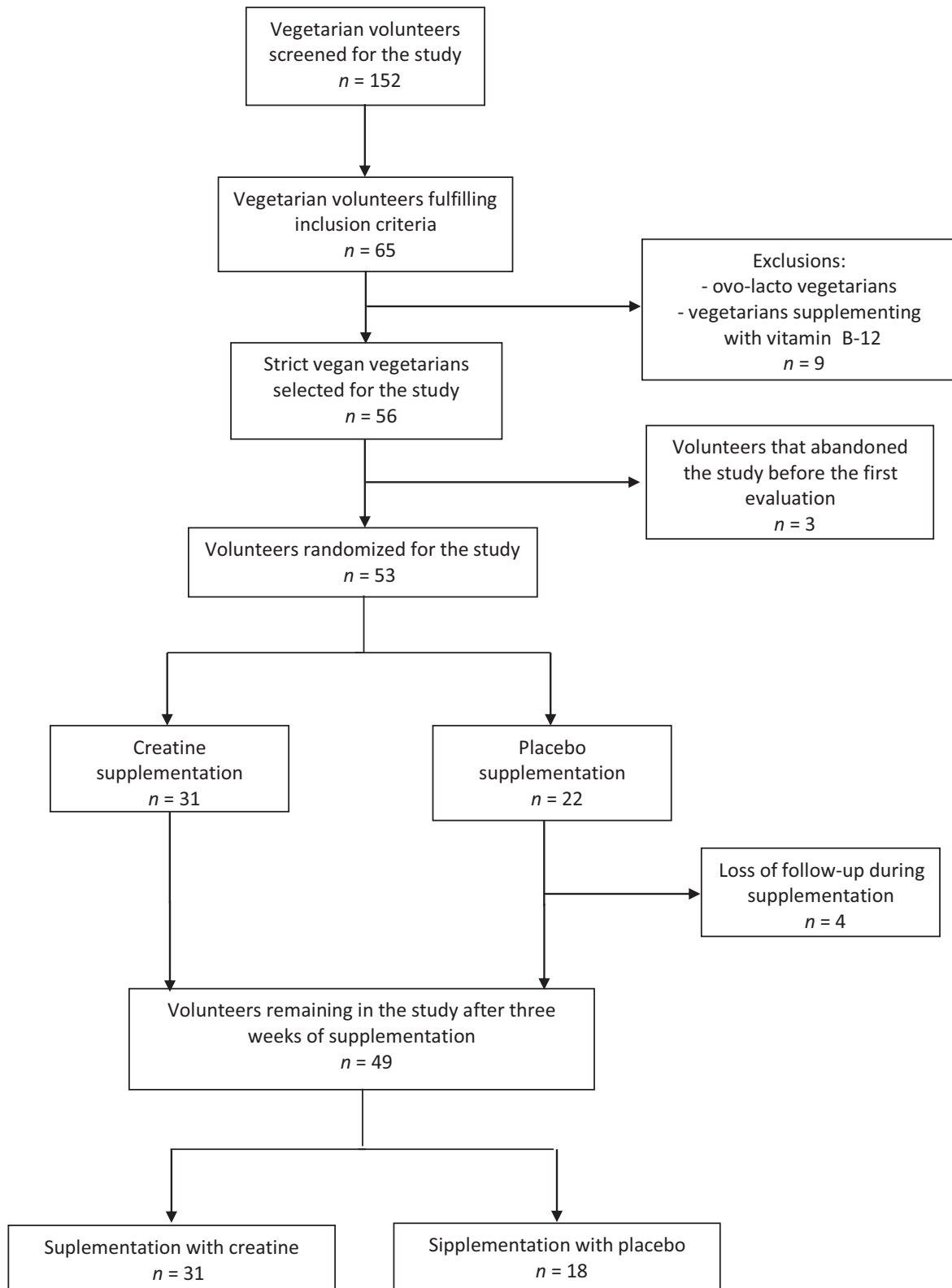


Figure 1 Flowchart of the study population selection.

increase with a standard deviation of 7 capillaries/mm² during PORH between the groups. The calculations were made using classical power calculations with the formula $n = f(\alpha, \beta) \cdot \frac{2s^2}{\delta^2}$, where α is the significance level, β is the power of the test, $f(\alpha, \beta)$ is a value calculated from α and β (in this case, 7.9), δ is the difference in the means that we should be able to detect, and s is the standard deviation found in these previous studies.

The evaluation of microvascular reactivity and capillary density was performed immediately before and after supplementation with placebo or creatine. All of the evaluations were performed in the morning, between 8 A.M. and 12 P.M., after a 12-h fast. First, blood specimens were collected. Then, the subjects rested in a quiet environment with a constant temperature of 23 °C ± 1 °C before the microvascular reactivity tests for 20 min. The subjects were also asked to refrain from smoking and abstain from caffeine- and alcohol-containing beverages for 12 h before the study. All of the procedures followed the same sequence, beginning with the collection of blood samples, followed by the clinical and physical evaluations, and concluding with the microcirculatory evaluation by laser speckle contrast imaging (LSCI) and skin video-capillaroscopy.

