EFFECTS OF INCORPORATING DARK CHOCOLATE AND A COCOA BEVERAGE INTO A WEIGHT LOSS DIET ON INFLAMMATORY STATUS AND BONE TURNOVER

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by
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ABSTRACT

Individuals who are overweight or obese are at increased risk for developing multiple co-morbidities. Losing body weight (BW) is a proven method to reduce excess fat mass (FM) and ameliorate risk factors for chronic disease. Lack of long-term dietary adherence to a weight loss plan can lead to unsuccessful maintenance of BW loss, weight regain and the persistence of health concerns. Complicated dietary patterns that restrict certain macronutrients or food groups may be difficult to follow and can contribute to the lack of long-term dietary adherence. However, a diet composed of whole grains, fruits, vegetables and lean proteins can provide adequate nutrients and has additional energy remaining for the accommodation of an individual’s favorite snack food. Certain snack foods may also provide additional health benefits. Cocoa and chocolate products contain potent antioxidants called flavanols. Cocoa’s high antioxidant content and potential to reduce oxidative stress and inflammation have been credited with the improvement of blood pressure (BP), lipid concentrations and insulin sensitivity. Because several of the health effects observed with cocoa intake are similar to those benefits observed after BW loss, there may be an additive effect of consuming cocoa while reducing BW. Therefore, this research project was conducted to evaluate the effects of incorporating two daily dark chocolate (DC) snacks and high-flavanol (HF) cocoa beverage into an energy-restricted diet (ERD) on BW, body composition, BP, metabolic parameters, oxidative stress and inflammatory proteins, markers of bone and mineral metabolism and bone mineral density (BMD).

Sixty overweight and obese (body mass index ≥25<43 kg/m^2) premenopausal women between the ages of 25 and 45 years were recruited in two waves for this 18-week randomized dietary intervention. All women followed an ERD while being further randomized to consume either a twice daily snack of DC and a HF cocoa beverage (DC; n=30) or a twice daily snack of
flavanol-free (FF) licorice and a FF, cocoa-free vanilla beverage (NC; n=30). At baseline, and at weeks 6, 12 and 18 of the intervention, women underwent anthropometric measurements, a fasting blood draw and provided a 24-hour urine sample. At baseline and week 18, participants completed 4-day food and 7-day physical activity records and underwent dual-energy X-ray absorptiometry to measure body composition and BMD. Intention-to-treat analysis using data from all women who completed baseline measurements (n=60) and an efficacy analysis using only data from the women that completed the intervention (n=51) were conducted. Differences in characteristics at baseline between groups and between study-completers and those who withdrew were also analyzed using analysis of variance (ANOVA). A 2 x 2 x 4 ANOVA with repeated measures on the time factor was performed to assess the main effects of cohort (cohort 1 and 2) and group (DC and NC) on all variables over the four intervals. Interactions of cohort and group (Treatment) by interval (Time) were also assessed using 2 x 4 ANOVA.

According to the intention-to-treat analysis, the DC and NC groups significantly reduced energy intake by 379 and 437 kcals (both $p<0.001$), respectively. Weight loss was $4.4 \pm 3.3$ kg (mean $\pm$ SD) ($p<0.001$) and $5.0 \pm 4.9$ kg ($p<0.001$) for the DC and NC groups, respectively. Both groups similarly and significantly reduced waist and hip circumferences (both groups $p<0.001$), fat mass (FM) (both groups $p<0.001$) and abdominal FM (DC: $p<0.001$, NC: $p<0.01$). Women in the NC group also experienced a decrease in fat-free mass (FFM) ($p<0.05$). There were no statistically significant differences in anthropometrics or body composition measurements between groups at any interval throughout the study or in the change over time.

The DC group reduced systolic and diastolic BP by 2.7 mmHg ($p<0.05$) and 2.7 mmHg ($p<0.01$), respectively, and the NC group reduced systolic and diastolic BP by 3.4 and 4.2 mmHg (both $p<0.01$), respectively. The DC and NC groups, respectively, reduced glucose and insulin
concentrations, respectively, by 13.0 mg/dL ($p<0.001$) and 1.9 µU/mL ($p<0.01$) and 14.9 mg/dL ($p<0.001$) and 1.9 µU/mL ($p<0.01$). The NC group demonstrated an increase in total cholesterol concentration ($p<0.05$) while the DC group experienced no changes in lipid parameters. There were no differences in metabolic parameters between groups at any interval throughout the study or in the change over time.

