Effect of cocoa powder on the modulation of inflammatory biomarkers in patients at high risk of cardiovascular disease[1–4]

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ABSTRACT

Background: Epidemiologic studies have suggested that flavonoid intake plays a critical role in the prevention of coronary heart disease. Because atherosclerosis is considered a low-grade inflammatory disease, some feeding trials have analyzed the effects of cocoa (an important source of flavonoids) on inflammatory biomarkers, but the results have been controversial.

Objective: The objective was to evaluate the effects of chronic cocoa consumption on cellular and serum biomarkers related to atherosclerosis in high-risk patients.

Design: Forty-two high-risk volunteers (19 men and 23 women; mean ± SD age: 69.7 ± 11.5 y) were included in a randomized crossover feeding trial. All subjects received 40 g cocoa powder with 500 mL skim milk/d (C+M) or only 500 mL skim milk/d (M) for 4 wk. Before and after each intervention period, cellular and serum inflammatory biomarkers related to atherosclerosis were evaluated.

Results: Adherence to the dietary protocol was excellent. No significant changes in the expression of adhesion molecules on T lymphocyte surfaces were found between the C+M and M groups. However, in monocytes, the expression of VLA-4, CD40, and CD36 was significantly lower ($P = 0.005$, 0.028, and 0.001, respectively) after C+M intake than after M intake. In addition, serum concentrations of the soluble endothelium-derived adhesion molecules P-selectin and intercellular adhesion molecule-1 were significantly lower (both $P = 0.007$) after C+M intake than after M intake.

Conclusions: These results suggest that the intake of cocoa polyphenols may modulate inflammatory mediators in patients at high risk of cardiovascular disease. These antiinflammatory effects may contribute to the overall benefits of cocoa consumption against atherosclerosis. This trial was registered in the Current Controlled Trials at London, International Standard Randomized Controlled Trial Number, at controlled-trials.com as ISRCTN75176807. *Am J Clin Nutr* 2009;90:1144–50.

INTRODUCTION

Evidence based on epidemiologic studies suggests that dietary flavonoids may play a critical role in the prevention of coronary heart disease (CHD) (1). Cocoa and its derived products, such as chocolate, represent a very rich source of dietary flavonoids, which contain a higher content per serving than tea or red wine (2). The health benefits associated with cocoa consumption have been related to their capacity to improve the lipid profile and insulin sensitivity, diminish blood pressure, reduce platelet activity and function, and ameliorate endothelial dysfunction (3–5).

On the other hand, over the past years, evidence linking inflammation with the initiation and progression of atherosclerosis has been accumulated (6). The process of atherosclerosis initiates with a stage characterized by endothelium dysfunction followed by penetration of LDL particles into the subendothelium. Circulating leukocytes, particularly T lymphocytes and monocytes, migrate into this layer as a result of the upregulation of adhesion molecules on both the endothelium and cellular membranes. Besides the antiinflammatory effects on the lipoxygenase pathway (7, 8), several in vitro studies have shown that cocoa polyphenols can modulate the transcription and secretion of proinflammatory cytokines in human peripheral blood mononuclear cells (PBMCs) (9–13), macrophages (14), and lymphoid cell lines (15). However, few human feeding trials have focused on the study of antiinflammatory effects of cocoa, and the results obtained have been contradictory, reporting either neutral effects or changes in a single inflammatory biomarker, including endothelial-derived adhesion molecules, proinflammatory cytokines, and high-sensitivity C-reactive protein (hs-CRP) (17–20).

We embarked, therefore, on a randomized, crossover, controlled clinical trial to evaluate the effects of chronic cocoa consumption on the expression of soluble adhesion molecules and proinflammatory cytokines related to the early stages of atherosclerosis in a series of subjects at high risk of CHD. Among other inflammatory biomarkers, we also studied the effect on the

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expression of adhesion molecules on leukocyte surface—a substance not considered in previous cocoa feeding trials.