The anthropometric evaluation consisted of measurements of the weight, height, waist circumference (cm), and calculated body mass index (kg/m²) of each subject. The systolic, diastolic, and mean blood pressures were determined using a sphygmomanometer. The brachial systolic (SAP) and diastolic (DAP) blood pressures were measured twice, 1 min apart, using a mercury sphygmomanometer, and the mean values are recorded as the patients' clinical blood pressure. The mean arterial pressure (MAP) was calculated as $DAP + 1/3(SAP - DAP)$.

Laboratory measurements

Blood specimens were collected before and after the three-week creatine supplementation, and the plasma samples were stored at -80 °C until their utilization. Fasting glucose, total cholesterol, HDL cholesterol, triglycerides, creatinine, uric acid, transaminases, homocysteine, vitamin B-12, and folic acid levels were determined by a photometric colorimetric optical system (Cobas Mira Systems, Roche Diagnostic Corporation, Indianapolis, IN, USA). LDL cholesterol was calculated by Friedewald's formula. The plasma levels of homocysteine were determined using an ELISA kit according to the manufacturer's instructions (Cayman Chemical, Ann Arbor, MI, USA).

Oral creatine supplementation

The subjects received 5 g/day once daily of commercially available micronized creatine monohydrate with a 99% purity (Bioderm, Rio de Janeiro, Brazil) for three weeks immediately after lunch or dinner. This study protocol has already been shown to significantly increase the plasma and intramuscular levels of creatine without causing important side effects [19,20]. The placebo group was supplemented with maltodextrin (Bioderm, Rio de Janeiro, Brazil) for the same time period [21]; creatine and placebo sachets were blinded for appearance and color of the substance. During the study protocol, creatine supplementation was monitored by weekly phone calls to the volunteers; all volunteers included in the analyses adhered to the supplementation schedule.

Evaluation of skin microvascular flow and reactivity

Microvascular reactivity was evaluated using an LSCI system with a laser wavelength of 785 nm (PeriCam PSI System, Perimed, Järfälla, Sweden), which allowed for noninvasive and continuous measurements of cutaneous microvascular perfusion changes to be measured. The measurements are expressed in arbitrary perfusion units (APU). The images were analyzed using PIMSoft software (Perimed, Järfälla, Sweden). One skin site on the ventral surface of the forearm was randomly chosen for the recordings. Hair, broken skin, areas of skin pigmentation, and visible veins were avoided, and two drug-delivery electrodes were installed using adhesive discs (LI 611, Perimed, Järfälla, Sweden). The following two measurement areas were identified: a measurement area within the electrode (ACh) and a measurement area (baseline control) adjacent to the electrode. A vacuum cushion (AB Germa, Kristianstad, Sweden) was used to minimize recording artifacts generated by the arm movements. ACh 2% w/v (Sigma Chemical CO, St. Louis, MO, USA) iontophoresis was performed using a micropharmacology system (PF 751 PeriIont USB Power Supply, Perimed, Sweden) using increasing anodal currents of 30, 60, 90, 120, 150, and 180 μ A, which were administered for 10-s intervals and spaced 1 min apart. The total charges for the above currents were 0.3, 0.6, 0.9, 1.2, 1.5, and 1.8 mC, respectively. The dispersive electrode was attached at approximately 15 cm from the electrophoresis chamber. The pharmacological test results are expressed as the peak values representing the maximal vasodilation observed after the highest ACh dose

was administered. Skin blood flow measurements, expressed in arbitrary perfusion units (APU), were divided by the mean arterial pressure to yield the cutaneous vascular conductance (CVC) in APU/mmHg.

Capillaroscopy by intravital microscopy

Microcirculatory tests were performed in a room with a stable defined temperature (23 ± 1 °C) after a 20-min rest in the supine position. The dorsum of the nondominant middle phalanx was used for image acquisition while the patient remained in a comfortable seated position. The room temperature was monitored and, if necessary, was adjusted using air conditioning, considering that the outside temperature was usually >25 °C. The arm was positioned at the level of the heart and immobilized using a vacuum cushion (AB Germa).

The capillary density, that is, the number of perfused capillaries per square millimeter area of skin, was assessed by high-resolution intravital color microscopy (Moritex, Cambridge, UK), as previously described and validated by our research team [22–24]. A video-microscopy system was used with an epi-illuminated fiberoptic microscope containing a 100-W mercury vapor lamp light source and an M200 objective with a final magnification of $200\times$. The images were acquired and saved for posterior offline analysis using a semi-automatic integrated system (Microvision Instruments, Evry, France). For the postocclusive reactive hyperemia (PORH), a blood pressure cuff was then applied around the patient's arm and inflated to a suprasystolic pressure (50 mmHg greater than the systolic arterial pressure) to completely interrupt the blood flow for 3 min. This time of occlusion already has been shown to effectively recruit capillaries in an endothelium-dependent manner. After the cuff release, images were acquired again and recorded over the subsequent 60–90 s, during which the maximal hyperemic response was expected to occur.

