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**File: ■ Cocoa (*Theobroma cacao*)**
**■ Cardiovascular Disease Risk**
**■ DNA Methylation**

**HC 091311-492**

**Date: March 14, 2014**

**RE: Cocoa Consumption Decreases Global DNA Methylation in Subjects with Cardiovascular Disease Risk Factors**

Crescenti A, Solà R, Valls RM, et al. Cocoa consumption alters the global DNA methylation of peripheral leukocytes in humans with cardiovascular disease risk factors: A randomized controlled trial. *PLoS One*. 2013;8(6):e65744. doi: 10.1371/journal.pone.0065744.

One of the major processes associated with epigenetics (changes in DNA expression outside of changes in the genes themselves) is DNA methylation. It has been shown that global DNA methylation levels in peripheral lymphocytes are associated with cardiovascular disease (CVD) and risk factors of CVD; however, environmental and dietary factors can modify these epigenetic effects. Polyphenolic-rich foods such as cocoa (*Theobroma cacao*) may prevent or alter epigenetic changes associated with certain disease states. The aim of this randomized, controlled study was to assess the effects that cocoa consumption has on DNA methylation in subjects with risk factors for CVD and to determine if gene expression of enzymes associated with DNA methylation are affected by cocoa.

This study took place at 3 primary care centers in 3 cities across Spain (Alcover, Vic, and Centelles). There were 104 subjects in the control group (males: n=46; females: n=58) and 110 subjects in the treatment group (males: n=45; females: n= 65). Subjects included in the study were > 20 years of age; pre-hypertensive (systolic blood pressure [BP]: 120–139 mmHg or diastolic BP: 80–89 mmHg); stage 1 hypertensive (systolic BP: 140–159 mmHg or diastolic BP: 90–99 mmHg); with low-density lipoprotein cholesterol (LDL-C) ≥ 130 mg/dL and ≤ 189 mg/dL; and with at least 1 other major CVD risk factor such as age (males: > 45; females > 55 years), smoking habit, low high-density lipoprotein cholesterol (HDL-C) (males: ≤ 40 mg/dL; females: ≤ 46 mg/dL), or a family history of premature heart disease. Exclusion criteria were diabetes, chronic disease, any hypolipemic treatment, body mass index (BMI) > 35 kg/m$^2$, or a history of CVD.

The tolerance to cocoa was first evaluated in the treatment group for 2 weeks (6 g cocoa/d; 465 Kcal/d) while the subjects were on an isocaloric diet (13% of energy from saturated fatty acids). The control group also consumed an isocaloric diet during this period. They did not consume any chocolate in the diet. After 2 weeks of treatment with the cocoa cream product (manufacturer unknown), fasting blood samples were obtained from the treated and untreated subjects for the isolation of peripheral leukocyte DNA. The global methylation levels of peripheral leukocyte DNA from blood samples and cultured peripheral blood

mononuclear cells (PBMCs) were assessed from measurements of 5-methylcytosine (5mC). Several single nucleotide polymorphisms (SNPs) were also assessed in 3 different types of DNA methyltransferases (DNMTs), in methylenetetrahydrofolate reductase (MTHFR), and in methionine synthase reductase (MTRR) genes.

Additionally, PBMCs from 6 of the subjects (3 male, 3 female) were treated with a cocoa extract (consisting mostly of proanthocyanidins [catechin, 2.374 mg/g and epicatechin, 5.638 mg/g] measured by liquid chromatography-mass spectrometry [LC-MS]) that was similar to the cocoa product used in the human intervention trial. The mRNA levels of DNMT, MTHFR, and MTRR genes were analyzed from PBMCs treated with 25 mg/L and 50 mg/L of cocoa extract for 3 h and 24 h. Global methylation was evaluated by treatment with 50 mg/L of cocoa extract for 72 hours. Moreover, cell viability was measured in PBMCs treated with the cocoa extract.

There were no statistical differences in demographics or body measurements between the control and treatment groups. The treated subjects had significantly lower %5mC in peripheral leukocyte DNA compared to the control subjects (2.991 ± 0.366 vs. 3.909 ± 0.380; $P<0.001$). Gender did not contribute to significant differences, and age did not correlate with methylation.

It was also found that subjects with the A66G polymorphism in the MTRR gene and men with the A1298C polymorphism in the MTHFR gene had more global peripheral leukocyte DNA methylation in comparison with those without polymorphisms in these genes. In addition, it was found that there was an association between cocoa consumption and the A38946G polymorphism in DNMT3B genes on global peripheral leukocyte DNA methylation in men.

No effects were found in methylation genes of the PBMCs after 3 hours; however, after 24 hours of treatment at both 25 mg/L and 50 mg/L, the mRNA expression of the DNMT1, DNMT3A, DNMT3B, MTHFR, and MTRR genes were significantly lower compared to the control (vehicle only) cells ($P\leq0.05$). It was also found that global DNA methylation in the PBMCs treated with the cocoa extract (50 mg/L) had lower, although non-significant, %5mC levels compared to the control cells (3.824 ± 0.971 vs. 4.599 ± 0.683, $P=0.09$).

The authors conclude that cocoa consumption decreases global DNA methylation of peripheral leukocytes in humans with CVD risk factors. These effects were also further validated in vitro (in PBMCs) by the inhibitory effects of cocoa extracts on the expression of genes that play a role in DNA methylation, suggesting a mechanism for the hypomethylation effects of cocoa. This is one of the first studies about the epigenetic effects of cocoa consumption in human health; further studies will help to better understand the mechanisms of the active components of cocoa and their impact on epigenetic changes and disease outcomes.

*—Laura M. Bystrom, PhD*

Referenced article can be found at
www.plosone.org/article/info%3Adoi%2F10.1371%2Fjournal.pone.0065744.

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