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Original Article

Effects of Dietary Supplementation with Brazil Nuts on Microvascular Endothelial Function in Hypertensive and Dyslipidemic Patients: A Randomized Crossover Placebo-Controlled Trial

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ABSTRACT

Objective: To investigate the effects of dietary supplementation with GBNs on microvascular endothelial function in hypertensive and dyslipidemic patients.

Methods: Ninety-one patients of both sexes aged 62.1 ± 9.3 years received 13 g/day of GBNs or a placebo for three months with a washout period of one month between treatments. Microvascular endothelial function was assessed using LSCI coupled with iontophoresis of ACh and PORH. We also used skin video capillaroscopy to measure capillary density and recruitment at rest and during PORH. Plasma concentrations of NO*x* were also measured as a marker of nitric oxide bioavailability.

Results: Supplementation with GBNs significantly increased the plasma levels of Se ($p < 0.05$) and NO*x* ($p < 0.05$). However, we did not observe any effects of GBN consumption on microvascular vasodilator responses to ACh or PORH ($p > 0.05$), and GBNs did not improve capillary density at baseline or recruitment during PORH ($p > 0.05$).

Conclusions: Supplementation with GBNs induced significant increases in the plasma Se concentration and systemic bioavailability of nitric oxide. Nevertheless, GBN supplementation did not lead to any improvement in systemic microvascular reactivity or density in patients with arterial hypertension and dyslipidemia who were undergoing multiple drug therapies.

Key words: Brazil nuts, systemic microcirculation, endothelial function, hypertension, dyslipidemia, randomized controlled trial

Abbreviations used: ACE, angiotensin-converting enzyme; ACh, acetylcholine; AMI, acute myocardial infarction; APUs, arbitrary perfusion units; ASA, acetylsalicylic acid; AUC, area under the curve; BMI, body mass index; CABG, coronary artery bypass grafting; CHD, coronary heart disease; CVC, cutaneous vascular conductance; DBP, diastolic blood pressure; GBNs, granulated Brazil nuts; HDL-c, high-density lipoprotein-cholesterol; LDL-c, low-density lipoprotein-cholesterol; LSCI, laser speckle contrast imaging; MAP, mean arterial pressure; NO*x*, nitrite and nitrate; PORH, post-occlusive reactive hyperemia; SBP, systolic blood pressure; Se, selenium; TEV, total energy value; WC, waist circumference.

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INTRODUCTION

The dietary consumption of nuts has been associated with beneficial effects on cardiovascular health [33], including improved vascular endothelial function [7], reduced serum lipids [38], and a reduced risk of hypertension [26]. Therefore, nuts are currently considered to be a component of a cardioprotective diet [29]. Brazil nuts (*Bertholletia excelsa*, Lecythidaceae) are thought to be the food that contains the highest content of unadulterated Se [14]. Se is a key component that is incorporated into selenoproteins, which have antioxidant and anti-inflammatory properties and participate in the metabolism of thyroid hormones [32]. The consumption of Brazil nuts can significantly increase the activity of glutathione peroxidase, which is a selenoprotein involved in the dismutation of hydrogen peroxide [45]. In

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addition, oxidative stress is associated with endothelial dysfunction in patients with hypertension [12] and hypercholesterolemia [30]. Partially defatted GBNs are produced after partial extraction of extra virgin oil from the nuts, and the solid compound resulting from this lipid extraction is crushed.

Prospective studies in humans have demonstrated that endothelial dysfunction is an independent predictor of adverse cardiovascular events and leads to poor long-term prognosis [41]. Hypertensive and dyslipidemic individuals demonstrate decreased endothelial function compared with healthy controls [8], and both diseases are predictors of damage to the vascular endothelium, which is directly related to endothelial dysfunction [3]. Moreover, there is an association between improvement in endothelial function and an increase in the survival rate of patients with coronary artery disease [13].

A previous study observed improved brachial arterial flow in hypercholesterolemic subjects after four weeks of a diet enriched with nuts [34], and similar results have also been reported after the consumption of a single meal enriched with nuts [9]. The only study to date that has examined Brazil nut consumption and microvascular function was conducted on obese adolescents, and the results showed improved skin nutritive capillary function after supplementation [25].

Therefore, the primary aim of this study was to investigate the effects of partially defatted GBNs in combination with individual diet on microvascular endothelial function in severely hypertensive and dyslipidemic patients. Our hypothesis was based on the protective effects of the bioactive compounds present in Brazil nuts and their use as an adjunctive therapy for the treatment of hypertension and dyslipidemia to improve endothelial dysfunction.

The evaluation of the microcirculatory effects resulting from dietary supplementation with Brazil nuts was achieved using LSCI coupled with physiological and pharmacological provocations in the evaluation of cutaneous microvascular reactivity, and intra-vital video microscopy was used to evaluate skin capillary density and reactivity.

MATERIALS AND METHODS

Subjects

One hundred and thirty-seven subjects were screened at the Clinic of Atherosclerosis and Cardiovascular Disease Prevention of the National Institute of Cardiology, Rio de Janeiro, Brazil from September 2011 to September 2012. Of the 137 subjects screened, 125 were eligible for the study. Males and females aged >20 years with referred diagnoses of dyslipidemia and hypertension who had been taking medication for their condition for at least three months were included in this study. The exclusion criteria were as follows: patients with a food allergy to Brazil nuts, those who were pregnant or breastfeeding, those on a low calorie diet, those using dietary supplements containing antioxidant vitamins or minerals, those using corticoid substances, and those with thyroid disease, chronic renal failure, liver disease, cancer, rheumatic disease, or systemic connective tissue disease. This study was approved by the Institutional Review Board of the National Institute of Cardiology (protocol #0316/11) and was registered at ClinicalTrials.gov (NCT01990391).

Study Design and Dietary Intervention

A randomized placebo-controlled, double-blind crossover trial was performed. Subjects were provided with either partially defatted GBNs or placebo along with nutritional counseling for dyslipidemia and hypertension [6,43]. Each supplementation period lasted for three months, as described in a previous study [25], with monthly follow-ups, when the supplements were given to patients in a white bottle with a standard spoon. No specific time during which to consume the supplements was specified. Nevertheless, the subjects were advised to take the supplements with meals or snacks. A previous study showed that after 16 weeks of Brazil nut consumption, functional changes were induced in the microvascular reactivity of obese adolescents [25]. After the first step of the intervention, the subjects underwent a washout period of one month, during which they did not receive any supplements. After the washout period, the patients underwent a second step of supplementation (crossover design). The randomization was simple, in blocks of 10 and based on a table of random numbers that were blinded from all researchers except for one who encoded the bottles of supplements and had no contact with the center at which the study was conducted.

The subjects being treated with GBNs were advised to consume a standard portion of 13 g per day (containing an average of 227.5 μg of Se) of partially defatted GBNs (Ouro Verde Amazônia®, Mato Grosso, Brazil). The amount of GBNs was calculated to provide an average dose of 200 μg of Se, based on a previous study demonstrating that higher doses could impair glucose homeostasis [45]. Partially defatted GBNs were used in this study rather than the Brazil nut kernel because these two nut products contain similar amounts of Se (227.5 versus 249.21 μg, respectively). Because the main purpose of this study was to study the effects of Se supplementation, rather than the single consumption of Brazil nuts, we assessed the Se content in all batches of partially defatted GBNs and found it to always be $\approx$200 μg of Se. Adherence to the consumption of the supplement was monitored using the evaluation of plasma Se concentrations because this is considered to be a good marker of Se intake [44]. Thus, the monthly rates of plasma Se were calculated using basal values plasma Se and values after each month of

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intervention. The equation for the calculation of the slope of the regression line was: $b = \frac{\sum(x-\bar{x})(y-\bar{y})}{\sum(x-\bar{x})^2}$. We considered adherent to the intervention the patients who showed increasing monthly rates of plasma Se (>0). Patients who showed monthly rates of plasma Se ≤0 were considered as non-adherent. Using this standard, in our study 91.2% (n = 83) of the patients were considered to be adherent to the supplementation.