The mean number of spontaneously perfused skin capillaries at rest was considered to represent the functional capillary density, as previously described [23–25]. On the other hand, the number of perfused capillaries during postocclusive reactive hyperemia was considered to represent the functional capillary recruitment resulting from the release of endothelial mediators and the consequent arteriolar vasodilation [25]. The mean capillary density for each patient was calculated as the arithmetic mean of visible (i.e., spontaneously perfused) capillaries in three contiguous 1 mm^2 microscopic fields. The capillary counting was performed by two investigators who were blinded to

the patients' characteristics, and the final values of the capillary density represent the means of the individual counts. Reproducibility was assessed by examining an identical area of the skin, while intra-observer repeatability of the data analysis was assessed by blindly reading the same images on two separate occasions ($n = 15$, coefficient of variability: 4.3%).

Statistical analysis

The results are presented as the mean \pm SD. Variables without a Gaussian distribution, which was determined with the Shapiro–Wilk normality test, are presented as the median (25th–75th percentile). Comparisons of parameters obtained from placebo and creatine supplementation groups were performed using two-tailed paired Student's *t*-test or the Mann–Whitney test, for parametric or nonparametric variables, respectively. Comparisons of parameters obtained before and after supplementation with placebo or creatine were performed using two-way ANOVA followed by multiple comparisons (Sidak's multiple comparison test), where we considered the interactions of time (pre- and post-treatment) and treatment (placebo or creatine). The dose dependency of the effects of acetylcholine on microvascular vasodilation was tested using repeated measures ANOVA, followed by Dunnett's test for multiple comparisons. *P* values <0.05 were considered statistically significant. The identification of potential outliers was performed using the (robust regression and outlier removal) ROUT method, which is based on the false discovery rate (FDR), with a specified value of $Q = 1\%$. The statistical package used for the statistical analyses is Prism version 7.0 (GraphPad Software Inc., La Jolla, CA, USA).

RESULTS

Clinical, anthropometric, and laboratory data

Tables I and II show the baseline clinical characteristics of the placebo (PLA) and creatine supplementation (CrS) groups. There were no significant differences in age, time on the vegan diet, physical activity levels, or laboratory parameters between the PLA and CrS groups. *Table II* shows that after the three-week creatine supplementation, there were increases in the total body mass and body mass index only in the CrS group. Despite the CrS group having slightly higher diastolic blood pressures than the PLA group, there were no statistically significant differences in blood pressure or heart rate before and after CrS or PLA treatment.

Table I Basal clinical and biochemical characteristics of volunteers from the placebo and creatine supplementation groups.

Characteristics	Vegans + Placebo (n = 18)	Vegans + Creatine (n = 31)	P value
Age (years)	32 ± 9	33 ± 10	0.8772
Diet time (years)	6 (2–17)	10 (3–16)	0.7322
Male sex, n (%)	9 (50)	18 (58)	0.5843
Weekly frequency of physical activity (days)	3.4 ± 1.2	3.4 ± 1.4	0.9749
Sedentary, n (%)	3 (17)	11 (35)	0.1817
Triglycerides (mg/dL)	87.8 ± 28.1	100 ± 68.7	0.4886
Cholesterol (mg/dL)	146 (126–165)	152 (133–175)	0.1776
HDL (mg/dL)	44.2 ± 11.4	50.5 ± 12.6	0.0751
LDL (mg/dL)	82.8 ± 18.3	88.9 ± 33.1	0.0909
Vitamin D (ng/mL)	26.7 ± 9.1	23.1 ± 8	0.2637
Folic acid (ng/mL)	13.5 ± 4.9	13.1 ± 5.6	0.8658
Vitamin B-12 (pg/mL)	315 (205–628)	288 (164–391)	0.1554
Homocysteine (μmol/L)	11.89 (8.23–15.35)	12.11 (8.57–17.12)	0.3837

The results are shown as the mean ± standard deviation or as the median (25–75%) for parametric or nonparametric variables, respectively. Statistical analyses were performed using two-tailed paired Student's *t*-tests or the Mann–Whitney tests, for parametric and nonparametric variables, respectively. HDL, high-density lipoprotein; LDL, low-density lipoprotein.

Table II Anthropometric and haemodynamic parameters before (PRE) and after (POST) oral placebo (PLA) or creatine (CrS) supplementation.

Characteristics	Vegans + Placebo (n = 18)		Vegans + Creatine (n = 31)		P value
	PRE-PLA	POST-PLA	PRE-CrS	POST-CrS	
Body mass (kg)	64.4 ± 13.3	64.3 ± 13.4	66.1 ± 10.3	67 ± 10.5***	0.0064
BMI (Kg/m ²)	22.9 ± 2.7	22.9 ± 2.8	23.3 ± 3.4	23.6 ± 3.5***	0.0096
SBP (mmHg)	115 ± 12	118 ± 14	118 ± 14	120 ± 15	0.7683
DBP (mmHg)	68 ± 5	67 ± 6	75 ± 8	74 ± 10	0.4063
MBP (mmHg)	84 ± 6	84 ± 6	89 ± 9	89 ± 11	0.6163
HR (bpm)	64 ± 7	64 ± 9	67 ± 11	69 ± 9	0.4896

The results are shown as the mean ± standard deviation or as the median (25–75%) for parametric and nonparametric parameters, respectively. Statistical analyses were performed using two-way ANOVA followed by multiple comparisons (Sidak's multiple comparisons test), where we considered the interactions of time (pre- and post-treatment) and treatment (placebo or creatine). *P* values in bold depict statistically significant values. BMI, body mass index; DBP, diastolic blood pressure; HR, heart rate; MBP, mean blood pressure; SBP, systolic blood pressure.

