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Improvement of Endothelial Function With Dietary Flavanols Is Associated With Mobilization of Circulating Angiogenic Cells in Patients With Coronary Artery Disease

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Objectives In patients with coronary artery disease (CAD) medically managed according to currently accepted guidelines, we tested whether a 1-month dietary intervention with flavanol-containing cocoa leads to an improvement of endothelial dysfunction and whether this is associated with an enhanced number and function of circulating angiogenic cells (CACs).

Background Dietary flavanols can improve endothelial dysfunction. The CACs, also termed endothelial progenitor cells, are critical for vascular repair and maintenance of endothelial function.

Methods In a randomized, controlled, double-masked, cross-over trial, 16 CAD patients (64 ± 3 years of age) received a dietary high-flavanol intervention (HiFl [375 mg]) and a macronutrient- and micronutrient-matched low-flavanol intervention (LoFl [9 mg]) twice daily in random order over 30 days.

Results Endothelium-dependent vasomotor function, as measured by flow-mediated vasodilation of the brachial artery, improved by 47% in the HiFl period compared with the LoFl period. After HiFl, the number of $CD34^+/KDR^+$-CACs, as measured by flow cytometry, increased 2.2-fold as compared with after LoFl. The CAC functions, as measured by the capacity to survive, differentiate, proliferate, and to migrate were not different between the groups. The HiFl led to a decrease in systolic blood pressure (mean change over LoFl: −4.2 ± 2.7 mm Hg), and increase in plasma nitrite level (mean change over LoFl: 74 ± 32 nM). Applying a mixed-effects linear regression model, the results demonstrated a significant increase in flow-mediated vasodilation and a decrease in systolic blood pressure with increasing levels of $CD34^+/KDR^+$-CACs.

Conclusions Sustained improvements in endothelial dysfunction by regular dietary intake of flavanols are associated with mobilization of functional CACs. (Effect of Cocoa Flavanols on Vascular Function in Optimally Treated Coronary Artery Disease Patients: Interaction Between Endothelial Progenitor Cells, Reactivity of Micro- and Macrocirculation; NCT00553774). (J Am Coll Cardiol 2010;56:218–24) © 2010 by the American College of Cardiology Foundation

Flavanols, a subgroup of phytochemicals called flavonoids, have gained increasing attention, as epidemiological investigations revealed an inverse correlation between the intake of flavanol-containing foods and coronary artery disease (CAD) mortality (1). Major flavanol sources in the Western diet include tea, wine, cocoa products, and various fruits and

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vegetables. Several controlled human dietary intervention studies that have utilized flavanol-containing foods and beverages in concert with nutrient-matched, low-flavanol controls have demonstrated flavanol-dependent effects, including the recovery of endothelial function, decreased blood pressure, and reductions in platelet aggregation (2). The potential mechanisms of action proposed to underlie the pharmacological properties of flavanols are various, and may include enhanced endothelial homeostatic repair.

Recent studies have demonstrated that bone marrow-derived circulating angiogenic cells (CACs), also referred to as early endothelial progenitor cells (3), contribute to the recovery and maintenance of endothelial function (4). Primary and secondary preventive life-style interventions and statin therapy can enhance endogenous endothelial repair mechanisms by stimulating CAC mobilization (5). It is unknown whether improvements in cardiovascular function, as previously observed in flavanol-based dietary interventions, involve CAC-dependent vascular regenerative processes. In the present study, we tested, in CAD patients who are medically managed according to currently accepted guidelines, whether a 30-day dietary intervention with flavanol-containing cocoa leads to an improvement of endothelial dysfunction, and whether this improvement is associated with an enhanced number and function of CACs.