SUBJECTS AND METHODS

Subjects

A total of 47 high-risk subjects aged ≥55 y were recruited for the study in the outpatient clinic of the Internal Medicine Department of our Institution. The subjects included had diabetes mellitus or had ≥3 of the following cardiovascular disease risk factors: tobacco smoking, hypertension, plasma LDL cholesterol ≥160 mg/dL, plasma HDL cholesterol ≤35 mg/dL, obesity [body mass index (in kg/m^2) ≥30], and/or family history of premature CHD. Exclusion criteria included documented CHD, stroke or peripheral arteriopathy, and a history of allergic reactions to any cocoa components. The institutional review board of the hospital approved the study protocol, and all participants gave written consent before participation in the study.

Study design

The study was designed as a randomized, crossover, and controlled clinical trial consisting of two 4-wk periods. After a 2-wk lead-in diet, the subjects received two 20-g sachets of soluble cocoa powder (Nutrexpa, Barcelona, Spain) (C) per day (one for breakfast and another for the afternoon snack or after dinner) (total: 40 g/d) with 250 mL skim milk (Lactalis, Barcelona, Spain) each (total: 500 mL/d) (C+M intervention) or only 500 mL skim milk/d (M intervention) for 4 wk in a random order. Half of the subjects received C+M as the first intervention and the other half received only M. None consumed multivitamin or vitamin E supplements. The nutritional composition of the soluble cocoa powder (defatted and sugar-free) used in the study is detailed in **Table 1**. The total phenolic and total proanthocyanidin contents of the soluble cocoa powder was determined with the Folin-Ciocalteu (21) and Bathe-Smith (22) methods, respectively. Individualized phenolic compounds were determined by HPLC as described by Andrés-Lacueva et al (23). The mean degree of flavanol polymerization (mDP) in the soluble cocoa powder was 8, as estimated by thiolysis (24).

Diet monitoring

All of the participants in the study followed an isocaloric Mediterranean-type diet, which was designed according to their personal preferences. Subjects were asked to exclude all other cocoa-containing foods throughout the study and to limit the intake of foods with a high polyphenol content, such as virgin olive oil, red wine, tea, fruit, and vegetables. At the beginning of the study and after each intervention period, a 3-d food record questionnaire (2 weekdays and 1 weekend day), which has been validated in our population (25), was used to assess nutrient intake and to monitor adherence to the study protocol. This information was converted into dietary data by using the Professional Diet Balancer software (Cardinal Health Systems Inc, Edina, MN). The nutritional variables analyzed included total energy, total protein, carbohydrates, dietary fiber, sugars, total lipids, saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs), polyunsaturated fatty acids (PUFAs), cholesterol, vitamin C, vitamin A, vitamin E and total polyphenols. Subjects were asked to maintain their lifestyle habits and to report any illness or abnormality presented during the study period.

Clinical and laboratory measurements

Anthropometric and blood pressure measurements were performed with standardized methods (26). Fasting blood samples and a 24-h urine specimen were collected at baseline and after each intervention (C+M and M). Immunophenotyping of PBMCs was performed. Serum, EDTA-plasma, and urine samples were stored at −80°C until assayed. The clinical investigators and laboratory technicians were blinded to the interventions. For each subject, the analytes determined in frozen samples of whole serum or plasma, as appropriate, were as follows: blood glucose with the glucose oxidase method; cholesterol and triglycerides with enzymatic procedures; HDL cholesterol after precipitation with phosphotungstic acid and magnesium chloride; hs-CRP with particle-enhanced immunonephelometry; soluble intercellular adhesion molecule-1 (ICAM-1), vascular

TABLE 1
Nutritional composition of the soluble cocoa powder (40 g) used in the study[1]

Nutrient	Mean value
Total carbohydrates (g)	26.3
Starch (g)	6.4
Dietary fiber (g)	7.6
Total fat (g)	2.1
SFA (g)	1.3
MUFA (g)	0.69
PUFA (g)	0.07
Cholesterol (mg)	26.3
Proteins (g)	6.8
Potassium (mg)	492
Phosphorous (mg)	349
Calcium (mg)	285
Magnesium (mg)	119
Sodium (mg)	72.8
Iron (mg)	17.6
Zinc (mg)	1.6
Copper (μg)	1.1
Thiamine (mg)	0.11
Riboflavin (mg)	0.18
Vitamin B-3 (mg)	0.72
Vitamin B-5 (mg)	0.60
Vitamin B-9 (μg)	8.0
Theobromine (g)	0.44
(+)-Catechin (mg)	10.41
(−)-Epicatechin (mg)	46.08
Procyanidin B-2 (mg)	36.54
Vanillin (mg)	37.79
Isoquercetrin (mg)	2.23
Quercetin (mg)	0.22
Quercetin-3-arabinoside (mg)	0.70
Quercetin-3-glucuronide (mg)	0.10
Total polyphenols (mg)	495.2
Total proanthocyanidins (mg)	425.7