****P* < 0.001 vs. PRE-CrS.

Table III shows that CrS but not PLA significantly reduced the homocysteine (Hcy) plasma levels in the subjects. Although the mean baseline Hcy levels of the CrS group were considered normal (5 to 15 μmol/L), 42% of the vegan subjects presented with hyperhomocysteinemia (H-Hcy: Hcy >15 μmol/L, *n* = 16). From this group of vegan subjects, two of them were classified as having mild H-Hcy (16 to 30 μmol/L), and two were classified as having moderate H-Hcy (31 to 100 μmol/L). After the CrS period, the Hcy levels of all of the vegan subjects normalized. As verified by normal levels of T3 and T4 before and after PLA or CrS, no hypothyroid condition was found in this group of

individuals. The creatinine levels in the CrS group increased significantly from the baseline values after the three-week CrS treatment, but the creatinine levels of the PLA group did not show any changes (Table III). The folic acid levels showed the same trend, increasing only after CrS (Table III). There were no significant differences in the other biochemical parameters in the PLA and CrS groups before or after supplementation (Table III). The individual values of the Hcy plasma levels before and after CrS are described in Figure 2c; these data show that in our study around 71% of the subjects are responders to CrS concerning the decrease in Hcy plasma levels.

Table III Laboratory data before (PRE) and after (POST) oral placebo (PLA) or creatine (CrS) supplementation.

Laboratory parameters	Vegans + Placebo (n = 18)		Vegans + Creatine (n = 31)		P value ANOVA
	PRE-PLA	POST-PLA	PRE-CrS	POST-CrS	
Homocysteine ($\mu\text{mol/L}$)	11.89 (8.23–15.35)	11.23 (8.46–13.32)	12.11 (8.57–17.12)	10.58 (8.15–13.22)*	0.0282
PCR (mg/dL)	0.07 (0.04–0.31)	0.05 (0.02–0.13)	0.11 (0.05–1.10)	0.20 (0.07–0.46)	0.6226
Glucose (mg/dL)	85 (79–88)	87 (82–91)	88 (83–89)	85 (82–93)	0.6800
Urea (mg/dL)	17.7 \pm 5.5	17.5 \pm 6.7	17.9 \pm 4.1	18.9 \pm 3.9	0.3111
Creatinine (mg/dL)	0.75 \pm 0.15	0.80 \pm 0.16 [#]	0.71 \pm 0.1	0.91 \pm 0.19***	0.0232
Uric acid (mg/dL)	4.9 \pm 1.4	4.9 \pm 1.2	4.5 \pm 1.2	4.3 \pm 1.2	0.3676
Creatine kinase (μL)	61 (48–76)	64 (53–98)	59 (47–102)	58 (48–80)	0.9725
Triglycerides (mg/dL)	87.8 \pm 28.1	76.3 \pm 24.5	100.0 \pm 68.7	88.9 \pm 53.1	0.9613
Cholesterol (mg/dL)	146 (126–165)	143 (132–168)	152 (133–175)	147 (132–163)	0.0788
HDL (mg/dL)	44 \pm 11	47 \pm 12	50 \pm 12	51 \pm 12	0.2314
LDL (mg/dL)	83 \pm 18	88 \pm 30	89 \pm 33	83 \pm 28	0.1100
T3 (ng/mL)	1.28 (1.15–1.39)	1.18 (1.07–1.35)	1.16 (1.05–1.36)	1.18 (1.06–1.33)	0.4561
T4 (ng/dL)	0.88 \pm 0.14	0.89 \pm 0.14	1.05 \pm 0.61	0.96 \pm 0.12	0.2073
Vitamin B-12 (pg/mL)	315 (204–628)	291 (209–602)	288 (164–391)	252 (181–366)	0.9983
Vitamin D (ng/mL)	26.7 \pm 9.1	26.9 \pm 8.8	23.1 \pm 9.8	24.1 \pm 8.3	0.6187
Folic acid (ng/mL)	13.5 \pm 4.9	15.0 \pm 4.8	13.1 \pm 5.6	15.8 \pm 6.4**	0.0013

The results are shown as the mean \pm standard deviation or as the median (25–75%) for parametric and nonparametric parameters, respectively. Statistical analyses were performed using two-way ANOVA followed by multiple comparisons (Sidak's multiple comparisons test), where we considered the interactions of time (pre- and post-treatment) and treatment (placebo or creatine). P values in bold depict statistically significant values. HDL, high-density lipoprotein; LDL, low-density lipoprotein; PCR, high-sensitivity C-reactive protein; T3, triiodothyronine; T4, thyroxine.