Abbreviations and Acronyms

BA = brachial artery
CAC = circulating angiogenic cell
CAD = coronary artery disease
FMD = flow-mediated dilation
HiFI = high-flavanol intervention
LDL = low-density lipoprotein
LoFI = low-flavanol intervention
NO = nitric oxide
SCF = stem cell factor
SDF = stromal cell-derived factor
VEGF = vascular endothelial growth factor

Methods

Study design. High-flavanol cocoa (HiFI [375 mg cocoa flavanols]) and a matched low-flavanol cocoa (LoFI [9 mg]) were consumed by CAD patients (Table 1) twice daily over a 30-day period, using a randomized, double-masked, cross-over study design (Fig. 1) with 1 week of wash-out between interventions. Measurements were taken before initiation (day 0, pre-intervention), and the day after completion of each intervention (day 30, post-intervention). Pre- and post-intervention measurements were taken after overnight fasting and after 30 min of supine rest.

Endothelial vasomotor function assessed as flow-mediated dilation (FMD), as well as CAC numbers and function were defined as primary and secondary outcomes, respectively. Plasma nitrite and nitrate levels, blood pressure (GE Dinamap, Fairfield, Connecticut), heart rate, and circulating cytokines involved in CAC mobilization (vascular endothelial growth factor [VEGF], stromal cell-derived factor [SDF]-1 α, and stem cell factor [SCF]) were measured as tertiary outcomes.

Inclusion criteria (Fig. 1) comprised a diagnosis of CAD and the presence of medical therapy according to American Heart Association/American College of Cardiology (AHA/ACC) secondary prevention guidelines (6). From the 20 patients initially included, 16 patients completed the study and were included in data analyses. Randomization and cocoa drink dispensations were performed by the Department of Pharmacology, University of California–San Francisco. Patients and investigators were masked throughout the study with regard to flavanol content of the test drinks. The study protocol was approved by the University of California–San Francisco Committee on Human Research, and subjects gave written informed consent.

Flavanol-containing test material. Cocoa drinks (Mars, Inc., McLean, Virginia) were standardized for flavanol content and profile, and closely matched for macronutrient content, micronutrient content, caloric load, and theobromine and caffeine levels. The HiFI was made with Cocoapro cocoa powder. While minor differences with regard to the content of individual constituents cannot be excluded, the drinks are matched to a degree that reflects the current state of the art (Table 2). All drinks were similar in taste, supplied in individual sachets labeled with a 3-digit code, and prepared for consumption by mixing with 250 ml skim milk or water. Sachets either contained 9 mg (LoFI) or 375 mg (HiFI) of cocoa flavanols. The amount of flavanols referenced here is

Table 1 Baseline Characteristics of Study Population

Characteristic	Value
n (male/female)	16 (13/3)
Age, yrs	64 ± 3
Body mass index, kg/m^2	27.8 ± 1.8
Diabetes mellitus	38
Hypertension	88
Hyperlipidemia	94
Prior smoking	63
ACEI/ARB	100
Aspirin*	94
Beta-blocker†	88
Statin‡	94
Heart rate, beats/min	60 ± 2
Systolic blood pressure, mm Hg	132 ± 2
Diastolic blood pressure, mm Hg	75 ± 2
Total cholesterol, mg/dl	147 ± 8
LDL cholesterol, mg/dl	80 ± 7
HDL cholesterol, mg/dl	46 ± 3
Triglycerides, mg/dl	107 ± 14
Fasting glucose, mg/dl	104 ± 9
White blood cells, per μl	5,900 ± 400
hsCRP, mg/l	1.8 ± 0.4
Flow-mediated dilation	4.6 ± 0.3

Data given as n, mean ± SEM, or %. *One patient received clopidogrel instead of aspirin because of a history of gastric ulcers. †Two patients did not receive beta-blockers because of intolerance and hypotension. ‡One patient did not receive a statin because of asymptomatically elevated creatine phosphokinase levels.

ACEI = angiotensin-converting enzyme inhibitor; ARB = angiotensin-receptor blocker; HDL = high-density lipoprotein; hsCRP = highly sensitive C-reactive protein; LDL = low-density lipoprotein.