[1] Total polyphenols were determined by using the Folin-Ciocalteu reagent, and total proanthocyanidins were determined by using the Bathe-Smith method. SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid.

cell adhesion molecule (VCAM-1), E-selectin, P-selectin, and monocyte chemoattractant protein (MCP-1) by using standard enzyme-linked immunosorbent assay (BenderMedSystems, Vienna, Austria); and interleukin-6 (IL-6) with high-sensitivity enzyme-linked immunosorbent assay (detection limit: 0.08 pg/mL; BenderMedSystems).

In addition, epicatechin metabolites derived from phase II metabolism (ie, epicatechin-*O*-glucuronide, epicatechin sulfate, *O*-methyl epicatechin-*O*-glucuronide, and *O*-methyl epicatechin sulfate) were measured in 24-h urine samples by liquid chromatography tandem mass spectrometry (27, 28) as a biochemical marker of compliance.

PBMC immunophenotyping

PBMCs were isolated from whole blood by density-gradient centrifugation over Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) (29). The expression of adhesion molecules on the surface of PBMCs was analyzed via double direct immunofluorescence with the use of commercial monoclonal antibodies. Cell counting and fluorescence analysis were performed in a FACScan Clinical Cytometer (Becton-Dickinson, San Jose, CA) by using CellQuest software. The adhesion molecules analyzed were as follows: VLA-4 (very late activation antigen-4, CD49-d) (Cytogmos, Barcelona, Spain), LFA-1 (lymphocyte function-associated antigen-1. CD11a) (Bender MedSystems), Mac-1 (CD11b/CD18) (Bender MedSystems), and SLe^x (Sialil-Lewis X, CD15s) (Beckman Coulter, Fullerton, CA). CD40 (Caltag Laboratories, Burlingame, CA), another related molecule, was also measured. Monocytes and T lymphocytes were identified separately by using anti-CD14 and anti-CD2 (Caltag Laboratories) monoclonal antibodies, respectively.

Statistical analysis

For a parallel design, statistical power calculations indicated that to detect mean differences of 10 mean fluorescence units (MFI) in monocyte CD49d expression with a conservative SD of 10 MFI, assuming a maximum loss of 10% participants, 21 subjects per group would need to complete the study (α risk = 0.05, power = 0.8) (ENE 2.0 statistical program; GlaxoSmithKline, Brentford, United Kingdom). However, to obtain greater differences, the sample size was doubled. CD49d was used to set the sample size, but changes in all endpoints were of equal interest in this study.

Statistical analysis was performed by using the SPSS Statistical Analysis System (version 14.0; SPSS Inc, Chicago, IL). Descriptive statistics (mean ± SD) were used for the baseline characteristics of the participants. For analysis of laboratory variables, the average of the 2 measures taken after each intervention was used in the comparison between the 2 interventions. Values with a skewed distribution (CRP, VCAM-1, ICAM-1, and IL-6) were transformed to their natural logarithm for analyses. One-factor analysis of variance (ANOVA) for repeated measures with the Bonferroni post hoc test, adjusted by age and sex, was used to compare changes in outcome variables in response to the intervention treatments. Within- and between-group differences are expressed as means and 95% CIs. *P* was considered significant when <0.05.