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.0001$ vs. PRE-CrS; # $P < 0.05$ vs. PRE-PLA.

Figure 2a presents the stratified data of the H-Hcy and N-Hcy vegan subjects who participated in the study. Although the CrS treatment had no significant

effects on the N-Hcy vegan subjects, it reduced the homocysteine levels of H-Hcy vegan subjects by approximately 39%. The cutoff point for the strata in the PLA and creatine groups was clinical reference values >13.00 μmol/L. Although PLA or CrS did not alter the plasma levels of vitamin B-12, these levels were marginally and significantly lower in the H-Hcy vegan subjects, than in the subjects of the other groups. CrS significantly increased the folic acid levels only in H-Hcy vegan subjects (Figure 2b).

Microcirculatory parameters

Video-capillaroscopy

Figure 3 shows that the basal (functional) capillary density of the vegan subjects was significantly increased in those who had the three weeks of CrS but that it was not significantly increased in those in the PLA group. An increase in capillary recruitment during postocclusive reactive hyperemia was also observed only in the CrS group (Figure 3). Figure 4 shows that increases in basal capillary density and capillary recruitment were observed in both the N-Hcy and H-Hcy individuals in the CrS group.

Microvascular flow and reactivity

Microvascular responses to acetylcholine (ACh) stimulation and postocclusive reactive hyperemia (PORH)

PLA and CrS did not change the peak values of cutaneous vascular conductance (CVC) induced by skin iontophoresis of ACh and the global ACh effect represented by the area under the curve (Table IV). However, the stratification of the CrS group demonstrated that the area under the curve values were significantly reduced in the N-Hcy individuals compared to those in the other subjects (Table IV). In this way, after three weeks of CrS, the H-Hcy vegan subjects had a significantly higher area under the curve than did the N-Hcy individuals (Table IV). There was no difference in the CVC Max after PORH for both the PLA and CrS groups

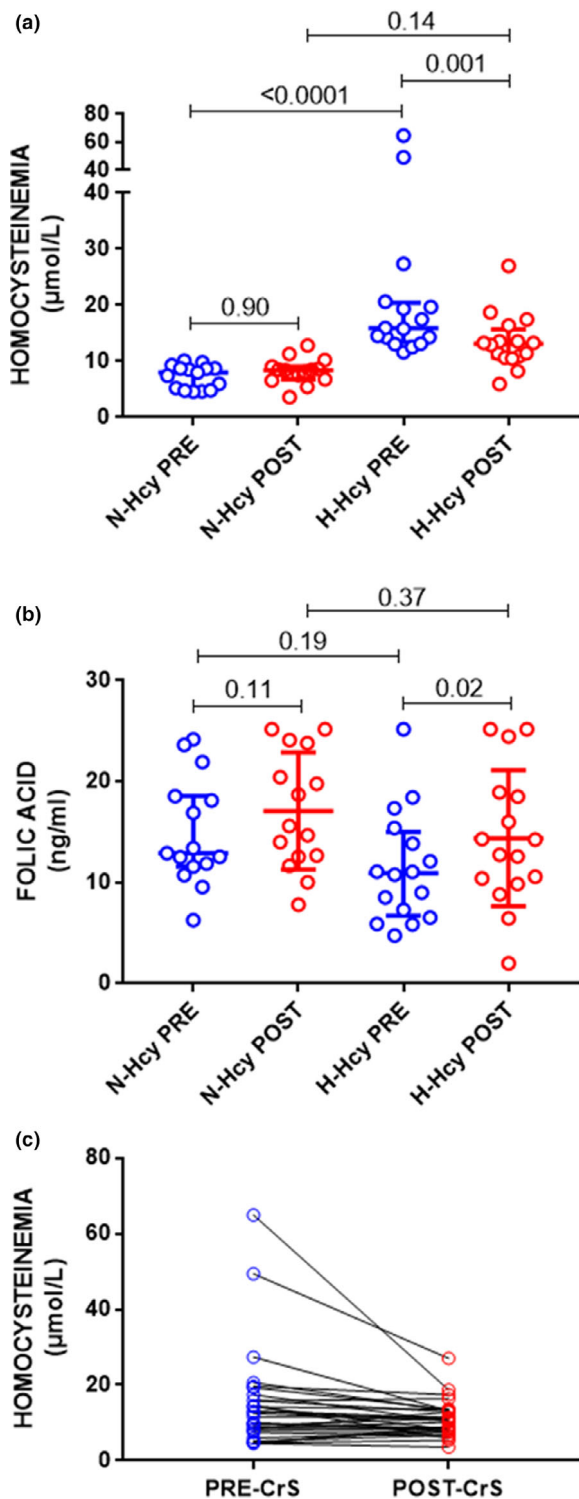


Figure 2 Effects of oral creatine supplementation (CrS) on homocysteine (a) and folic acid (b) plasma levels. (c) Individual values of plasma levels of homocysteine before and after CrS (n = 31). In Figure 2a and b, vegan subjects were stratified as hyperhomocysteinemic (H-Hcy, n = 16) or normohomocysteinemic (N-Hcy, n = 15). In Figure 1a, the y-axis was divided into two segments to improve the clarity of the presentation of results. Abbreviations: POST, after CrS; PRE, before CrS.