The placebo (Mane do Brazil Indústria e Comércio Ltda, Rio de Janeiro, Brazil) was composed of flavored cassava flour (which has a nutty aroma) and was lightly stained (with natural caramel pigment) to approximate the appearance and smell of GBNs. During the placebo intervention, the patients were advised to consume a standard portion of 10 g of placebo daily. The calculation of the diet was based on TEV formulas for men and women according to their nutritional status, age, and a physical activity coefficient of 1.0 related to physical inactivity [2]. The distribution of macronutrients was balanced and followed the guidelines for hypertension and dyslipidemia [6,43].

The sample size calculation was performed in a pilot study comprised of the first 15 participants, considering a 13% increase in skin microvascular blood flow induced by PORH after treatment with Brazil nuts. A power of 80% and a bilateral confidence interval of 90% were used. The calculated sample size was 112, and this number was increased by 11% (to a total of 125 subjects) to account for possible losses.

The excellent reproducibility of the skin PORH responses has been systematically investigated by Cracowski and colleagues [28,35–37]. The coefficients of variation and intra-class correlation coefficients of PORH peak responses using LSCI were 8% and 0.76, respectively, showing that the reproducibility is much higher than that obtained with the earliest methodologies such as single point laser Doppler flowmetry (30% and 0.54, respectively) [37]. We have previously studied the day-to-day repeatability of PORH peak responses using LSCI in eight healthy volunteers (four men) aged 33 ± 9 years (data not published). The increases in CVC observed during PORH in the first and second days were of 0.56 ± 0.14 and 0.52 ± 0.11 APU/mmHg, respectively (p = 0.2645; Table 1). The coefficients of variation and intra-class correlation coefficients of PORH peak responses were 11% and 0.69, respectively, demonstrating the excellent reproducibility of the method.

Table 1. Day-to-day repeatability of microvascular parameters in healthy volunteers (n = 8) using LSCI

Hemodynamic and microvascular parameters	Day 1	Day 2	p Values
MAP (mmHg)	84 ± 7	88 ± 9	0.7215
Increase in CVC induced by ACh iontophoresis (APU/mmHg)	0.40 ± 0.1	0.37 ± 0.2	0.5753
AUC of ACh iontophoresis (APU/sec)	8,201 ± 3,046	6,150 ± 4,026	0.1836
Peak CVC during PORH (APU/mmHg)	0.88 ± 0.2	0.85 ± 0.1	0.5944
Increase in CVC during PORH (APU/mmHg)	0.56 ± 0.14	0.52 ± 0.11	0.2645

The results are expressed as the mean ± SD; Paired two-tailed Student's t-test.

Compositions of Supplements

Thirteen grams of partially defatted GBNs contained 64.4 kcal, 2.8 g carbohydrate, 3.4 g protein, 5.6 g total fat, and 2.6 g dietary fiber. Ten grams of the placebo was composed of [49] 36.5 kcal, 8.92 g carbohydrate, 0.12 g protein, 0.03 g total fat, 0.65 g dietary fiber, and 0.07 μg Se.

Analysis of Se Content in Partially Defatted GBNs

The Se content in the partially defatted Brazil nuts was analyzed according to Benicasa *et al.* [4] and adapted for use with 0.3 g of sample, 6 mL of bi-distilled nitric acid and 3 mL of hydrogen peroxide (both from Merck®, Darmstadt, Germany). Samples were decomposed in a microwave oven (model DGT 100 plus; Provecto Analitica, Jundiaí, São Paulo, Brazil). The resulting solutions were transferred to polyethylene flasks and diluted to 50 mL with distilled and deionized water (minimum resistivity of 18 MΩ cm, MilliQ System; Millipore, Bedford, MA, USA). The reading of the ^{77}Se isotope was performed by ICP-MS (Agilent 7500 CX series, Santa Clara, CA, USA). Accuracy was evaluated by recovery tests and analysis of a dogfish liver certified reference material for trace metals (DOLT-3; National Research Council Canada Standard, Canada). Recoveries of approximately 100% were observed. Each batch of the partially defatted GBNs was analyzed, and the amount of Se measured was 17.5 ± 0.2 μg/g, which corresponded to 227.5 μg in 13 g of GBNs.

Evaluation Questionnaires

A questionnaire was used to obtain information pertaining to socio-demographic parameters, medical history, lifestyle, and the use of medication. To assess physical activity, a previously validated questionnaire was used [16]. Patients who performed at least 150 minutes of moderate intensity exercise per week, according to international recommendations, were considered physically active [11].

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Anthropometric and Blood Pressure Assessments

Anthropometric evaluation was performed at baseline and after supplementation. This evaluation included measurements of weight (kg), height (m), and WC (cm) and calculation of BMI (kg/m^2). BMI was classified according to the WHO.

SBP and DBP were measured in the supine position using a sphygmomanometer twice by a trained professional, with a one-minute interval between the two measurements. The SBP and DPB were measured immediately before microvascular examination, at baseline, and after supplementation, and the average value was used as the patient's blood pressure. The MAP was calculated as DBP + 1/3 (SBP − DBP) and used in the calculation of CVC.

Laboratory Measurements

Blood samples were collected after 12 hours of overnight fasting at baseline and after intervention, and laboratory evaluations were performed by an automated method (ARCHITECT *ci*8200, Abbott ARCHIECT®; Abbott Park, Chicago, IL, USA) using commercial kits (Abbott ARCHITECT *c*8000®, Abbott Park, IL, USA). Serum triglyceride levels, total cholesterol, and HDL-c were evaluated. LDL-c was calculated using the formula described by Friedewald, Levy, and Fredrickson [15].

Plasma Se levels were determined in plasma samples collected in NH Trace Element tubes with sodium heparin (VACUETTE®; Vacuette do Brasil LTDA, Americana, SP, Brazil) and stored at −20°C until analysis. Analysis was performed in an inductively coupled plasma mass spectrometer (NexIon™ 300X; PerkinElmer, Waltham, MA, USA) according to a method adapted from [20]. The plasma samples (0.5 g) were combined with 0.5 mL nitric acid and diluted with water to a final volume of 5.0 mL. For this experiment, the most abundant ^{80}Se isotope was monitored, due to the low Se concentrations found in the plasma samples, and analysis was performed in DRC mode with 0.75 mL/min of methane to circumvent the interferences. The plasma Se level was used as a marker of adherence to the consumption of the supplement. Plasma Se was considered to be low when the plasma level was <90 μg/L [32].

The evaluation of total plasma NO*x* ($NO_2 + NO_3$) concentrations was performed by a colorimetric assay (Cayman Inc., Ann Arbor, MI, USA) with a sensitivity of 2.5 μM and a 2.7% intra-assay coefficient of variation.

Assessment of Microvascular Endothelial Function

Skin microvascular flow and reactivity. Microcirculatory tests were performed after a 20-minute period of rest in the supine position in a temperature-controlled room (23 ± 1°C) approximately one hour after a light breakfast. Microvascular endothelial function was assessed at the beginning and end of each study phase using a LSCI system (PeriCam PSI system, Perimed, Järfälla, Sweden) coupled to physiological and pharmacological reactivity tests, including iontophoresis of ACh (dissolved in deionized water) and PORH, as previously described [8]. 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) with increasing anodal currents of 30, 60, 90, 120, 150, and 180 μA for 10-seconds intervals spaced one minute apart (Figure 1); after the last current, microvascular blood flow was recorded during 120 seconds, resulting in a total recording time of 540 seconds for the whole ACh curve. The dispersive electrode was attached approximately 15 cm away from the electrophoresis chamber. The image acquisition rate was eight images/sec, and the distance between the laser head and the skin surface was fixed at 20 cm, as recommended by the manufacturer's manual. Measurement skin areas (circular regions of interest) of approximately 80 mm^2 were used, and the mean skin microvascular blood flow was calculated at the peak of each dose of ACh. The AUC of ACh iontophoresis was normalized for the baseline blood flow of each patient. The amplitudes of the PORH responses were expressed as the peak CVC values minus the baseline CVC values. Skin conductance, as measured by the iontophoresis system PeriIont, did not vary significantly among patients. We have previously reported that iontophoresis charges up to 1.5 mC, which were used in this study, do not induce nonspecific effects on skin microvascular flow [8,50].