RESULTS

Baseline characteristics, intervention compliance, diet, exercise monitoring, and side effects

Of the 47 eligible subjects attended to in the outpatient clinic of our department during the study period, 5 declined to participate. Thus, 42 volunteers were included in the study (19 men and 23 women with a mean ± SD age of 69.7 ± 11.5 y). The baseline data of the participants are detailed in **Table 2**. The subjects were randomly assigned to 1 of the 2 interventions (C+M or M), and all subjects completed both phases of the study. Compliance was assessed according to the participants' reports, and none was classified as noncompliant. In addition, as another measure of compliance, epicatechin metabolites derived from phase II metabolism were measured in 24-h urine samples. Regular consumption of 40 g cocoa powder with milk per day resulted in a urinary excretion of 18.38 ± 4.26 μmol/d of total phase II metabolites of epicatechin (Σ epicatechin-*O*-glucuronide, epicatechin sulfate, *O*-methyl epicatechin-*O*-glucuronide, and *O*-methyl epicatechin sulfate) in 24 h, which represented a global increment of 458% ($P < 0.001$) in comparison with that observed after the intake of milk (3.29 ± 1.13 μmol/L).

Self-reported dietary surveys were similar to the recommended diets, and the daily energy and nutrient intakes estimated from the 3-d food record questionnaire did not differ after each intervention period (**Table 3**). Except for one subject who reported constipation during the C+M period, no other participant reported any side effects during either phase of the study. The constipation reported was solved by increasing fiber intake during both intervention periods.

Cardiovascular disease risk factors

Body weight was slightly higher after the C+M intervention than after the M intervention; the mean increase was 0.50 kg (P = 0.013) (Table 2). No significant changes were observed in systolic and diastolic blood pressure or in heart rate between the 2 interventions. Of the biochemical variables, serum concentrations of fasting glucose, total cholesterol, LDL cholesterol, and triglycerides did not change significantly after the 2 interventions (Table 2). However, the HDL-cholesterol concentration after C+M intake was modestly, albeit significantly, higher (mean increase: 2.2 mg/dL; P = 0.033) than after M intake (Table 2).

Expression of cell adhesion molecules on leukocyte cell surfaces

Changes in the expression of cell adhesion molecules on the surface of T lymphocytes and monocytes recorded after the C+M and M interventions are presented in **Table 4**. Although the expression of LFA-1 (−3.0%) and SLe^x (−5.9%) on T lymphocyte surfaces after C+M intake was slightly lower than that after M intake, these changes were not statistically significant (Table 4). Similarly, the expression of the remaining molecules (Mac-1, VLA-4, and CD40) on T lymphocyte surfaces remained practically constant after C+M intake when compared with M intake (Table 4). In contrast, the expression of VLA-4 (−7.4%; P = 0.005), CD40 (−6.6%; P = 0.028), and CD36 (−21.2%; P = 0.001) on the surface of monocytes after C+M intake was significant lower than that after M intake (Table 4). For the

TABLE 2
Body weight, blood pressure, and serum concentrations of glucose and lipids in the 42 subjects at baseline and after both interventions[1]

	Estimated mean (95% CI)			
	Baseline	C+M intervention	M intervention	*P*
Weight (kg)	73.6 (69.6, 77.7)[a]	73.7 (69.8, 77.6)[a]	73.2 (69.2, 77.2)[b]	0.037
BMI (kg/m^2)	27.6 (26.0, 29.1)[a]	27.7 (26.2, 29.3)[a]	27.4 (25.8, 28.9)[b]	0.042
Systolic blood pressure (mm Hg)	138 (130, 146)[a]	138 (131, 141)[a]	135 (127, 142)[a]	0.361
Diastolic blood pressure (mm Hg)	84 (80, 88)[a]	82 (78, 86)[a]	81 (77, –85)[a]	0.322
Heart rate (beats/min)	73 (67, 78)[a]	74 (69, 79)[a]	75 (69, 80)[a]	0.388
Glucose (mg/dL)	121 (109, 133)[a]	129 (111, 148)[a]	123 (108, 139)[a]	0.176
Total cholesterol (mg/dL)	225 (212, 238)[a]	222 (208, 236)[a]	221 (208, 233)[a]	0.410
Triglycerides (mg/dL)	127 (106, 146)[a]	118 (98, 138)[a]	119 (101, 137)[a]	0.176
LDL cholesterol (mg/dL)	177 (165, 188)[a]	174 (165, 182)[a]	171 (159, 182)[a]	0.473
HDL cholesterol (mg/dL)	51.8 (47.7, 55.9)[a]	54.4 (50.1, 58.6)[b]	52.2 (48.0, 56.3)[a]	0.035