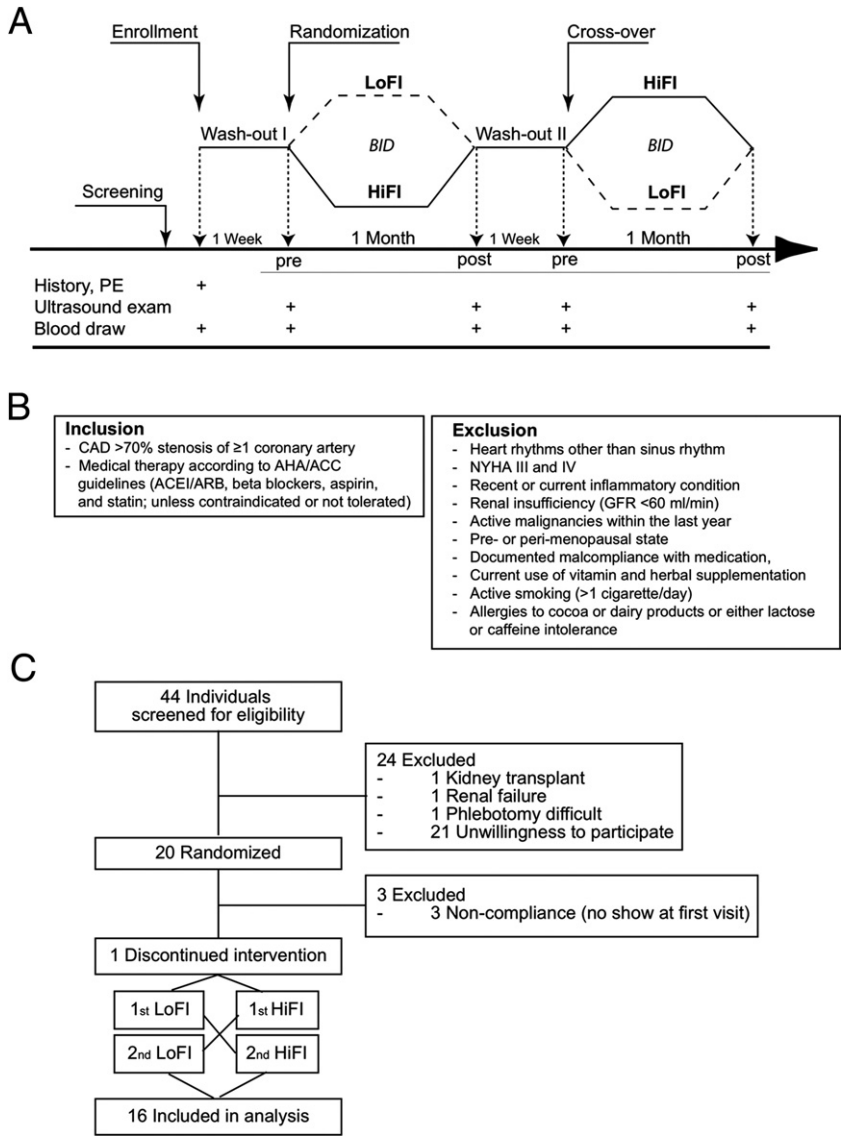

Figure 1 **Protocol, Inclusion/Exclusion Criteria, and Study Flow**

(A) Protocol. **(B)** Inclusion/exclusion criteria. **(C)** Study flow diagram. ACEI = angiotensin-converting enzyme inhibitor; AHA/ACC = American Heart Association/American College of Cardiology; ARB = angiotensin-receptor blocker; BID = twice a day; BP = blood pressure; CAD = coronary artery disease; GFR = glomerular filtration rate; HiFI = high-flavanol intervention (375 mg); HR = heart rate; LoFI = low-flavanol intervention (9 mg); NYHA = New York Heart Association functional class; PE = physical examination.

defined as the sum of all monomeric flavanols and their oligomeric derivatives (2 to 10 monomeric subunits).

FMD. Brachial artery (BA) FMD was measured by ultrasonography (13-MHz transducer, Sonosite Micromax, Bothell, Washington) in combination with an automated analysis system (Medical Imaging Applications, Iowa City, Iowa) in a 21°C temperature-controlled room after 30 min of supine rest (7). A forearm blood-pressure cuff was placed distal to the antecubital fossa and inflated to 250 mm Hg for 5 min. Diameter and Doppler-flow velocity were measured at baseline and immediately after cuff deflation, at 20, 40, 60, and 80 s. The FMD was expressed as: $(\text{diameter}_{max} - \text{diameter}_{baseline})/\text{diameter}_{baseline} \times 100$.