A vacuum cushion (AB Germa, Kristianstad, Sweden) was used to reduce recording artifacts generated by arm movements. During the PORH test, arterial occlusion was performed with supra-systolic pressure (50 mmHg above systolic arterial pressure) using a sphygmomanometer for three minutes. Following the release of pressure, maximum flux was measured. Images were analyzed using the manufacturer's software (PIMSoft; Perimed), and continuous measurements of cutaneous microvascular perfusion changes were expressed in APUs. Measurements of skin blood flow were divided by the MAP to give the CVC in APU/mmHg. The amplitudes of the PORH responses were expressed as the peak CVC minus the baseline CVC.

Skin capillaroscopy by intra-vital microscopy. Capillary density, which was defined as the total visible number of capillaries per mm^2 of skin area, was assessed on the dorsum of the non-dominant middle phalanx by high-resolution intra-vital color microscopy (Moritex, Cambridge, UK). We used a video microscopy system with an epi-illuminated fiber optic microscope containing a 100-W mercury vapor lamp light source and an M200 objective with a final magnification of 200×. The patient was seated comfortably in a constant temperature environment (23 ± 1°C), and the arm was

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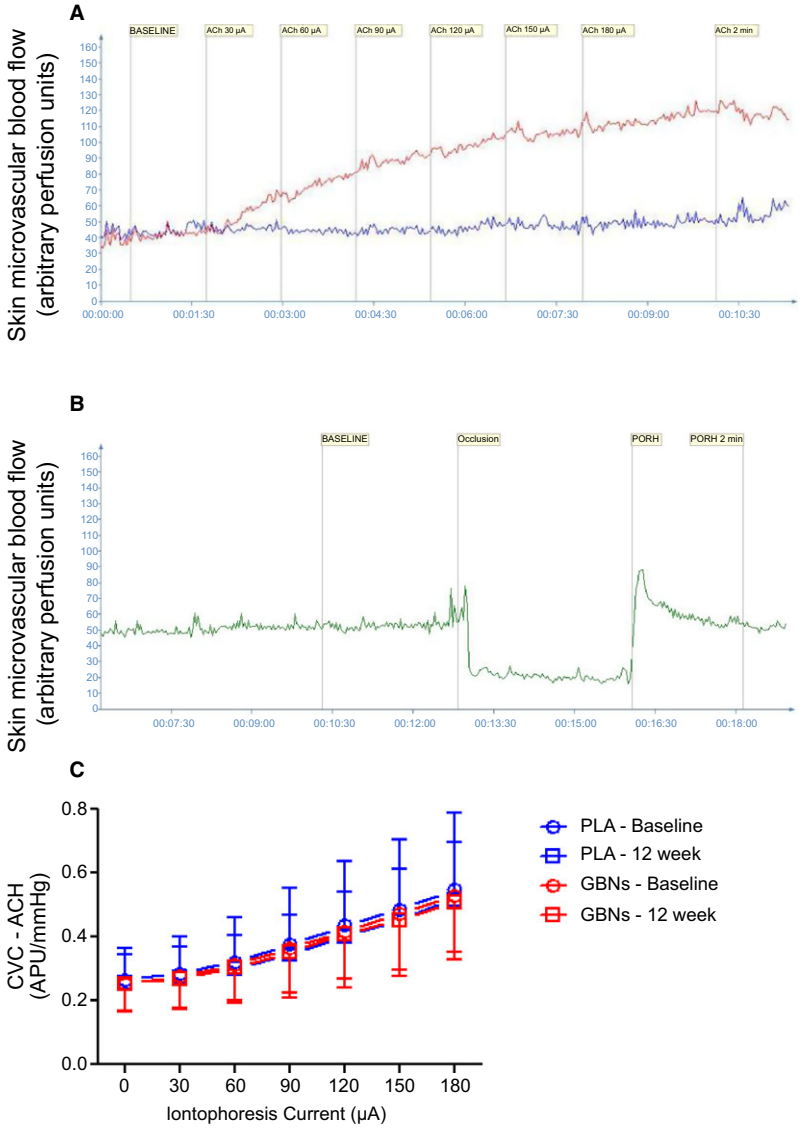

Figure 1. Typical recordings of skin microvascular blood flow by LSCI during iontophoresis of ACh (red line) using increasing anodal currents of 30, 60, 90, 120, 150, and 180 μA during 10-seconds intervals spaced one minute apart (**A**) and during PORH (**B**). The blue line represents microvascular flow in a non-stimulated region of the skin. (**C**) Mean effects of forearm skin iontophoresis of ACh on CVC (expressed in APUs, divided by MAP in mmHg) before (Baseline) and after 12 weeks of supplementation with placebo (PLA) or GBNs.

positioned at the level of the heart and immobilized using a vacuum cushion (a specially constructed pillow filled with polyurethane foam that can be molded to any desired shape by creating a vacuum; AB Germa). We evaluated the variability in skin video capillaroscopy in a previous study [46]. The reproducibility of this methodology was assessed by examining an identical area of the finger skin; intra-observer repeatability of data analysis was assessed by reading the same images in a blinded manner on two separate occasions (n = 20; coefficient of variability, 4.3%). To assess inter-observer repeatability, a second observer independently assessed the capillary density in the same images (n = 15; coefficient of variability, 3.3%).

Acquired images were saved for posterior off-line analysis with a semi-automatic integrated system (Microvision Instruments, Evry, France). The mean capillary density was calculated as the arithmetic mean of the number of visible capillaries in three contiguous microscopic fields of 1 mm^2 each. A blood pressure cuff was then applied to the patient's arm and inflated to supra-systolic pressure (50 mm Hg above systolic arterial pressure) to completely interrupt blood flow for three minutes. After cuff release, images were again acquired and recorded during the following 60–90 seconds, during which the maximal hyperemic response was expected to occur [18].

Statistical Analysis

Statistical analyses of the results obtained from the patients who concluded the study were conducted with IBM® SPSS® Statistics software version 21 (Armonk, New York, USA). The results are presented as the mean ± SD or median (25th–75th percentiles). The normality of the variables was investigated