The combined effect of the BW loss and additional flavanol consumption on markers of oxidative stress and inflammation, markers of bone formation and resorption and mineral metabolism and BMD was assessed using data from women who completed the intervention and provided blood and urine samples at all four testing intervals. At baseline, there were significant differences in physical activity ($p<0.05$) and N-telopeptide of bone type I collagen (NTx) concentration ($p<0.05$) between cohort 1 and 2; however, there were no differences between flavanol groups.

There was a significant main effect of cohort on tumor-necrosis factor-alpha (TNF-α) and interleukin-1β (IL-1β) concentrations. TNF-α concentration was significantly greater in cohort 1 at weeks 6 and 12 (both $p<0.001$) and IL-1β was significantly greater in cohort 2 at week 6 ($p<0.05$). The decrease in TNF-α from baseline to week 18 was only significant in cohort 1 ($p<0.01$) while the decrease in IL-1β concentration was significant for both cohorts ($p<0.001$). However, the overall change from baseline to week 18 in these biomarkers was not significantly different between cohorts.

The effect of time was significant within groups in markers of inflammation. The DC and NC groups had significant decreases in IL-6 (both groups $p<0.001$) and IL-1β (both groups $p<0.001$) from baseline to week 18. The DC group also had significant decreases in TNF-α ($p<0.01$). Neither group had significant changes in C-reactive protein or urinary 8-epi-prostaglandin F$_{2a}$ concentrations.
from baseline to week 18. There were no significant differences between groups in markers of oxidative stress or inflammation at any interval throughout the intervention or in the change over time.

The effect of cohort was also significant for serum osteoprotegerin (OPG), NTx and receptor activator for nuclear factor κB ligand (RANKL) concentrations. OPG concentration was significantly greater in cohort 2 at weeks 6 ($p<0.001$) and 12 ($p<0.01$). The change in OPG from baseline was significantly different between cohorts ($p<0.001$) and the increase in OPG concentration from baseline to week 18 was significant for cohort 2 ($p<0.001$) but not for cohort 1. NTx concentration was significantly higher in cohort 1 at baseline ($p<0.001$) and at weeks 6 ($p<0.01$) and 12 ($p<0.05$). Cohort 1 experienced a decrease while cohort 2 experienced an increase in NTx concentration from baseline to week 18. The change in NTX from baseline was significantly different between cohorts ($p<0.01$) and the decrease in NTx concentration from baseline to week 18 was significant for cohort 1 ($p<0.05$) but not for cohort 2. Cohort 2 had a significantly higher RANKL concentration at week 6 ($p<0.001$) while cohort 1 had significantly higher RANKL concentration at week 12 ($p<0.01$). The change in RANKL from baseline to week 18 was not significantly different between cohorts and the change from baseline to week 18 was not significant for either cohort.

The effect of time was significant within groups in markers of bone turnover. Serum OPG significantly increased in the DC ($p<0.01$) and NC ($p<0.001$) groups with no significant overall change from baseline to week 18 in osteocalcin, NTx, or RANKL concentrations. By week 18, serum phosphorus concentration ($p<0.001$) had significantly increased in the NC group while the DC group had no significant changes in mineral metabolism. There were no significant differences
between groups in markers of bone turnover or mineral metabolism at any interval throughout the intervention or in the change over time.

Finally, there was a significant effect of cohort on forearm (FA) and hip BMD. FA BMD was significantly greater in cohort 1 at week 18 (p<0.01). The changes from baseline for hip (p<0.01) and FA BMD (p<0.001) were significantly different between cohorts and the increases from baseline to week 18 for hip (p<0.01) and FA BMD (p<0.001) were significant only in cohort 1. The effect of time was significant within groups for BMD. The DC group had significant increases in hip (p<0.01) and FA BMD (p<0.01) and the NC group had a significant increase in FA BMD (p<0.001) from baseline to week 18. There were no significant differences between groups in BMD at baseline or week 18 or in the change over time.