[1] Values were adjusted for age and sex. C+M, cacao + milk; M, milk. Changes in outcome variables in response to the intervention treatment were determined by repeated-measures ANOVA adjusted for age and sex. Values in a row with different superscript letters are significantly different, $P < 0.05$ (Bonferroni post hoc test).

remaining molecules (LFA-1, Mac-1, and SLe^x), no significant differences were observed on the monocyte surface.

Changes in circulating inflammatory markers

Changes in circulating inflammatory markers after both interventions are shown in **Table 5**. Significantly lower concentrations of P-selectin (−10.8%; $P = 0.007$) and ICAM-1 (−9.7%; $P = 0.007$) were found after the C+M intervention than after the M intervention. VCAM-1 and MCP-1 concentrations were also lower, but the changes were not statistically significant. Finally, concentrations of E-selectin and of the proinflammatory cytokine IL-6 and hs-CRP remained practically constant after C+M compared with M intake (Table 5).

DISCUSSION

In the current study, the expression of VLA-4, CD40, and CD36 on monocyte surfaces was significantly lower after the 4-wk C+M intervention than after the M intervention. Serum concentrations of soluble adhesion molecules were in consonance with the data obtained in the cellular analysis. Soluble P-selectin and ICAM-1 concentrations were significantly lower after C+M intake than after M intake. However, no changes were observed for the proinflammatory cytokine IL-6 or hs-CRP. In addition, besides the changes observed in the inflammatory variables, a significantly higher concentration of serum HDL cholesterol was observed as a consequence of C+M intake.

The interaction of T lymphocytes and monocytes with endothelium, through adhesion molecules, is the first event in atheroma plaque formation. Several steps may be observed in this process: rolling, tethering, firm adhesion, and transmigration of circulating mononuclear cells in which different adhesion molecules participate (6). Selectins and SLe^x exert their function during the rolling phase, whereas integrins, ICAM-1, and VCAM act during firm adhesion and transmigration (30). Our results suggest that regular consumption of nutritional doses of

TABLE 3
Daily energy and dietary intakes in the 42 subjects studied at baseline and after both interventions[1]

	Estimated mean (95% CI)			
	Baseline	C+M intervention	M intervention	*P*
Energy (kcal/d)	1721.3 (1583.8, 1858.7)[a]	1661.5 (1501.6, 1821.4)[a]	1706.4 (1573.2, 1839.6)[a]	0.176
Total protein (g/d)	86.9 (79.8, 94.0)[a]	94.2 (87.7, 100.7)[a]	96.5 (89.4, 103.6)[a]	0.364
Carbohydrates (g/d)	201.6 (180.4, 222.7)[a]	181.8 (161.0, 202.4)[a]	191.8 (89.4, 103.6)[a]	0.552
Dietary fiber (g/d)	24.9 (20.9, 28.9)[a]	19.6 (16.8, 22.4)[a]	20.8 (17.7, 23.9)[a]	0.545
Sugars (g/d)	75.7 (65.1, 86.4)[a]	80.5 (70.3, 90.6)[a]	85.1 (77.1, 93.1)[a]	0.270
Total lipids (g/d)	63.2 (56.6, 69.7)[a]	58.5 (51.9, 66.2)[a]	58.2 (52.3, 63.6)[a]	0.271
SFA (g/d)	17.3 (14.9, 19.6)[a]	14.3 (12.4, 16.2)[a]	14.4. (12.6, 16.2)[a]	0.198
MUFA (g/d)	30.9 (27.5, 34.6)[a]	29.2 (26.4, 33.6)[a]	29.7 (27.0, 32.4)[a]	0.063
PUFA (g/d)	9.6 (8.4, 10.8)[a]	9.5 (7.8, 11.2)[a]	9.2 (8.1, 10.4)[a]	0.185
Cholesterol (mg/d)	289.1 (243.9, 334.3)[a]	290.6 (250.9, 330.2)[a]	264.5 (233.8, 295.2)[a]	0.075
Vitamin C (mg/d)	171.1 (134.8, 207.4)[a]	171.8 (142.3, 201.2)[a]	169.7 (136.1, 203.4)[a]	0.832
Vitamin A (mg/d)	7440.9 (5555.2, 9326.7)[a]	8377.0 (6194.3, 10560.5)[a]	7019.2 (5207.8, 8830.6)[a]	0.316
Vitamin E (μg/d)	8.6 (7.4, 9.9)[a]	9.0 (7.6–10.4)[a]	8.4 (7.7, 9.2)[a]	0.196
Total polyphenols (mg/d)	307.1 (228.4, 385.8)[a]	334.3 (267.4, 401.1)[a]	349.1 (273.9, 424.4)[a]	0.810