Cell assays. The CAC number in whole blood was measured by flow cytometry as CD34/KDR and CD133/KDR double-positive cells in the lymphomononuclear cell gate (8).

Functional CAC characterization was performed after ex vivo expansion (8). Peripheral blood mononuclear cells were isolated on the basis of the Ficoll method (Vacutainer CPT, Becton Dickinson, Franklin Lakes, New Jersey) and cultured for 7 days on fibronectin-coated plates. To confirm the endothelial phenotype and survival, we performed fluorescent staining to detect lectin-binding and acetylated low-density lipoprotein (LDL) uptake. Chemotaxis toward a VEGF gradient (50 ng/ml in endothelial basal medium-2, 0.5% bovine serum albumin, Sigma-Aldrich, St. Louis,

Table 2 Composition of Matched Flavanol-Containing Test Materials Ingested Twice Daily

Composition	LoFI	HiFI
Cocoa flavanols, monomers-decamers, mg	9	375
Monomers, mg	3	65
Epicatechin	1	59
Catechin	2	6
Dimers, mg	2	53
Trimers-decamers, mg	3	258
Theobromine, mg	96	93
Caffeine, mg	9	11
Energy, kcal	25	25
Fat, g	0.3	0.4
Carbohydrate, g	4.6	4.3
Protein, g	0.7	0.7

HiFI = high-flavanol intervention; LoFI = low-flavanol intervention.

Missouri) was quantified using a modified Boyden chamber. The CACs were plated in the upper of 2 chambers (Corning Transwell, Lowell, Massachusetts) and number of migrated cells counted on the lower side of the dividing membrane after 6 h. The proliferation fraction was determined in adherent CACs by immunofluorescent staining for Ki67. As positive control, VEGF (50 ng/ml) was added for 3 h to parallel samples.

Plasma analyses. Plasma levels of nitrite and nitrate were measured by Rgp chemiluminescence (7), and SDF-1α, VEGF, and SCF were assessed by high-sensitivity enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, Minnesota).

Statistical analysis. Results are expressed as mean ± SEM. Baseline data represent data of first visit. The primary test for an effect was 2-way repeated measurements analysis of variance (2 factors were drink [HiFI/LoFI] vs. time [pre/post]). Analysis of variance and Holm-Sidak post-hoc p values were computed with SigmaStat 3.5 software (Systat, Chicago, Illinois). We tested for linear relationships between $CD34^+/KDR^+$-CAC numbers and FMD using a random-effects linear regression model to account for within-person differences, allowing there to be random variation in the slopes and intercepts in the association of different biological outcomes, including CAC number (independent variable) on FMD (dependent variable) using Stata 9.2 xtmixed procedure (StataCorp, College Station, Texas). All p values and other results reported are for pooled-effects estimates across individual subjects.

Results

Characteristics of study group. The study group consisted of 16 CAD patients having achieved treatment goals in accordance with current AHA/ACC guidelines (6), as indicated by baseline characteristics and medication (Table 1). All individual medications and treatment paradigms, as well as body mass indexes remained unaltered throughout the study. The cocoa drinks were well tolerated, and none of the patients experienced major adverse events, cardiovascular-specific events, or hospitalization during the study period.