Results of this investigation demonstrate that a short-term ERD which incorporates either two daily snacks of DC or FF licorice can achieve BW loss and improve body composition, BP, metabolic parameters and inflammatory status without adversely affecting lipid concentrations, markers of bone turnover or BMD. Further, incorporating additional flavanols into an ERD in the form of two DC snacks and a HF cocoa beverage provided no greater benefit on metabolic parameters, markers of oxidative stress or inflammation or bone health following BW loss.

Based on these results, daily calorie-controlled sweet snacks can be included within the context of an ERD without exceeding the energy reduction necessary for the induction of BW loss. The act of reducing energy and losing BW provided multiple health improvements including a reduction in FM, BP, glucose and insulin concentrations and inflammatory status in healthy women independent of the type of snacks consumed. Further, BW and FM loss may reduce obesity-induced inflammation thereby attenuating excess stimulation of bone resorption and leading to a maintenance of BMD in otherwise healthy, premenopausal women. Therefore, overweight or obese yet otherwise
healthy women should be encouraged to lose BW to improve current health status and reduce risk factors for future conditions without substantial concern for harming short-term bone health.
# TABLE OF CONTENTS

List of Tables ............................................................... xii
List of Figures .............................................................. xiii
Acknowledgments ............................................................. xiv

Chapter 1. Introduction ......................................................... 1

Chapter 2. Literature Review .................................................. 10

Chapter 3. A reduced-calorie dietary pattern including a daily sweet snack promotes body weight reduction and body composition improvements in premenopausal women who are overweight and obese: a pilot study. .................. 70
  Abstract ................................................................. 71
  Introduction ............................................................. 72
  Methods ................................................................. 74
  Results/ Discussion .................................................... 77
  Conclusions ............................................................ 81
  References ............................................................ 82

Chapter 4. Changes in body weight, blood pressure and selected metabolic markers with an energy-restricted diet including a sweet snack. .................. 88
  Abstract ................................................................. 89
  Introduction ............................................................. 91

ix
Appendix B. Diet-Related Materials ......................................................... 201

exchange List by Calorie Level ...................................................... 202
LIST OF TABLES

Table 3.1 Characteristics of study participants at baseline and week 18 by dark chocolate and non-chocolate snack group ................................................................. 86

Table 4.1 Baseline characteristics of participants by snack group ........................................... 114

Table 4.2 Estimated dietary intakes by snack group and changes over time, including baseline values carried forward for cases of missing data ........................................... 115

Table 4.3 Anthropometric measurements by snack group and changes from baseline, including baseline values carried forward for cases of missing data ......................... 117

Table 4.4 Blood pressure measurements and metabolic profile by snack group and changes from baseline, including baseline values carried forward for cases of missing data ........................... 120

Table 4.5 Anthropometric measurements by snack group and changes from baseline, for women who completed the study ................................................................. 124

Table 4.6 Blood pressure measurements and metabolic profile by snack group and changes from baseline, for women who completed the study ................................................................. 126

Table 5.1 Baseline characteristics for all women and by flavanol group ........................................... 162

Table 5.2 Estimated daily dietary intake by flavanol group at baseline and week 18 ........................ 164

Table 5.3 Anthropometric measurements by flavanol group at all intervals ................................. 165

Table 5.4 Body composition and bone mineral density and by flavanol group at baseline and week 18 .............................................................................................................. 166

Table 5.5 Markers of oxidative stress and inflammation by flavanol group at all intervals ........ 168

Table 5.6 Markers of bone turnover and mineral metabolism by flavanol group at all intervals .... 174
LIST OF FIGURES

Figure 3.1 Changes in anthropometric and body composition measurements from baseline to week 18 by snack group. ................................................................. 87

Figure 4.1 Study flow diagram of participants. .................................................. 113

Figure 4.2 Body weight changes over time by snack group. ............................. 119

Figure 4.3 Serum glucose and insulin concentrations and HOMA-IR changes over time by snack group ................................................................. 122

Figure 5.1 Change in bone mineral density from baseline to week 18 by cohort. 167

Figure 5.2 Tumor-necrosis factor-α at each interval by cohort. ............................... 169

Figure 5.3 Interleukin-1β at each interval by cohort. ............................................ 170

Figure 5.4 Osteoprotegerin at each interval by cohort. ............................................. 171

Figure 5.5 Cross-linked N-telopeptide of type I collagen at each interval by cohort .... 172

Figure 5.6 Receptor Activator for Nuclear Factor κB Ligand at each interval by cohort 173
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CHAPTER 1

INTRODUCTION
Study Rationale

An estimated 68% of adults in the United States (US) have a body mass index (BMI) that classifies them as either overweight or obese. With the numbers in other industrialized nations beginning to mirror our own, obesity has become a universal health crisis (1). Obesity is a condition that is characterized by the excess accumulation of energy as fat mass (FM). Obesity reduces an individual’s quality of life, decreases work productivity (2) and increases the risk of a variety of chronic morbidities including diabetes, hypertension, hyperlipidemia and certain types of cancer (3). With the significant health and economic burdens of obesity, methods for preventing and treating this disease are necessary (4).