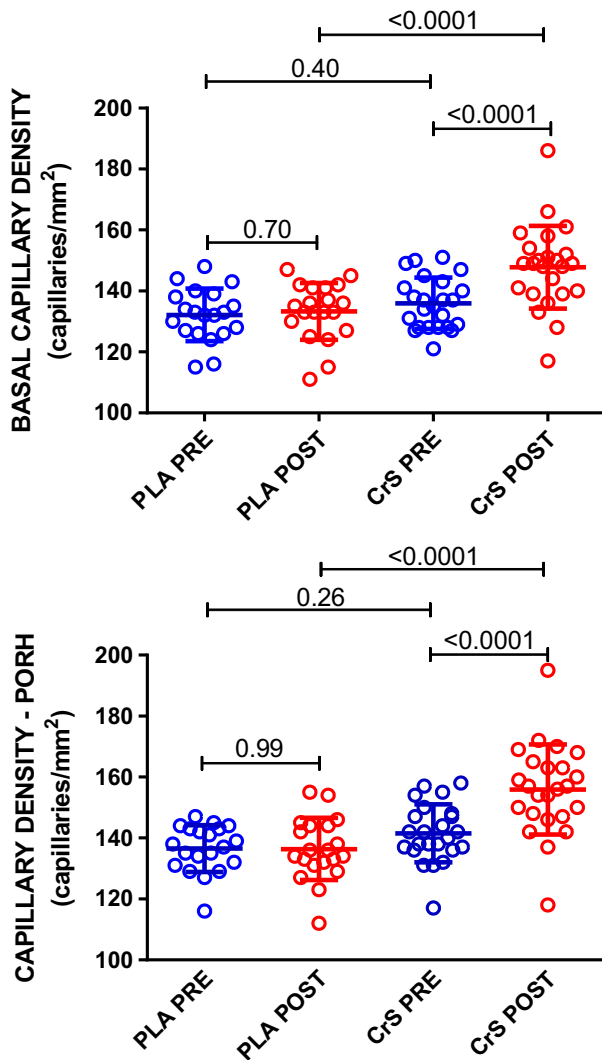


Figure 3 Functional (basal) capillary density and capillary recruitment during postocclusive reactive hyperemia (PORH) in the PLA and CrS groups. Abbreviations: CrS, oral creatine supplementation; PLA, oral placebo supplementation; POST, after supplementation; PRE, before supplementation.

(Table IV). In fact, the delta and peak values of cutaneous vascular conductance after skin iontophoresis of ACh were significantly higher in the H-Hcy individuals than in the N-Hcy individuals after the CrS period.

DISCUSSION

The main findings of this study are as follows: i) In strict vegan subjects, CrS significantly increased the body weight, body mass index, and creatinine levels; ii) CrS significantly reduced the plasma homocysteine

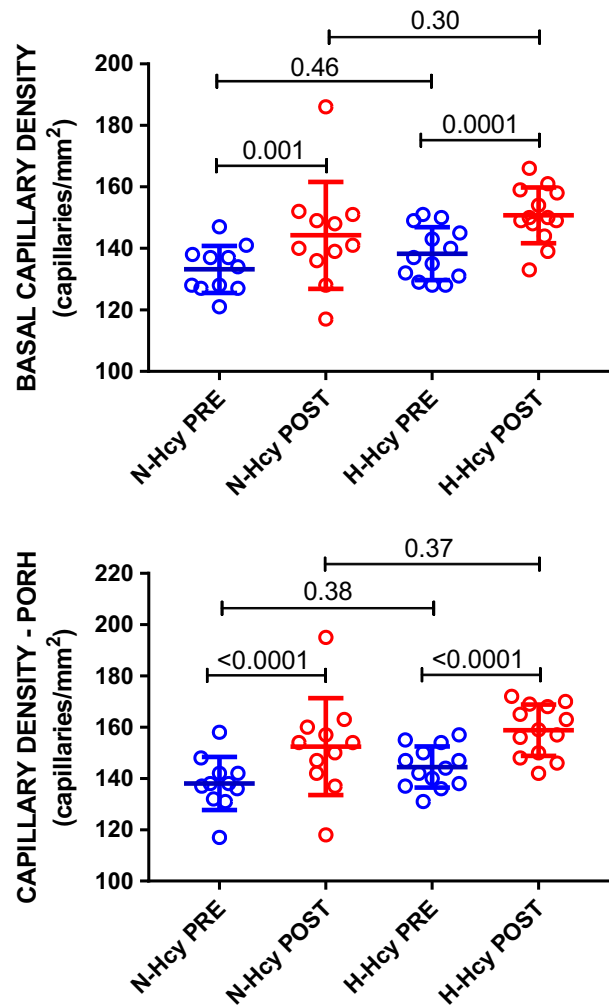


Figure 4 Functional (basal) capillary density and capillary recruitment during postocclusive reactive hyperemia (PORH) stratified by the hyperhomocysteinemic (H-Hcy) and normohomocysteinemic (N-Hcy) vegan subjects. Abbreviations: CrS, oral creatine supplementation; POST, after supplementation; PRE, before supplementation.