Improvement of FMD. The average baseline FMD values were 4.4 ± 0.5% and 4.6 ± 0.5% (p = 0.125) before starting the LoFI or HiFI regimen, respectively (Fig. 2). By the end of the 30-day periods, FMD values significantly increased to 5.7 ± 0.5% (LoFI) and 8.4 ± 0.8% (HiFI [each $p < 0.001$ vs. pre-intervention values]), and the post-HiFI values were significantly greater than post-LoFI values (p = 0.001 between groups). The baseline diameter of the brachial artery (LoFI: 5.11 ± 0.30 mm, 4.85 ± 0.33 mm; HiFI: 5.43 ± 0.24 mm, 4.86 ± 0.43 mm; p = 0.569), the baseline and hyperemic blood flow velocities (LoFI: 181 ± 25 cm/s and 840 ± 115 cm/s, 210 ± 30 cm/s and 904 ± 79 cm/s; HiFI: 212 ± 34 cm/s and 946 ± 107 cm/s, 165 ± 14 cm/s and 862 ± 76 cm/s; p = 0.811 and p = 0.735), as well as corresponding blood flow at baseline and at reactive hyperemia (LoFI: 239 ± 43 ml/min and 1,240 ± 228 ml/min, 259 ± 34 ml/min and 1,178 ± 79 ml/min; HiFI: 278 ± 34 ml/min and 1,270 ± 142 ml/min, 201 ± 34 ml/min and 1,114 ± 191 ml/min; p = 0.827 and p = 0.929) were unaffected by treatments.

Mobilization of functionally unaltered CACs. After 30-day HiFI, $CD34^+/KDR^+$-CACs increased 2.2-fold and $CD133^+/KDR^+$-CACs increased 8.0-fold, relative to LoFI control (each $p < 0.001$) (Fig. 3). Similar to FMD effects, $CD34^+/KDR^+$-CACs, but not $CD133^+/KDR^+$-CACs, were significantly increased after intake of LoFI, as compared with baseline.

There was no change throughout the study in cell survival after ex vivo expansion as determined by the total number of cells positive for acetylated LDL uptake and Ulex europaeus agglutinin-1 (lectin) binding. Neither the VEGF-

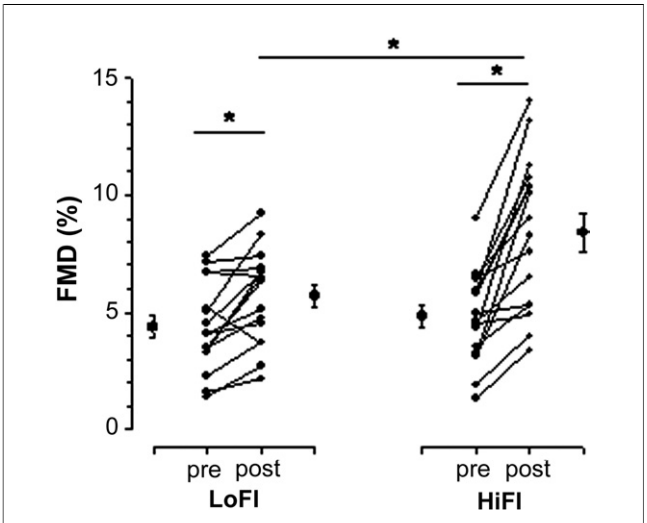

Figure 2 FMD Before and After 30-Day HiFI and LoFI

Flow-mediated vasodilation (FMD) before and after 30-day twice a day HiFI (375 mg) and LoFI (9 mg). **Lines** = individual subjects, **circles** = mean ± SEM. *$p < 0.05$. Abbreviations as in Figure 1.