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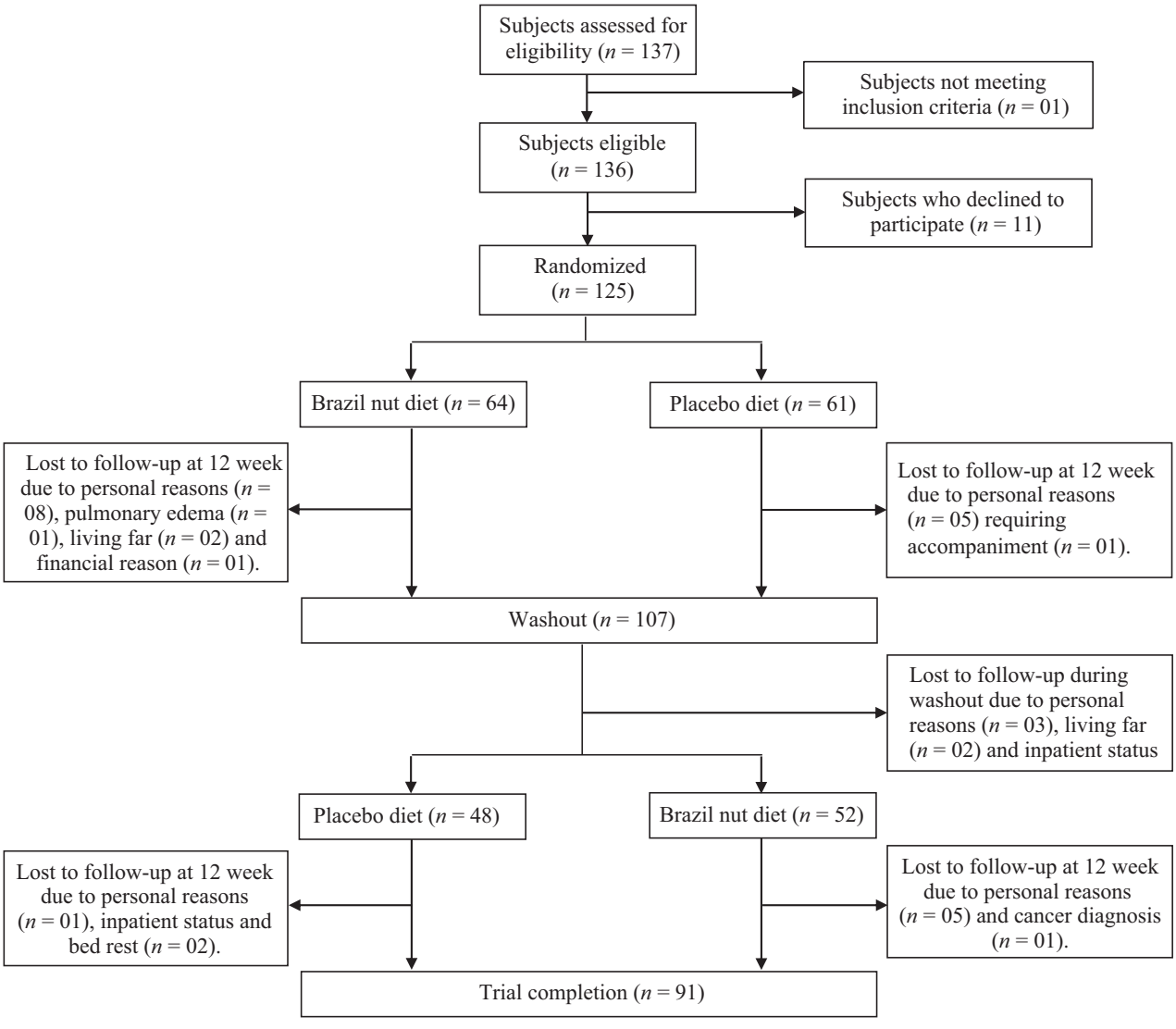

Figure 2. Flowchart of the patients during each study phase.

using the Kolmogorov–Smirnov test. To assess differences in categorical variables between the patients that completed the study protocol and follow-up losses, the chi-square test was used. To assess the effects within each intervention (GBNs and placebo) on the anthropometric, clinical, and laboratory measurements, a paired two-tailed Student's *t*-test or the Wilcoxon signed ranks test were used, whereas the effects between interventions were assessed by the Mann–Whitney *U* or Student's *t*-tests according to the distribution of variables. A $p < 0.05$ was considered significant.

RESULTS

Of the initial 125 patients, 91 completed all stages of the study, resulting in a loss of 27.2% of the patients. Figure 2 shows the flow and losses throughout the study. The dropout rate during follow-up was higher during supplementation with partially defatted GBNs, but there was no report of withdrawal due to the taste of the supplement. The mean age of the patients who completed the study (62.1 ± 9.3 years) was significantly higher than that of the patients who did not complete the study (56.9 ± 10.5 years, $p = 0.008$).

Table 2 shows the characteristics of the patients who completed the study. The medications most commonly used by the study participants were statins (81.3%), fibrates (33%), oral hypoglycemic agents (48.4%), sympatholytics (72.5%), ACE inhibitors (53.8%), diuretics (49.5%), calcium channel blockers (40.7%), AT1 receptor blockers (36.3%), vasodilators (12.1%), and ASA (65.9%). There were no changes in the patients' medication during the study.

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Table 2. Clinical and laboratory variables of the subjects who completed the study (n = 91)

Age (years)	62.1 ± 9.3
Male, *n* (%)	47 (51.6)
Diabetic, *n* (%)	42 (46.2)
Overweight/Obese, *n* (%)	61 (67.0)
Stroke, *n* (%)	6 (6.6)
AMI, *n* (%)	39 (42.9)
PCI, *n* (%)	22 (24.2)
CABG, *n* (%)	29 (31.9)
Angina, *n* (%)	36 (39.6)
Duration of diabetes (years)	10.6 ± 11.2
Duration of hypertension (years)	11.8 ± 9.7
Duration of dyslipidemia (years)	10.1 ± 9.7
Smoker, *n* (%)	23 (25.3)
Alcohol use, *n* (%)	32 (37.2)
Sedentary, *n* (%)	68 (74.7)
BMI (kg/m^2)	28.8 ± 5.1
WC (cm)	100.1 ± 12.3
SBP (mmHg)	146.5 ± 28.7
DBP (mmHg)	81.8 ± 13.7
Glucose (mg/dL)	109.0 (90.0–137.0)
Triglycerides (mg/dL)	153.0 (119.0–226.0)
Total cholesterol (mg/dL)	204.4 ± 61.7
HDL-cholesterol (mg/dL)	39.7 ± 12.3
LDL-cholesterol (mg/dL)	125.8 ± 54.8

The results are expressed as the mean ± SD or median (interquartile intervals).
PCI, percutaneous coronary intervention.

Although all of the patients received drug treatment for dyslipidemia and hypertension, 60.4% had high blood pressure (systolic ≥140 mmHg or diastolic ≥90 mmHg). Regarding the levels of serum lipids, 94.5% of patients showed abnormalities in their lipid profile early in the study, including 52.7% with high triglycerides (≥150 mg/dL), 60.4% with high LDL-c (>100 mg/dL), and 33.3% with high triglycerides and high LDL-c.

Evaluation of Plasma Levels of Se

The basal Se plasma concentration in the study group was 87.0 ± 16.8 μg/L, and 57.1% of those in the study group (n = 52) had low plasma Se (<90 μg/L). The consumption of GBNs led to a significant increase in plasma Se levels (first step 195.8 ± 67.4 μg/L, $p < 0.001$ and second step 168.0 ± 67.4 μg/L, $p < 0.001$).

Evaluation of Skin Microvascular Density and Capillary Recruitment

The baseline capillary density value of 94 (84–109) capillaries/mm^2 before placebo intake was not significantly different from the baseline value of 97 (82–111) capillaries/mm^2 before GBN supplementation (p = 0.7194, Figure 3). The same was true for the baseline value after 12 weeks of placebo

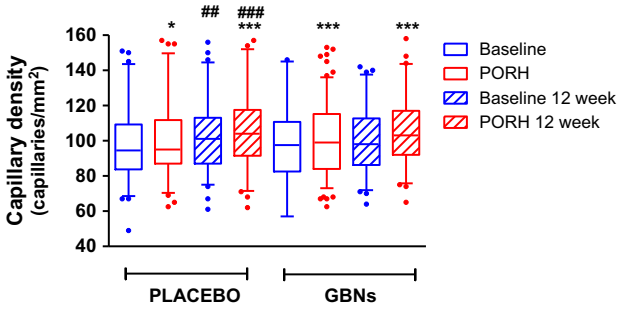

Figure 3. Box plots representing skin capillary densities at baseline and during PORH before (open boxes) and after 12 weeks (hatched boxes) of supplementation with placebo or GBNs. The values in the boxes represent the median and interquartile intervals, the whiskers represent the 5th and 95th percentiles, and the outliers are plotted as individual values. *$p < 0.05$ and ***$p < 0.001$ vs. baseline values; paired two-tailed Student's t-test. ##$p < 0.01$ and ###$p < 0.001$ vs. values before treatment; paired two-tailed Student's t-test.

intake, which was 101 (87–113) capillaries/mm^2, and that after GBN intake, which was 98 (86–113) capillaries/mm^2 (p = 0.4708). The baseline capillary density increased after intake of the placebo, rising from 94 (84–109) capillaries/mm^2 before intake to 101 (87–113) capillaries/mm^2 after intake (p = 0.0031); additionally, this value showed an increasing trend after GBN consumption, rising from 97 (82–111) capillaries/mm^2 before intake to 98 (86–113) capillaries/mm^2 after consumption (p = 0.0568).