[1] The data represent the daily energy and dietary intakes, exclusive of the intake of the tested products, adjusted for age and sex. C+M, cacao + milk intervention; M, milk intervention; SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid. Changes in outcome variables in response to the intervention treatment were determined by repeated-measures ANOVA adjusted for age and sex. Values in a row with different superscript letters are significantly different, $P < 0.05$ (Bonferroni post hoc test).

TABLE 4
Expression of adhesion molecules on the surface of T lymphocytes and monocytes in the 42 subjects studied at baseline and after both interventions[1]

	Estimated mean (95% CI)			
	Baseline	C+M intervention	M intervention	*P*
T lymphocytes (MFI)				
LFA-1	73.03 (65.70, 78.57)[a]	76.34 (71.93, 80.75)[a]	78.72 (74.86, 82.58)[a]	0.380
VLA-4	53.42 (52.55, 54.30)[a]	54.07 (53.24, 54.89)[a]	53.96 (53.05, 54.87)[a]	0.359
SLe^x	127.83 (111.54, 138.12)[a]	125.99 (111.23, 140.75)[a]	133.87 (123.82, 143.92)[a]	0.361
CD40	60.46 (54.23, 66.70)[a]	57.70 (53.36, 62.05)[a]	56.03 (52.02, 60.04)[a]	0.461
Monocytes (MFI)				
LFA-1	32.20 (29.96, 34.44)[a]	30.83 (28.96, 32.69)[a]	30.01 (28.27, 31.76)[a]	0.310
Mac-1	33.65 (30.56, 34.44)[a]	35.76 (32.97, 38.54)[a]	35.11 (32.93, 37.29)[a]	0.405
VLA-4	25.17 (23.92, 26.42)[a]	22.96 (22.14, 23.78)[b]	24.79 (23.85, 25.74)[a]	0.039
SLe^x	57.53 (52.43, 62.63)[a]	60.70 (54.69, 66.71)[a]	61.29 (55.92, 66.65)[a]	0.420
CD40	24.53 (23.59, 25.47)[a]	23.31 (22.52, 24.05)[b]	24.95 (23.86, 26.02)[a]	0.031
CD36	26.01 (24.01, 28.01)[a]	22.61 (20.84, 24.57)[b]	28.69 (26.60, 30.88)[a]	0.038

[1] Values are adjusted for age and sex. C+M, cacao + milk; M, milk; MFI, mean fluorescence unit; LFA-1, lymphocyte function-associated antigen-1, CD11a; VLA-4, very late activation antigen-4, CD49-d; Mac-1, CD11b/CD18; SLe^x, Sialil-Lewis X, CD15s. Changes in outcome variables in response to the intervention treatment were determined by repeated-measures ANOVA adjusted for age and sex. Values in a row with different superscript letters are significantly different, $P < 0.05$ (Bonferroni post hoc test).

cocoa may have an effect on all initial phases of the atherosclerotic process in subjects at high risk of CHD.