The primary approach for the reduction of excess FM is through body weight (BW) loss (5). Reducing BW can decrease blood pressure (BP) and lipid concentrations and increase insulin sensitivity, thus ameliorating the main risk factors in the development of many obesity-related chronic conditions. Unfortunately, BW loss of as little as 5% has also been associated with decreases in bone mineral density (BMD) (6). Low BMD is a risk factor for osteoporosis, a chronic condition that increases one’s risk for fracture. Osteoporosis is a disease that affects 10 million Americans, 8 million of which are women (7). Osteoporosis-related fractures cost the US approximately 19 billion dollars per year in healthcare expenses (7). There are many factors beside BW that influence BMD including physical activity, nutrient adequacy of the diet (8,9), body composition (10) and inflammatory status (11,12).

In the interest of providing the general public with safe methods to lose BW, nutrition professionals have invested a great deal of time researching and designing effective (13) and cost-efficient (14) dietary interventions that focus on lifestyle modification and improvement of essential nutrient intake. Unfortunately, with the strong desire to lose weight in our society, fad diet programs
have become exceedingly popular and eclipsed these research-based techniques. The appeal of popular diets is their promise of quick weight loss within a rather short time period. These diet plans are often based on a specific combination of macronutrients or the restriction of certain food groups (15-18). When followed closely, these diets can induce significant short-term BW loss (19-21). However, long-term adherence to these highly restrictive diets is difficult (17). Over time, recidivism in dietary adherence is common leading to a stall in BW loss and rebound weight gain (22). The majority of the BW regained is typically in the form of FM which can increase an individual’s risk of negative health effects due to increased adiposity-induced oxidative stress and inflammation (23). Restriction of certain food groups commonly recommended by these popular commercial diets can also lead to poor essential nutrient intake and nutrient deficiencies if followed for a long period of time (24).

To improve dietary adherence, an individual’s weight-loss plan should be designed to be easily incorporated into their current dietary and lifestyle pattern (17). For example, the incorporation of commonly consumed and highly enjoyed foods into an individual’s energy-restricted (ERD) may make it easier to comply with a reduction in energy. If a nutrient-dense ERD, high in whole grains, fruits, vegetables and lean protein sources is consumed, it can fulfill daily essential nutrient recommendations while leaving extra calories to be used for a regular snack without exceeding energy requirements. The inclusion of a commonly consumed portion-controlled sweet snack in an ERD may be a less dramatic alteration in the dietary pattern for some individuals, making it easier to remain adherent for a longer period of time, potentially increasing the magnitude of BW lost (17).

Snacks not only have the potential to aid in dietary adherence, but certain sweet snacks may confer additional health benefits. Cocoa and chocolate products, in particular, can provide essential
nutrients including potassium, magnesium, copper and manganese (25,26) that are often lacking in
the typical American diet. In addition, cocoa is high in flavanols which are potent antioxidants. In
vitro, cocoa flavanols have been shown to protect against oxidative stress (27), reduce secretion of
the pro-inflammatory cytokines tumor-necrosis factor-α (TNF-α), interleukin (IL)-1β (28) and IL-12
(29) and increase the activity of the anti-inflammatory protein, IL-4 (30). Cocoa products have also
been shown to increase flow mediated dilation, reduce BP (31), and improve insulin sensitivity (32)
while remaining essentially lipid-neutral (31) when added isocalorically to the diet. In combination
with BW loss, cocoa and high-flavanol (HF) dark chocolate (DC) products may provide additional
health benefits beyond those acquired from BW loss alone.