PORH consistently induced significant increases in capillary density not only before but also after the intake of placebo or GBNs, indicating that endothelium-dependent capillary recruitment in the study subjects was preserved (Figure 3). The capillary density value during PORH of 95 (87–112) capillaries/mm^2 before placebo supplementation was not significantly different from the value before GBN intake, which was 99 (84–115) capillaries/mm^2 (p = 0.5086, Figure 3). The same was true for the capillary density during PORH after 12 weeks of placebo intake, which was 104 (91–117) capillaries/mm^2, and that after GBN intake, which was 103 (92–117) capillaries/mm^2 (p = 0.6421). The capillary density during PORH increased after placebo intake from 95 (87–112) capillaries/mm^2 before to 104 (92–118) capillaries/mm^2 after intake (p = 0.006) but did not change after the consumption of GBNs—from 99 (84–115) capillaries/mm^2 before to 103 (92–117) capillaries/mm^2 after consumption (p = 0.1288).

Evaluation of Skin Microvascular Reactivity

The values of MAP used in the calculation of CVC are depicted in Table 3. There were no significant differences in microvascular parameters at baseline and post intervention according to obesity status and the presence of diabetes (data not shown). We did not observe any effect of the intake of GBNs on microvascular endothelial function as evaluated by

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Table 3. Anthropometric, blood pressure, and laboratory measures before and after supplementation in hypertensive and dyslipidemic patients

	Diet + placebo		Diet + GBNs	
	Baseline	**12 weeks**	**Baseline**	**12 weeks**
Weight (kg)	74.9 ± 14.7	74.9 ± 15.2	75.4 ± 15.1	75.4 ± 15.5
BMI (kg/m^2)	28.3 ± 4.9	28.3 ± 5.1	28.4 ± 5.1	28.4 ± 5.3
WC (cm)	98.1 ± 14.6	99.0 ± 11.9	99.5 ± 13.5	99.3 ± 12.5
SBP (mmHg)	144.3 ± 28.8	142.8 ± 27.5	143.1 ± 27.7	143.3 ± 25.9
DBP (mmHg)	79.7 ± 13.9	78.6 ± 12.3	78.1 ± 12.5	78.4 ± 12.0
MAP (mmHg)	101 ± 16	100 ± 15	99 ± 15	101 ± 16
Fasting glucose (mg/dL)	109.0 (92.0–139.0)	105.0 (93.5–142.5)	105.0 (92.5–130.0)	108.0 (94.0–142.0)
Total cholesterol (mg/dL)	199.2 ± 60.7	201.21 ± 63.8	204.0 ± 63.2	199.5 ± 66.1
HDL-cholesterol (mg/dL)	38.9 ± 13.3*	41.2 ± 12.8*	38.7 ± 11.9†	40.8 ± 14.7†
LDL-cholesterol (mg/dL)	110.0 (87.5–144.5)	111.0 (83.0–147.0)	114.0 (87.0–143.0)	109.0 (87.0–144.0)
Triglycerides (mg/dL)	153.0 (112.0–230.0)	166.0 (102.0–226.0)	159.0 (122.0–236.0)	165.0 (116.0–263.0)

Mean ± SD, median (25th–75th percentiles). Paired two-tailed Student's *t*-test or Wilcoxon test.
*,†$p < 0.05$.

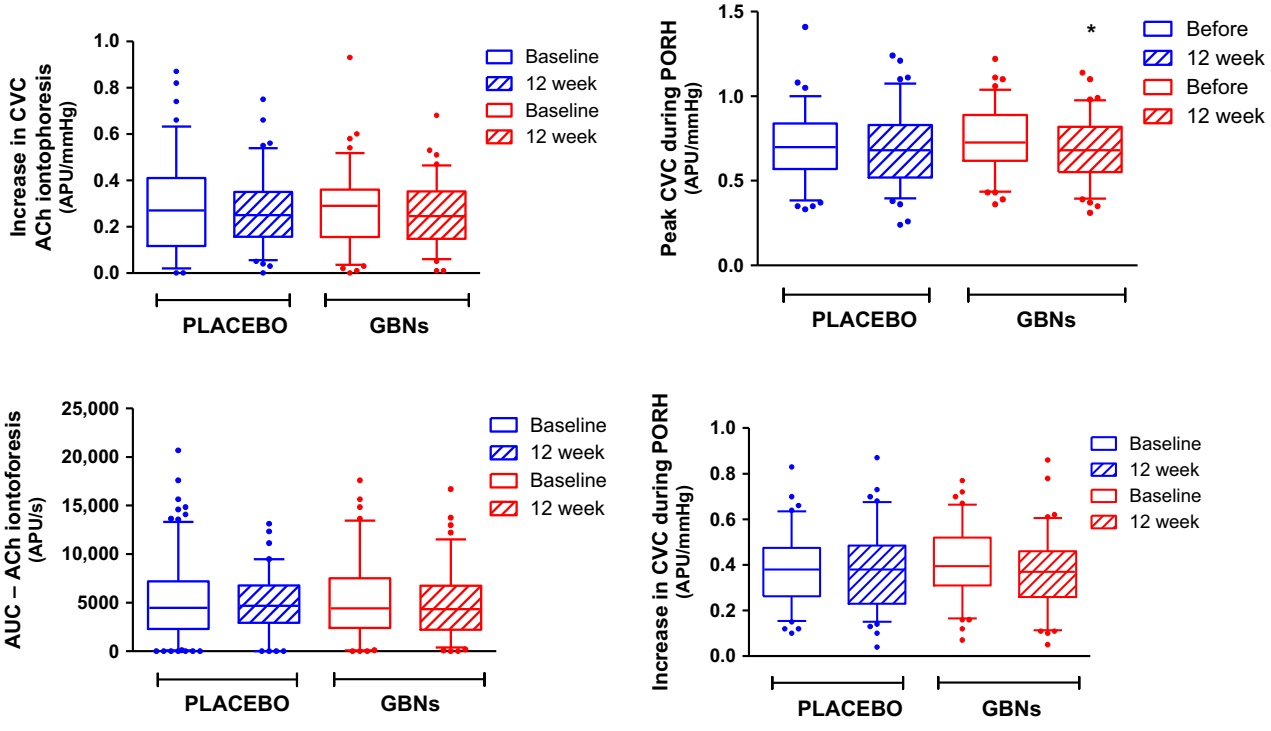

Figure 4. Box plots representing different parameters of microvascular reactivity before (open boxes) and after 12 weeks (hatched boxes) of supplementation with placebo or GBNs. The values in the boxes represent the median and interquartile intervals, the whiskers represent the 5th and 95th percentiles, and the outliers are plotted as individual values. *$p < 0.05$ vs. baseline values; paired two-tailed Student's *t*-test.

pharmacological stimulation with ACh (Figures 1 and 4). The peak increases in CVC and the AUC of ACh-induced vasodilation did not change significantly after placebo or GBN intake compared with the basal values (Figure 4). The maximum increases in CVC resulting from iontophoresis of ACh were 0.25 (0.16–0.35) and 0.24 (0.15–0.35) APUs/mmHg after placebo or GBN intake, respectively (Figure 4). The increases in the AUC resulting from iontophoresis of ACh were of 4671 (2931–6791) and 4344 (2221–6764) APUs/s after placebo or GBN intake, respectively (Figure 4). On the

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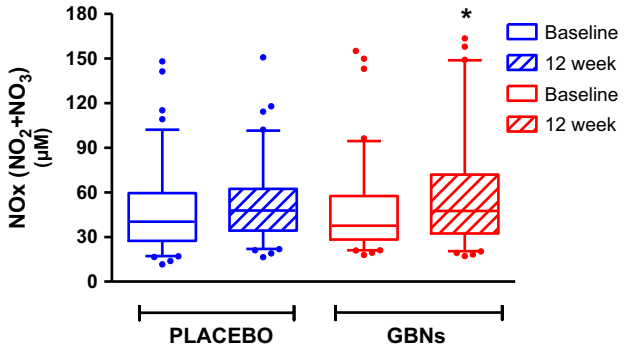

Figure 5. Box plots representing total plasma concentrations of NO*x* before (open boxes) and after 12 weeks (hatched boxes) of supplementation with placebo or GBNs. The values in the boxes represent the median and interquartile intervals, the whiskers represent 5th and 95th percentiles, and the outliers are plotted as individual values. *$p < 0.05$ vs. baseline values; paired two-tailed Student's *t*-test.

other hand, the peak values of CVC during PORH did not change after placebo intake and were slightly but significantly reduced after GBN intake compared with the basal values ($p = 0.0282$; Figure 4). The maximum increases in CVC during PORH were 0.68 (0.52–0.83) and 0.68 (0.55–0.82) APUs/mmHg after placebo or GBN intake, respectively (Figure 4). The increases in CVC from the baseline values during PORH did not significantly change after placebo or after GBN intake compared with the basal values (Figure 4). The maximum increases in CVC during PORH were 0.38 (0.23–0.48) and 0.37 (0.26–0.46) APUs/mmHg after placebo or GBN intake, respectively (Figure 4).