Outcomes from previous cocoa intervention studies are mixed and contradictory. To date, no study has analyzed the effects of cocoa powder on cellular adhesion molecules related to atherosclerosis; therefore, our results can only be compared with those obtained in studies of soluble adhesion molecules. Mathur et al (16) found no changes in the concentrations of IL-1β, IL-6, tumor necrosis factor-α, hs-CRP, and P-selectin when healthy subjects were supplemented with cocoa (36.9-g dark chocolate bar and 30.9-g cocoa powder drink, ≈651 mg procyanidins/d) for 6 wk. Similarly, Farouque et al (17) reported that consumption of a flavanol-rich chocolate bar and a cocoa beverage (444 mg flavanols/d) over a 6-wk period did not modify the concentrations of ICAM-1, VCAM-1, E-selectin, or P-selectin in subjects with CHD. Likewise, more recently, Allen et al (20) found no changes in hs-CRP, ICAM-1, or sCD40L after consumption of 2 chocolate bars containing sterols (360 mg flavanols/d) in a normotensive population with hypercholesterolemia. In contrast, Wang-Polagruto et al (18) found that chronic consumption (6 wk) of flavanol-rich cocoa (446 mg flavanols/d) significantly decreased plasma concentrations of VCAM-1 (11% reduction) in hypercholesterolemic postmenopausal women, but did not produce changes in other cellular adhesion markers, such as E-selectin or P-selectin. In another recent study, circulating ICAM-1 concentrations also significantly decreased (10% reduction) after consumption of dark chocolate (41 g/d, polyphenol content not reported) in healthy women, but no significant changes were observed for VCAM-1 or hs-CRP (19).

Our results agree with some of the data of Kurlandsky and Stote (19), who also found a decrease in the soluble adhesion molecule ICAM-1, and with the data of Mathur et al (16) and Kurlandsky and Stote (19) in that no changes were observed in the concentrations of IL-6 and hs-CRP after cocoa intake. However, our study is the first to report a positive effect of cocoa

TABLE 5
Circulating inflammatory markers in the 42 subjects studied at baseline and after both interventions[1]

	Estimated mean (95% CI)			
	Baseline	C+M intervention	M intervention	*P*
Soluble adhesion molecules				
P-selectin (ng/mL)	255.02 (203.39, 306.49)[a]	235.39 (187.03, 283.75)[b]	263.87 (215.45, 312.28)[a]	0.031
E-selectin (ng/mL)	45.41 (36.91, 53.90)[a]	46.02 (36.33, 55.72)[a]	45.72 (37.55, 53.96)[a]	0.211
ICAM-1 (ng/mL)	359.07 (316.13, 402.00)[a]	331.47 (285.30, 377.64)[b]	367.23 (317.31, 417.15)[a]	0.034
VCAM-1 (ng/mL)	992.07 (846.55, 1137.58)[a]	986.31 (850.18, 1122.43)[a]	1026.90 (856.91, 1196.90)[a]	0.349
MCP-1 (ng/mL)	289.99 (224.83, 355.15)[a]	280.11 (225.54, 332.68)[a]	294.24 (234.76, 353.72)[a]	0.239
Proinflammatory cytokines				
IL-6 (pg/mL)	1.02 (0.67, 1.37)[a]	1.07 (0.75, 1.39)[a]	1.13 (0.74, 1.52)[a]	0.253
Other inflammatory markers				
hs-CRP (mg/dL)	0.52 (0.30, –0.73)[a]	0.50 (0.36, 0.65)[a]	0.53 (0.34, 0.71)[a]	0.726

[1] Values are adjusted for age and sex. Changes in outcome variables in response to the intervention treatment were determined by repeated-measures ANOVA adjusted for age and sex. Values in a row with different superscript letters are significantly different, $P < 0.05$ (Bonferroni post hoc test). C+M, cacao + milk; M, milk; ICAM-1, intercellular adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1; MCP-1, monocyte chemoattractant protein-1; IL-6, interleukin-6; hs-CRP, high-sensitivity C-reactive protein.

consumption on P-selectin concentrations, in contrast with the results found by Mathur et al (16) and Farouque et al (17).

Another positive outcome of our study was the higher HDL-cholesterol concentration after C+M intake than after M intake. Although outcomes on lipid metabolism from cocoa feeding trials are still scarce, this finding seems to be in accordance with that of several studies that have reported higher HDL-cholesterol concentrations after cocoa or chocolate intake but no changes in LDL cholesterol (31–33).