To date, well-designed studies that demonstrate the feasibility and effectiveness of
incorporating a daily energy-controlled sweet snack into an ERD are lacking. In addition, there are
no published interventions that evaluate the effect of including a DC snack during BW loss on
metabolic parameters, markers of oxidative stress and inflammation and bone health. Therefore, this
dietary intervention aimed to evaluate the effects of an ERD that incorporated a daily DC snack and
HF cocoa beverage and or a daily non-chocolate (NC) licorice snack and flavanol-free (FF) vanilla
beverage on BW loss and clinical and biochemical outcomes.

**Study Aims and Hypotheses**

The first objective of this 18-week research project was to test the feasibility of purposefully
incorporating two different types of energy-controlled daily sweet snacks into an ERD on selected
health outcomes in premenopausal women who were overweight or obese. The second objective
was to evaluate the effect of incorporating two daily DC snacks or two daily NC licorice snacks into
an ERD on BW, body fat percent, BP, glucose and insulin concentrations and lipid levels in
premenopausal women who were overweight or obese. The third objective was to determine the
effect of consuming additional flavanols in the form of two daily DC snacks and HF cocoa beverage compared to two daily NC, FF snacks and FF vanilla beverage on markers of oxidative stress and inflammation after BW loss in premenopausal women who were overweight or obese. The final objective was to determine the effect of consuming additional flavanols in the form of two DC snacks and HF cocoa beverage on markers of bone formation and resorption, mineral metabolism and BMD after BW loss in premenopausal women who were overweight or obese.

The first hypothesis was that overweight or obese premenopausal women who followed an ERD containing either two daily DC or two daily NC snacks could achieve an energy deficit and experience significant positive changes in anthropometric and body composition measurements. The second hypothesis was that women consuming an ERD that contained either two daily DC or two daily NC snacks would experience BW loss, improvements in body fat percent and BP and reductions in glucose and insulin concentrations without a negative effect on lipid levels. The third hypothesis was that overweight and obese women who lost BW and FM would experience reductions in markers of oxidative stress and inflammation. A further hypothesis was that overweight and obese women, who consumed additional flavanols in the form of two daily DC snacks and a HF cocoa beverage would have a greater reduction in markers of both oxidative stress and inflammation. The final part of this hypothesis was that the women who consumed additional flavanols in the form of two daily DC snacks and a HF cocoa beverage would experience a greater decrease in markers of bone resorption, a greater increase in markers of bone formation and maintenance of mineral metabolism and BMD following BW loss.

Within this dissertation, chapter 2 contains a literature review which provides an overview of obesity as well as the dietary approaches designed to reduce BW, FM and the risk factors for chronic diseases such as hypertension, hyperlipidemia and insulin resistance. Chapter 2 also discusses the
effect of BW loss on BMD and emerging research on the negative aspects of excess FM on BMD. Finally, Chapter 2 discusses the health benefits of cocoa products and the potential benefits of incorporating them into an ERD as a snack. Chapter 3 presents a pilot study that examines the feasibility of reducing energy intake, losing BW and improving body composition while consuming two different sweet snacks daily. Chapter 4 explores the effects of incorporating either two DC or NC snacks into an ERD on BW, anthropometric and BP measurements, glucose and insulin concentrations and lipid levels. Chapter 5 examines the effect of consuming additional flavanols in the form of two DC snacks and a HF beverage on markers of oxidative stress and inflammation, markers of bone formation and resorption, mineral metabolism and BMD. Chapter 6 summarizes the results of the study, discusses strengths and weakness of the study design and outlines possible areas for future research. Appendix A and B contain supporting documents used in the development and implementation of this research project.

The results of this research project provide novel information on the effects of incorporating two daily DC snacks and a HF cocoa beverage into an ERD on various outcomes of health following BW loss.
REFERENCES


CHAPTER 2

LITERATURE REVIEW
**Obesity - A Public Health Concern**

Overweight and obesity have become serious public health problems in our society as two-thirds of American adults are now affected (1). Categorizations of overweight and obesity are based on body mass index (BMI) which is calculated by dividing an individual’s body weight (BW) in kilograms (kg) by height in meters (m) squared (2). The World Health Organization defines overweight as having a BMI greater than 25 and obesity as having a BMI greater than 30 kg/m². BMI is used as a clinical indicator for chronic disease risk and on the population level, a greater BMI has been associated with an increased risk of developing diabetes, heart disease and hypertension (3-5). For the improvement of current health status and the reduction of future disease risk, the primary objective for individuals who are overweight or obese is to achieve and maintain BW loss (6). Losing BW has been shown to improve if not completely ameliorate hyperglycemia, hypertension and hyperlipidemia, all of which are major risk factors in the development of obesity-related chronic conditions (7).