Evaluation of Plasma Levels of NO*x*

Figure 5 shows the significant increase (26%) in total plasma NO*x* after the intake of GBNs. The plasma levels of NO*x* did not differ before—40.25 μM (27.32–59.43), versus after—47.85 μM (32.27–62.29, $p = 0.2357$), intake of the placebo; however, the intake of GBNs induced an increase in plasma levels of NO*x* from 37.57 μM (28.28–57.48) to 47.44 μM (32.38–71.87, $p = 0.0180$).

DISCUSSION

The main findings of this study are as follows: (i) dietary supplementation with GBNs for 12 weeks resulted in significant increases in plasma Se concentrations, (ii) the systemic bioavailability of nitric oxide was also increased after supplementation with GBNs, and (iii) GBN supplementation did not lead to any improvement in systemic microvascular reactivity or density in the patients with severe arterial hypertension and dyslipidemia who were receiving multiple drug therapies.

To date, no study in the literature has evaluated the effects of Brazil nut consumption on microvascular reactivity and density in hypertensive or dyslipidemic patients. Several studies have demonstrated the beneficial effects of the regular consumption of nuts in reducing the risks of cardiac events and death from CHD [38], serum lipid levels [39], and blood pressure [26]. For these reasons, nuts are considered to be a component of a cardioprotective diet [29]. The study of skin microcirculation is a non-invasive, affordable, and representative method for evaluating the vascular system for microvascular endothelial dysfunction, including endothelium-dependent vasodilation [8,24,36]. In this context, LSCI has demonstrated good spatial and temporal resolution and reproducibility [24,37]. Moreover, LSCI has been proven to be an effective technique for the evaluation of systemic microvascular reactivity in patients with cardiovascular and metabolic diseases [8].

Brazil nuts contain a high amount of L-arginine (2.15 g/100 g), which is a precursor of nitric oxide (a potent vasodilator) [31], and may have contributed to the significant increase in total plasma NO*x* levels. Other studies have shown that supplementation with L-arginine improves endothelial dysfunction associated with hypercholesterolemia [19] and hypertension [42], but these studies have used much higher amounts of L-arginine than those found in Brazil nuts.

Other clinical trials have tested the effects of the consumption of nuts on endothelium-dependent vasodilation and have shown a significant improvement after consumption of a diet enriched in nuts [9,23,34] and pistachios [40] in hypercholesterolemic, diabetic, and healthy subjects. In the abovementioned studies, the treatment period lasted for four weeks, and the doses ranged from 40 to 65 g/day for nuts and 60–100 g/day for pistachios. Individuals being treated were compared with those consuming a regular diet, the Mediterranean diet or a diet rich in olive oil.

The amount of GBNs used in this study was lower than the amounts used in other studies evaluating these nuts; thus, bioactive compounds other than Se were likely present in the GBNs in small amounts in the other studies and may have had some effects on endothelial function. The supplementation dose in this study was defined based on the Se content of GBNs and did not exceed the maximum daily tolerance limit (UL = 400 μg/day) [1].

Moreover, similar to this study, three other studies found no significant improvements in endothelial function in subjects with hypercholesterolemia or metabolic syndrome or in healthy men after the consumption of different types of nuts, and the consumption of nuts has been shown to reduce the vascular response during the postprandial period [5,22,27]. These studies evaluated endothelial function by assessing macrocirculation using different methods. This study investigated another aspect of endothelial function by assessing microvascular reactivity and showed similar out-

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comes. In our study, we observed a slight reduction in peak CVC during PORH after intervention with GBNs. Nevertheless, all other microvascular parameters, including ACh-induced vasodilation and increase in CVC induced by PORH did not change. Thus, we cannot conclude with certainty that the dietary supplementation with GBNs altered endothelium-dependent microvascular reactivity.

The placebo used in this study, but not the GBNs, contained cassava flour, which could have induced cardiovascular effects. In fact, the consumption of natural starchy carbohydrates taken with their full complement of fiber has been shown to have protective effects against hyperlipidemia and ischemic heart disease [47,48]. Consequently, it cannot be excluded that supplementation with cassava flour could have resulted in favorable microvascular effects.

Lipid-lowering drugs may have beneficial effects on endothelial function by reducing serum lipids. An increase of 6.86% in peripheral circulatory flow-mediated vasodilation associated with a significant reduction in LDL-cholesterol has been observed after treatment with simvastatin for eight weeks [21]. Capillary rarefaction and microvascular endothelial dysfunction in low-risk hypertensive subjects respond favorably to six months of anti-hypertensive drug treatment [18]. The population of this study, despite receiving pharmacological treatment for hypertension, hypercholesterolemia, and hypertriglyceridemia, largely presented with altered arterial pressure values (60.4% of individuals) and altered serum lipid levels (94.5%), which may have influenced their endothelial function. Furthermore, the duration of disease in these patients may also have contributed to the lack of change in the microvascular response after dietary intervention. Although supplementation with GBNs did not improve endothelial microvascular function or density, our study showed that capillary recruitment was preserved in patients under multiple drug cardiovascular therapies, most of which act to improve endothelial function. In fact, independent of the placebo or GBN treatment, PORH consistently induced significant increases in capillary density. Patients receiving placebo supplementation together with dietary intervention showed significant increases in functional capillary density and capillary recruitment at the end of the 12 weeks of supplementation.

The nutritional status of Se in this study population was low, as demonstrated by the finding that 57.1% of the subjects had a low plasma Se level (<90 μg/L). Individuals with a low plasma Se level may benefit from the consumption of GBNs. However, although these individuals increased their plasma Se levels by 88% after GBN supplementation, the endothelial microvascular response did not change significantly. The effect of Se on microvascular function was previously investigated in healthy men who received 300 μg Se/day for 48 weeks. No improvement was observed in endothelial function or the peripheral arterial response in the study group, and these subjects received adequate Se in their habitual diets [17]. Only one clinical study investigated microvascular function by nailfold capillaroscopy after consumption of Brazil nuts (15–25 g/day) in obese adolescents, observing a significant increase (13.9%) in average capillary blood flow and a 9.5% increase in maximum capillary blood flow in response to PORH after 16 weeks of supplementation [25], but these patients did not have any obesity-associated disease. In contrast to this study, these authors did not observe any change in baseline capillary density after consumption of Brazil nuts. In the abovementioned work performed by Professor Bouskela's team, the methodological approach was rather different from the one used in this study. In fact, these authors evaluated capillary density and reactivity in the terminal microvascular bed of the nailfold using video capillaroscopy. This technology is classically used in the context of rheumatologic diseases to study capillary morphological alterations. In contrast, in our study, we used video capillaroscopy to evaluate capillary density in the skin of the forearm, which is thought to represent systemic capillary density [10]. Consequently, the pathophysiological alterations of the microcirculation in the context of cardiometabolic diseases in these different vascular beds could be rather dissimilar.