Previous cocoa intervention studies have presented several limitations, including the recruitment of subjects in good health, small number of participants, large age differences among subjects, and the evaluation of only a few inflammatory variables. In this study we have tried to overcome some of these limitations by enrolling 42 subjects at high risk of CHD (19 men and 23 women with a mean ± SD age of 69.7 ± 11.5 y) and by including additional inflammatory variables such as the expression of adhesion molecules on the surface of leukocytes. In addition, we have used defatted and sugar-free cocoa powder and skim milk as test products to adhere to the dietary needs of the subjects and to minimize the possible effects of these components on the variables tested. It is worth noting that most of the abovementioned studies have been conducted with chocolate bars or water-based cocoa powder drinks, whereas the present study was conducted to provide nutritional doses of cocoa powder with milk, the form in which it is usually consumed. The effect of milk on the bioavailability of polyphenols has been a very contradictory issue. Serafini et al (34) found that consumption of chocolate with 200 mL milk decreased the plasma antioxidant capacity when compared with that obtained after consumption of 100 g dark chocolate, and no effect was observed after consumption of milk chocolate. However, Shroeter et al (35) described that the presence of milk in cocoa products did not counteract the absorption and biological activity of monomeric flavanols from cocoa products and did not affect plasma antioxidant capacity. More recent studies have suggested that the effect of milk on the bioavailability of cocoa polyphenols may depend on the intake of polyphenols per cocoa dose (36). In this sense, previous work by our research group has shown that the intake of soluble cocoa powder with milk at the same dose (40 g) and with a polyphenol content similar to that of the present study did not significantly affect the total amount of epicatechin metabolites excreted in urine compared with the intake of cocoa with water, although differences in the metabolite excretion profiles were observed, particularly in glucuronide and sulfate excretion rates and in the sulfation position (37, 38).

No washout periods between interventions were considered in the present work because previous studies have shown that changes in cellular and soluble adhesion molecules were already observed after 2 wk of intervention (39, 40). In addition, it has been reported that flavan-3-ols are eliminated from the organism in <1 d (41). Because the intervention period in the present study was 4 wk, the introduction of a washout period between interventions probably would not have modified the differences observed between treatments.

With respect to the side effects of cocoa, only one participant reported constipation during the C+M period, which was solved by increasing fiber intake. However, a weak but significant increase in body weight was observed after the intake of 40 g cocoa plus 500 mL milk/d for 28 d, which was probably due to the calories added to the diet because of the intervention. The number of additional calories due to cocoa intake was 136 kcal/d. These additional kilocalories produce a weight gain of ≈20 g/d, or 560 g/28 d (intervention period), almost the same value as the mean weight gain observed in the participants of our study (42). Because no changes in anthropometric variables were observed in other trials (4, 5), further studies are needed for a more in-depth exploration of the relation between body weight and cocoa intake in high-risk patients. Meanwhile, subjects introducing cocoa into their habitual diet should reduce their caloric intake to ensure maintenance of their body weight.

Finally, it is worth mentioning that the antiinflammatory effects derived from regular consumption of cocoa found herein seem to be very modest compared with those observed for other polyphenol-rich foods, such as wine, which also contains other modulating molecules such as ethanol. According to Estruch et al (39), changes induced in the expression of adhesion molecules (ie, LFA-1, Mac-1, VLA-4, and MCP-1) by moderate red wine intake were more significant on the surface of monocytes than of T lymphocytes, which also agrees with our results; however, positive changes were much higher (>25%) than those in the present work (<21%). In another study, moderate consumption of cava (white sparkling wine) produced a decrease of >10% in circulating adhesion molecules and other inflammatory biomarkers, such as hs-CRP (29). Nonetheless, albeit modest, changes induced by cocoa intake may also contribute to a reduction in cardiovascular disease risk factors in subjects prone to cardiovascular events.

In summary, the results of the present study suggest a positive influence of cocoa polyphenols on the modulation of inflammatory mediators in human subjects at high risk of CHD. These antiinflammatory effects, together with other previously reported effects, including those of antioxidant, anti-platelet, and positive vascular effects, may contribute to the overall benefits of cocoa consumption against atherosclerosis.

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