levels and increased folic acid levels only in the group of hyperhomocysteinemic subjects; iii) CrS was able to increase the perfusion of capillaries in the basal state and after hyperemia, which is an effect that was verified in both the normal and hyperhomocysteinemic vegan subjects.

Our results are in accordance with previous animal studies that have shown that CrS could reduce homocysteinemia and significantly increase folic acid serum levels, probably because it interferes with the availability of substrates in homocysteine synthesis and degradation routes [26,27]. In this way, the reduction in the

Table IV Effects of cutaneous iontophoresis of acetylcholine (Ach) and postocclusive reactive hyperemia (PORH) on cutaneous microvascular conductance (CVC), expressed in arbitrary perfusion units (APU per mean arterial pressure in mmHg), before (PRE) and after (POST) three weeks of oral placebo (PLA) or creatine (CrS) supplementation in vegan subjects (all subjects) and stratified by plasma homocysteine levels.

Parameters	Vegans + Placebo (<i>n</i> = 18)		Vegans + Creatine (<i>n</i> = 31)	
	PRE-PLA	POST-PLA	PRE-CrS	POST-CrS
CVC Max (APU/mmHg)	0.72 ± 0.21	0.79 ± 0.26	0.65 ± 0.18	0.64 ± 0.20
Delta CVC Ach (APU/mmHg)	0.43 ± 0.22	0.46 ± 0.21	0.38 ± 0.16	0.37 ± 0.20
AUC (APU/s)	8 632 ± 5 510	9 419 ± 7 427	7 187 ± 3 690	6 587 ± 4 313 [⊗]
CVC PORH (APU/mmHg)	0.96 ± 0.19	0.99 ± 0.17	0.86 ± 0.20 [#]	0.82 ± 0.27
Delta CVC PORH (APU/mmHg)	0.59 ± 0.18	0.61 ± 0.15	0.53 ± 0.16	0.52 ± 0.20

Parameters	N-Hcy (<i>n</i> = 15)		H-Hcy (<i>n</i> = 16)	
	PRE-CrS	POST-CrS	PRE-CrS	POST-CrS
CVC Max (APU/mmHg)	0.62 ± 0.13	0.55 ± 0.18	0.67 ± 0.20	0.72 ± 0.18 [#]
Delta CVC Ach (APU/mmHg)	0.36 ± 0.14	0.27 ± 0.17	0.40 ± 0.18	0.46 ± 0.17 ^{##}
AUC (APU/s)	6 928 ± 3 254	4 435 ± 3 463 [*]	7 429 ± 4 150	8 605 ± 4 127 ^{##}
CVC Max PORH (APU/mmHg)	0.84 ± 0.23	0.77 ± 0.31	0.89 ± 0.17	0.87 ± 0.21
Delta CVC PORH (APU/mmHg)	0.51 ± 0.21	0.46 ± 0.24	0.51 ± 0.12	0.51 ± 0.19

The results are shown as the mean ± standard deviation. Statistical analyses were performed using two-way ANOVA followed by multiple comparisons (Sidak's multiple comparisons test), where we considered the interactions of time (pre- and post-treatment) and treatment (placebo or creatine). AUC, area under the curve of vasodilation induced by Ach; CrS, oral supplementation with creatine; H-Hcy, hyperhomocysteinemic subjects; N-Hcy, normohomocysteinemic subjects.

**P* < 0.05 vs. PRE-CrS; [⊗]*P* < 0.05 vs. POST-PLA; [#]*P* < 0.05; ^{##}*P* < 0.01 vs. POST-CrS in N-Hcy.

methylation demand through CrS was paradoxically demonstrated to be able to reduce the DNA methylation level [28], exposing previously silenced genes that could influence the capillary reactivity to activation by transcription factors. In this sense, CrS is able to influence metabolic changes, including increases in the adenosine and folic acid levels that could change the DNA-methyltransferase activity and thereby alter the DNA methylation profile, inducing angiogenesis and anti-inflammatory effects [29–31]. In this way, CrS was already suggested to activate AMP-activated protein kinase (AMPK) and increase the enzyme activity of 5'-nucleotidase, which catalyzes the AMP deamination in adenosine and phosphate through the PKC pathway [17,32].