Limitations and Strengths of Study

This study has limitations that may have influenced the findings. Such limitations include the fact that part of the cohort of participants did not complete the study. Nevertheless, the losses in our study were not large, and there were no differences in baseline values between patients who completed or did not complete the study protocol, thus excluding inclusion bias. It is noteworthy that the original sample size calculation of the study was based on increases of 13% in skin microvascular blood flow induced by PORH; considering the drop out of 21 patients during the execution of the study, it appears that only larger differences should be expected to have statistical significance. Anyhow, in this study the dietary intervention with Brazil nuts did not have any influence on endothelial-dependent microvascular reactivity of the patients. In addition, in a previous methodological study of our research group, we observed that there was a difference of ≈28% on the microvascular response to PORH between hypertensive patients and healthy controls [8]. Moreover, we compared the microvascular parameters at baseline and post intervention, according to obesity status and the presence of diabetes, but there were no statistically significant differences. An additional limitation was the short intervention period and heterogeneity of the participants. Despite the use of multiple drug treatments, the majority of patients still had high blood pressure (60.4%) and high plasma lipid levels (94.5%) with several co-morbidities, including history of AMI (42.9%) and stroke (6.6%), which

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could have influenced their microvascular endothelial function, along with the extended durations of the evolution of arterial hypertension, dyslipidemia, and diabetes in these individuals (more than 10 years). The different pharmacological treatments used by the patients could have influenced the results of microcirculatory evaluation in our study. Nevertheless, we only initiated the dietary intervention three months after the beginning of medication, and there were no changes in the medication during the study. Moreover, the randomized placebo-controlled and crossover design allows for a patient to serve as his own control. Finally, it is not possible to withdraw treatment from hypertensive and dyslipidemic patients for medical and ethical reasons.

CONCLUSIONS

In conclusion, nutritional counseling along with the consumption of GBNs at a dose of 13 g/day led to a significant improvement in nitric oxide bioavailability, as measured by increased total plasma NO*x* levels. However, supplementation with GBNs did not improve endothelial microvascular reactivity in hypertensive and dyslipidemic patients undergoing pharmacological treatments with multiple drugs.

PERSPECTIVE

The present study showed that dietary supplementation with Brazil nuts during three months did not lead to any improvement in systemic microvascular reactivity or density in patients with arterial hypertension and dyslipidemia who were undergoing multiple drug therapies. Considering that the supplementation significantly increases systemic nitric oxide bioavailability, it could be beneficial in the prevention of cardiovascular and metabolic diseases.

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AUTHOR CONTRIBUTIONS

G.V.B.H., G.R., G.M.M.O., A.S.B.M., and E.T. conceived and designed the study. G.V.B.H., T.D.S.P., R.A.G., and E.T. performed the experiments. G.V.B.H., G.R., G.M.M.O., A.S.B.M., R.R.L., and E.T. analyzed the data and interpreted the results of the experiments. G.V.B.H., G.R., G.M.M.O., A.S.B.M. and E.T. drafted the manuscript. G.V.B.H., G.R., G.M.M.O., A.S.B.M. and E.T. edited and revised the manuscript; and E.T. approved the final version of the manuscript.

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

REFERENCES

1. Panel on Dietary Antioxidants and Related Compounds, Subcommittees on Upper Reference Levels of Nutrients and Interpretation and Uses of DRIs, Standing Committee on the Scientific Evaluation of Dietary Reference Intakes, Food and Nutrition Board, Institute of Medicine. *Dietary Reference Intakes for Vitamin C, Vitamin E, Selenium, Carotenoids*. Washington, DC: The National Academic Press, 2000. http://www.nap.edu/openbook [accessed on 1 June 2015].
2. Institute of Medicine (US) Panel on Macronutrients; Institute of Medicine (US) Standing Committee on the Scientific Evaluation of Dietary Reference Intakes. Dietary Reference Intakes for Energy, Carbohydrate, Fiber, Fat, Fatty Acids, Cholesterol, Protein, and Amino Acids. Washington, DC: The National Academic Press. Panel on Macronutrients, Panel on the Definition of Dietary Fiber, Subcommittee on Upper Reference Levels of Nutrients, Subcommittee on the Interpretation and Uses of Dietary Reference Intakes, and the Standing Committee on the Scientific Evaluation of Dietary Reference Intakes, Food and Nutrition Board, 2005. http://www.nal.usda.gov/fnic/DRI [accessed on 1 June 2015].
3. Ansell BJ. Evidence for a combined approach to the management of hypertension and dyslipidemia. *Am J Hypertens* 18: 1249–1257, 2005.
4. Benincasa C, Lewis J, Perri E, Sindona G, Tagarelli A. Determination of trace element in Italian virgin olive oils and their characterization according to geographical origin by statistical analysis. *Anal Chim Acta* 585: 366–370, 2007.
5. Berry SE, Tydeman EA, Lewis HB, Phalora R, Rosborough J, Picout DR, Ellis PR. Manipulation of lipid bioaccessibility of almond seeds influences postprandial lipemia in healthy human subjects. *Am J Clin Nutr* 88: 922–929, 2008.
6. Brazilian Society of Hypertension. VI Brazilian Guidelines on Hypertension. *Arq Bras Cardiol* 95: 1–51, 2010.
7. Casas-Agustench P, Lopez-Uriarte P, Ros E, Bullo M, Salas-Salvado J. Nuts, hypertension and endothelial function. *Nutr Metab Cardiovasc Dis* 21(Suppl. 1): S21–S33, 2011.
8. Cordovil I, Huguenin G, Rosa G, Bello A, Kohler O, de Moraes R, Tibirica E. Evaluation of systemic microvascular endothelial function using laser speckle contrast imaging. *Microvasc Res* 83: 376–379, 2012.
9. Cortes B, Nunez I, Cofan M, Gilabert R, Perez-Heras A, Casals E, Deulofeu R, Ros E. Acute effects of high-fat meals enriched with walnuts or olive oil on postprandial endothelial function. *J Am Coll Cardiol* 48: 1666–1671, 2006.
10. de Jongh RT, Serne EH, IJzerman RG, Stehouwer CD. Microvascular function: a potential link between salt sensitivity, insulin resistance and hypertension. *J Hypertens* 25: 1887–1893, 2007.