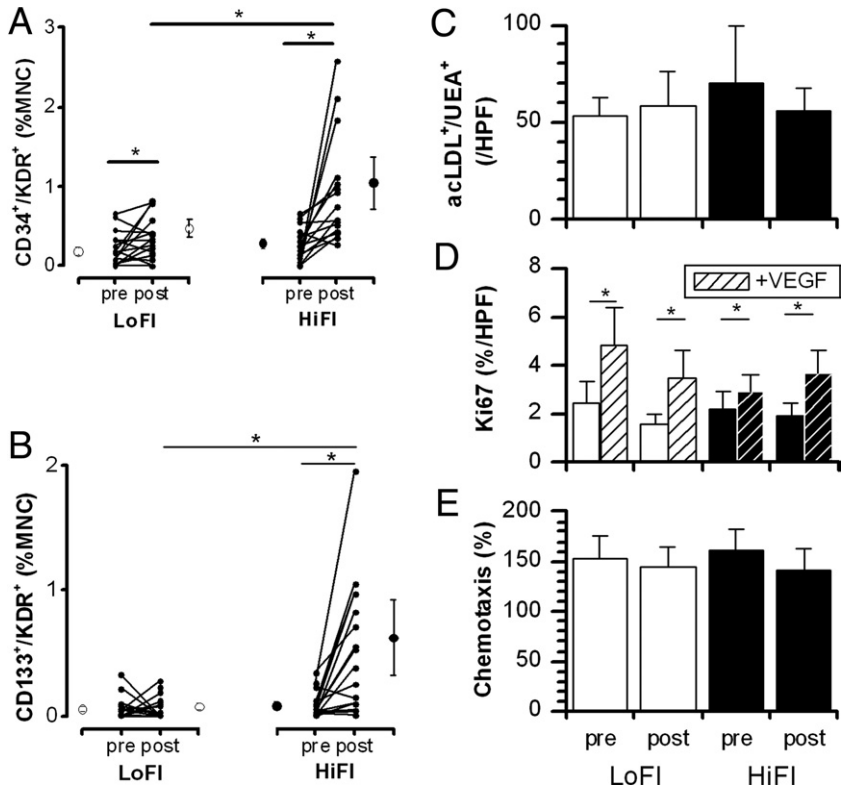

Figure 3 Mobilization of Functional CACs

Number and function of circulating angiogenic cells (CACs): **(A)** $CD34^+/KDR^+$; **(B)** $CD133^+/KDR^+$; **(C)** acetylated low-density lipoprotein $(acLDL)^+$/Ulex europaeus agglutinin 1 $(UEA\text{-}1)^+$; **(D)** proliferation; and **(E)** chemotaxis before and after 1 month of twice a day HiFI (375 mg) and LoFI (9 mg). *$p < 0.05$. HPF = high-power field; MNC = mononuclear cells; other abbreviations as in Figure 1.

stimulated nor the nonstimulated proliferation fraction ($Ki67^+$) of ex vivo expanded CACs was significantly altered by LoFI or HiFI consumption. Random cell movement (4.5 ± 1 cells per high-power field) and chemotaxis toward a VEGF gradient (7.1 ± 2.0 cells per high-power field, 152 ± 20%) were not different between the study visits.

We observed no significant changes in plasma VEGF, SDF-1α, or SCF and clinical laboratory parameters, including C-reactive protein, erythrocyte sedimentation rate, total cholesterol, LDL, high-density lipoprotein, glucose, triglycerides, and blood cell counts.

Increase in plasma nitrite and decrease in systolic blood pressure. Levels of plasma nitrite significantly increased after HiFI, but remained unaltered after LoFI (Fig. 4). Nitrate levels were not significantly affected by either treatment (average at first visit: 23 ± 5 μM).

Arterial blood pressure (BP) was 131 ± 1/76 ± 1 mm Hg and 132 ± 1/75 ± 1 mm Hg ($p = 0.710$, $p = 0.513$) at baseline, respectively. Whereas 30-day LoFI did not significantly change systolic blood pressure (SBP [129 ± 1 mm Hg, $p = 0.471$ vs. day 0]), HiFI significantly decreased SBP to 125 ± 1 mm Hg ($p = 0.013$ vs. day 0), corresponding to a 4.2 ± 0.3 mm Hg decrease as compared with LoFI. Mean heart rate and diastolic BP were not significantly affected by either treatment.

Linear relationship between $CD34^+/KDR^+$-CAC numbers and FMD, BA diameter, and SBP. To assess potential linear associations between $CD34^+/KDR^+$-CAC numbers (independent variable) and the observed effects (dependent variables: FMD, BA diameter, SBP, nitrite, and cholesterol), we performed random-effects linear regression analyses. For each 1% increase in $CD34^+/KDR^+$-CAC numbers, there was a significant increase in FMD of 4.6%, and significant decrease in BA diameter and SBP by 0.52 and 4.44 mm Hg, respectively.