The effect of CrS on plasma Hcy levels is still a controversial matter. The main reason for the discrepancies in the literature is probably the existence of responders and nonresponders to oral CrS. In the present study, approximately 71% of the volunteers presented with reductions in their Hcy plasma levels after CrS. There are also differences between humans and rodents, which are probably due to different characteristics among them, mainly because the human kidneys exhibit different enzymatic activities comparing to rats,

and the amount of protein-bound plasma Hcy is greater in humans when compared to rats [33]. Other factors that may influence these differences are linked to distinct study protocols along with different dosages, age, and gender of populations and especially to studies with reduced numbers of participants [34]. Yet, some studies in humans suggest that oral CrS does not alter vascular reactivity but instead causes significant elevations of plasma homocysteine levels in normohomocysteinemic subjects, despite that it causes significant reductions in hyperhomocysteinemic individuals [15,16].

Notwithstanding, recent data show that creatine supplementation may be a practical strategy to decrease plasma homocysteine levels and reduce vascular oxidative stress [9,13,14]. In fact, our group recently demonstrated that oral supplementation with 20 g per day of creatine for only one week was able to significantly increase skin capillary perfusion at rest and during postocclusive reactive hyperemia in young, healthy, and physically active nonvegetarian individuals [17]. In this study, we hypothesized that the possible increase in the creatine kinase–phosphocreatine ratio (Cr/PCr ratio) induced by CrS in endothelial cells could activate AMPK and increase adenosine levels

[31,32,35–37]. Thus, in our study, although the activation of AMPK still needs to be demonstrated, CrS may have activated the expression of vascular endothelial growth factor, independent of the release of nitric oxide, through changes in DNA methylation, thereby collaborating to increase capillary perfusion at rest, which is highly suggestive of the process of angiogenesis [38].

It is noteworthy that the capillary densities in all of the groups of the present study appeared to be significantly higher than those observed in omnivores of the same age-group in another study conducted by our group [17], suggesting that vegans have a higher degree of tissue perfusion than omnivorous subjects.

Even if CrS did not alter the microvascular vasodilation induced by ACh or reactive hyperemia, there was a significant reduction in the area on the ACh curve after CrS in the normohomocysteinemic vegan subjects, which could be associated with an increasing trend of the homocysteine level. This profile was not observed in previously hyperhomocysteinemic vegan subjects. In this sense, increases of 1 $\mu\text{mol/L}$ of homocysteine, as suggested by Jahandir *et al.*, are sufficient to impair the endothelium-dependent vasodilation response [15].

The enzyme S-adenosyl homocysteine hydrolase (SAHH) is known to turn S-adenosylhomocysteine (SAH) into homocysteine and adenosine and to be regulated by their reaction products [39]. During hyperhomocysteinemia, endothelial cells have been demonstrated to have an impaired ability to transport homocysteine to the outside of the cell, leading to SAH accumulation and SAHH inhibition, which could increase oxidative stress and inflammation [40,41]. Since CrS reduces the demand for methylation over S-adenosylmethionine (SAM) and increases the SAM/SAH ratio, CrS could enhance endothelial function and facilitate homocysteine and adenosine efflux to the outside of the endothelial cell, respectively, recovering the SAHH activity and contributing to capillary sphincter relaxation and perfusion enhancement [42,43].

As we had expected, CrS induced a significant increase in body mass and creatinine plasma levels that may be related to the intracellular augmentation of osmolarity due to cellular water retention and creatine metabolic degradation, respectively [44,45]. Although neither placebo nor CrS was able to alter the vitamin B-12 levels in normo- or hyperhomocysteinemic vegan subjects, these levels were marginally but significantly lower among the hyperhomocysteinemic vegan subjects. Our volunteers did not show any hypothyroidism

signs despite this micronutrient deficiency being a risk of vegan diets, and CrS did not change the T3 and T4 serum levels as it did with the omnivorous subjects in our previous study [17].

Limitations to this study must be considered. The study did not evaluate the food intake of the volunteers for food containing creatine, folate, and vitamin B-12. Nevertheless, volunteers who supplemented their diet with creatine or vitamin B-12 were not included in the study. Finally, the use of a randomized and single-blinded study design with parallel groups precludes the comparison of different treatments in the same subjects.

CONCLUSIONS

The present study demonstrates that three weeks of oral creatine supplementation is sufficient to enhance skin capillary perfusion in strict vegan subjects, which is paralleled by plasma increases in folic acid and reductions in homocysteine levels among only the hyperhomocysteinemic individuals.

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COMPETING INTERESTS

There are no conflicts of interest declared by the authors. The authors declare that there are no competing financial interests in relation to the present study.

AUTHOR CONTRIBUTIONS

R.d.M. and E.T. conceived and designed the study. D.V.B performed the experiments. R.d.M. and E.T. analyzed the data and interpreted the results of the experiments. R.d.M. drafted the manuscript. R.d.M., D.V.B., and E.T. edited and revised the manuscript. All of the authors approved the final version of the manuscript.

ABBREVIATIONS

ACh – acetylcholine
 APU – arbitrary perfusion units
 AUC – area under the curve
 CrS – creatine supplementation
 CVC – cutaneous vascular conductance
 H-Hcy – hyperhomocysteinemia
 LSCI – laser speckle contrast imaging
 N-Hcy – normohomocysteinemia
 PORH – postocclusive reactive hyperemia

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