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11. Department of Health and Human Services, Office of Disease Prevention and Health Promotion, Washington D.C. Physical activity guidelines advisory committee report, 2008. http://www.health.gov/paguidelines/report [accessed on 1 June 2015].
12. Ferroni P, Basili S, Paoletti V, Davi G. Endothelial dysfunction and oxidative stress in arterial hypertension. *Nutr Metab Cardiovasc Dis* 16: 222–233, 2006.
13. Fichtlscherer S, Breuer S, Zeiher AM. Prognostic value of systemic endothelial dysfunction in patients with acute coronary syndromes: further evidence for the existence of the "vulnerable" patient. *Circulation* 110: 1926–1932, 2004.
14. Freitas SC, Gonçalves EB, Antoniassi R, Felberg I, Oliveira SP. Meta-analysis of selenium content in Brazil nuts. *Braz J Food Technol* 11: 54–62, 2008.
15. Friedewald WT, Levy RI, Fredrickson DS. Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. *Clin Chem* 18: 499–502, 1972.
16. Gomes VB, Siqueira KS, Sichieri R. Physical activity in a probabilistic sample in the city of Rio de Janeiro. *Cad Saude Publica* 17: 969–976, 2001.
17. Hawkes WC, Laslett LJ. Selenium supplementation does not improve vascular responsiveness in healthy North American men. *Am J Physiol Heart Circ Physiol* 296: H256–H262, 2009.
18. Kaiser SE, Sanjuliani AF, Estato V, Gomes MB, Tibirica E. Antihypertensive treatment improves microvascular rarefaction and reactivity in low-risk hypertensive individuals. *Microcirculation* 20: 703–716, 2013.
19. Kawano H, Motoyama T, Hirai N, Kugiyama K, Yasue H, Ogawa H. Endothelial dysfunction in hypercholesterolemia is improved by L-arginine administration: possible role of oxidative stress. *Atherosclerosis* 161: 375–380, 2002.
20. Labat L, Dehon B, Lhermitte M. Rapid and simple determination of selenium in blood serum by inductively coupled plasma-mass spectrometry (ICP-MS). *Anal Bioanal Chem* 376: 270–273, 2003.
21. Ling MC, Ruddy TD, deKemp RA, Ukkonen H, Duchesne L, Higginson L, Williams KA, McPherson R, Beanlands R. Early effects of statin therapy on endothelial function and microvascular reactivity in patients with coronary artery disease. *Am Heart J* 149: 1137, 2005.
22. Lopez-Uriarte P, Nogues R, Saez G, Bullo M, Romeu M, Masana L, Tormos C, Casas-Agustench P, Salas-Salvado J. Effect of nut consumption on oxidative stress and the endothelial function in metabolic syndrome. *Clin Nutr* 29: 373–380, 2010.
23. Ma Y, Njike VY, Millet J, Dutta S, Doughty K, Treu JA, Katz DL. Effects of walnut consumption on endothelial function in type 2 diabetic subjects: a randomized controlled crossover trial. *Diabetes Care* 33: 227–232, 2010.
24. Mahe G, Humeau-Heurtier A, Durand S, Leftheriotis G, Abraham P. Assessment of skin microvascular function and dysfunction with laser speckle contrast imaging. *Circ Cardiovasc Imaging* 5: 155–163, 2012.
25. Maranhao PA, Kraemer-Aguiar LG, de OC, Kuschnir MC, Vieira YR, Souza MG, Koury JC, Bouskela E. Brazil nuts intake improves lipid profile, oxidative stress and microvascular function in obese adolescents: a randomized controlled trial. *Nutr Metab (Lond)* 8: 32, 2011.
26. Martinez-Lapiscina EH, Pimenta AM, Beunza JJ, Bes-Rastrollo M, Martinez JA, Martinez-Gonzalez MA. Nut consumption and incidence of hypertension: the SUN prospective cohort. *Nutr Metab Cardiovasc Dis* 20: 359–365, 2010.
27. Mercanligil SM, Arslan P, Alasalvar C, Okut E, Akgul E, Pinar A, Geyik PO, Tokgozoglu L, Shahidi F. Effects of hazelnut-enriched diet on plasma cholesterol and lipoprotein profiles in hypercholesterolemic adult men. *Eur J Clin Nutr* 61: 212–220, 2007.
28. Millet C, Roustit M, Blaise S, Cracowski JL. Comparison between laser speckle contrast imaging and laser Doppler imaging to assess skin blood flow in humans. *Microvasc Res* 82: 147–151, 2011.
29. Mozaffarian D, Appel LJ, Van Horn L. Components of a cardioprotective diet: new insights. *Circulation* 123: 2870–2891, 2011.
30. Palombo C, Lubrano V, Sampietro T. Oxidative stress, F2-isoprostanes and endothelial dysfunction in hypercholesterolemia. *Cardiovasc Res* 44: 474–476, 1999.
31. Preli RB, Klein KP, Herrington DM. Vascular effects of dietary L-arginine supplementation. *Atherosclerosis* 162: 1–15, 2002.
32. Rayman MP. Selenium and human health. *Lancet* 379: 1256–1268, 2012.
33. Ros E. Nuts and novel biomarkers of cardiovascular disease. *Am J Clin Nutr* 89: 1649S–1656S, 2009.
34. Ros E, Nunez I, Perez-Heras A, Serra M, Gilabert R, Casals E, Deulofeu R. A walnut diet improves endothelial function in hypercholesterolemic subjects: a randomized crossover trial. *Circulation* 109: 1609–1614, 2004.
35. Roustit M, Cracowski JL. Non-invasive assessment of skin microvascular function in humans: an insight into methods. *Microcirculation* 19: 47–64, 2012.
36. Roustit M, Cracowski JL. Assessment of endothelial and neurovascular function in human skin microcirculation. *Trends Pharmacol Sci* 34: 373–384, 2013.
37. Roustit M, Millet C, Blaise S, Dufournet B, Cracowski JL. Excellent reproducibility of laser speckle contrast imaging to assess skin microvascular reactivity. *Microvasc Res* 80: 505–511, 2010.
38. Sabate J, Ang Y. Nuts and health outcomes: new epidemiologic evidence. *Am J Clin Nutr* 89: 1643S–1648S, 2009.
39. Sabate J, Oda K, Ros E. Nut consumption and blood lipid levels: a pooled analysis of 25 intervention trials. *Arch Intern Med* 170: 821–827, 2010.
40. Sari I, Baltaci Y, Bagci C, Davutoglu V, Erel O, Celik H, Ozer O, Aksoy N, Aksoy M. Effect of pistachio diet on lipid parameters, endothelial function, inflammation, and oxidative status: a prospective study. *Nutrition* 26: 399–404, 2010.
41. Schachinger V, Britten MB, Zeiher AM. Prognostic impact of coronary vasodilator dysfunction on adverse long-term outcome of coronary heart disease. *Circulation* 101: 1899–1906, 2000.
42. Siani A, Pagano E, Iacone R, Iacoviello L, Scopacasa F, Strazzullo P. Blood pressure and metabolic changes during dietary L-arginine supplementation in humans. *Am J Hypertens* 13: 547–551, 2000.
43. Sposito AC, Caramelli B, Fonseca FA, Bertolami MC, Afiune Neto A, Souza AD, Lottenberg AM, Chacra AP, Faludi AA, Loures-Vale AA, Carvalho AC, Duncan B, Gelonese B, Polanczyk C, Rodrigues Sobrinho CR, Scherr C, Karla C, Armaganijan D, Moriguchi E, Saraiva F, Pichetti G, Xavier HT, Chaves H, Borges JL, Diament J, Guimaraes JI, Nicolau JC, dos Santos JE, de Lima JJ, Vieira JL, Novazzi JP, Faria Neto JR, Torres KP, Pinto Lde A, Bricarello L, Bodanese LC, Introcaso L, Malachias MV, Izar MC, Magalhaes ME, Schmidt MI, Scartezini M, Nobre M, Foppa M, Forti NA, Berwanger O, Gebara OC, Coelho OR, Maranhao RC, dos Santos Filho RD, Costa RP, Barreto S, Kaiser S, Ihara S, Carvalho T, Martinez TL, Relvas WG, Salgado W. IV Brazilian guideline for dyslipidemia and atherosclerosis prevention: Department of Atherosclerosis of Brazilian Society of Cardiology. *Arq Bras Cardiol* 88(Suppl. 1): 2–19, 2007.
44. Thomson CD. Assessment of requirements for selenium and adequacy of selenium status: a review. *Eur J Clin Nutr* 58: 391–402, 2004.
45. Thomson CD, Chisholm A, McLachlan SK, Campbell JM. Brazil nuts: an effective way to improve selenium status. *Am J Clin Nutr* 87: 379–384, 2008.

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46. Tibirica E, Rodrigues E, Cobas RA, Gomes MB. Endothelial function in patients with type 1 diabetes evaluated by skin capillary recruitment. *Microvasc Res* 73: 107–112, 2007.
47. Trowell H. Ischemic heart disease and dietary fiber. *Am J Clin Nutr* 25: 926–932, 1972.
48. Trowell H. Definition of dietary fiber and hypotheses that it is a protective factor in certain diseases. *Am J Clin Nutr* 29: 417–427, 1976.
49. United States Department of Agriculture, Agricultural Research Service, USDA. Dietary supplement ingredient database, 2013. Release 26. P. Pehrsson, Nutrient Data Laboratory Homepage. http://www.ars.usda.gov/ba/bhnrc/ndl [accessed on 1 June 2015].
50. Verri V, Brandao A, Tibirica E. The evaluation of penile microvascular endothelial function using laser speckle contrast imaging in healthy volunteers. *Microvasc Res* 99: 96–101, 2015.

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