Discussion

These outcomes demonstrate that a randomized, controlled dietary flavanol intervention results in improvements in endothelial dysfunction and BP, and that this is associated with the mobilization of functional CACs in patients with CAD. Despite the fact that our current patient population was medicated in accordance with current evidence-based standards, having reached their BP and LDL treatment goals, endothelial function was impaired as compared with

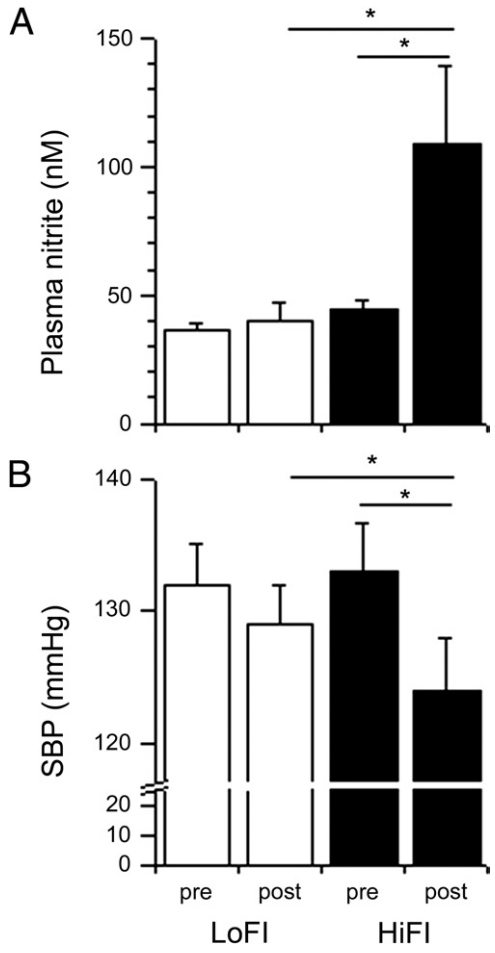

Figure 4 SBP and Plasma Nitrite

Systolic blood pressure (SBP) and plasma nitrite before and after 30-day twice a day HiFI (375 mg) and LoFI (9 mg). *$p < 0.05$. Abbreviations as in Figure 1.

age-matched controls without cardiovascular risk factors (8). Our data demonstrate that a further increase of endothelial function can be achieved by complementing standard treatments with a flavanol-based dietary intervention.

Physiologically, the mobilization of CACs contributes to the repair response after vascular injury (3,4). We demonstrated here that a flavanol-rich diet is capable of increasing CAC numbers more than 2-fold, suggesting that the effects are clinically relevant (3). The effect size observed here for CAC mobilization lies in a range similar to that reported for treatments with statins, estrogen, and changes in lifestyle factors, such as exercise and smoking cessation (5). Notably, during the course of the present study, our patients did not show any signs of augmented inflammation, or increases in the cytokines analyzed (VEGF, SDF-1α, and SCF).

A hallmark of endothelial dysfunction is an impairment of nitric oxide (NO) bioavailability. Corroborating previous investigations in healthy subjects (7,9), we observed an increase in plasma nitrite, which represents both a marker of NO bioavailability and a bioactive NO donor (10). In line with previous studies (2), we observed a flavanol intake-associated decrease in SBP, and our data extend these findings by showing that SBP-lowering effects of dietary flavanols may complement standard medical BP management. While the mechanisms that underlie this effect cannot be ascertained from the present study, it is tenable that increased endothelial function and NO bioavailability play a causal role in improving arterial blood pressure (10).

Differing in part from Balzer et al. (11), we observed small but significant increases in FMD and $CD34^+/KDR^+$-CAC after the LoFI regimen. Based on previous data, it seems unlikely that the small flavanol amounts present in LoFI would explain our findings, but the presence in both test drinks of bioactive compounds other than flavanols, for example, methylxanthines, as well as a general regression to the mean may need to be considered in this context.

Collectively, sustained improvements in endothelial dysfunction by regular dietary intake of flavanols are associated with the mobilization of functional CACs in CAD patients. Our data support the concept that dietary flavanols, in addition to improving cardiovascular functions, can facilitate endogenous repair mechanisms that act synergistically with current medical therapy. Long-term intervention trials examining the effects of high-flavanol diets on cardiovascular health and function are warranted.

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Key Words: nutrition ■ angiogenic cells ■ flow-mediated vasodilation ■ coronary artery disease ■ flavanols.