**Dietary Weight-Loss Interventions**

A variety of dietary interventions are available which have been developed to help individuals lose BW and improve health. The United States Department of Agriculture’s (USDA) Dietary Guidelines for Americans (DGAs) which are the current national guidelines advise that healthy BW loss begins with restricting energy intake and reducing portion sizes (8). The USDA DGAs also state that there is not one optimal macronutrient composition that has to be consumed for healthy BW loss or weight maintenance. Alternatively, the USDA recommends the Institutes of Medicine’s established Acceptable Macronutrient Distribution Ranges (AMDR) which are based on reducing chronic disease risk and consuming adequate essential nutrients (9). The AMDRs for American adults over the age of 19 years are 45–65% of total energy from carbohydrates, 20–35%
of energy from fat and 10–35% of energy from protein (8). The overarching message of the DGAs is that successful BW loss can be achieved by reducing energy intake (10) in the context of a diet high in whole grains, fruits, vegetables, lean protein and low-fat dairy products.

While the national recommendations are based on strong scientific research, alternative BW loss plans have evolved. Popular fad diets are widely available and often provide advice that has little clinical evidence of safety, efficacy or nutrient adequacy. Because many popular diets advocate for certain macronutrient combinations, these plans are usually referred to by their specific macronutrient targets (i.e., low-carbohydrate (LC), low-fat (LF) or high-protein (HP) diets) (11-14). Those who promote the diets encourage the consumption or restriction of certain macronutrients for greater BW loss and improvements in health status. Adding to the attractiveness of these diets is their promise of substantial BW loss within a rather short period of time (15).

With the pervasiveness of these fad diets in the media, it is important for nutrition professionals to be aware of the strengths and weaknesses of each diet plan. This will help identify a diet plan that works best for that individual’s health profile and eating pattern, thereby facilitating BW loss in a safe and effective manner. The first part of this review will summarize the various fad diet plans, then, it will compare the diet plan based on their ability to induce BW loss and improve chronic disease risk factors in randomized clinical trials. Finally, this review will assess the nutrient adequacy of each diet with long-term consumption.

**Low-Carbohydrate Diets**

The classic LC diet does not have a specific energy restriction component; instead its weight-loss is based on carbohydrate restriction (11). Because the Atkins diet is the most well-known LC diet, it will be the basis of this summary. The Atkins diet consists of 4 phases: induction, ongoing weight loss, pre-maintenance and lifetime maintenance. The induction phase is designed to jump-
start weight loss by switching the body from using carbohydrate as its main source of energy to using fat for energy. Individuals in this phase consume fewer than 20 g of carbohydrate per day with 12-15 grams in the form of high fiber, low-carbohydrate vegetables such as salad greens, broccoli and zucchini (11). The induction phase is a high-protein stage in which individuals are specifically instructed to get enough fats and to restrict all breads, pastas and fruit. There is no limit on fish, poultry and meat as they do not contain carbohydrates and eggs of any type are highly encouraged. Seafood and processed meats that contain carbohydrates are discouraged and cheese consumption is limited to 3-4 ounces per day as it contains a small amount of carbohydrate. Finally, oil and butter can be consumed freely during the induction phase, whereas alcohol intake is strictly prohibited.

The second phase of the Atkins diet is called “ongoing weight loss” (OWL). During the OWL phase, carbohydrate intake is slowly increased to 25 g per day. In this phase, individuals are encouraged to find their personal carbohydrate tolerance which is defined as the amount of carbohydrate they can consume without hindering further weight loss. Fluid and salt intake are closely monitored in the OWL phase as the body switches from using carbohydrate to fat for energy. Adequate water intake can prevent fatigue and nausea which are common side effects of the mild state of ketosis induced by the reduction in carbohydrate intake (16). Nuts, seeds, berries, low-carbohydrate fruits, dairy products and legumes are slowly added to diet in the OWL phase while still maintaining the limit of 25 g of total carbohydrate per day.

When individuals are within ten pounds of their goal BW, they enter the pre-maintenance phase of the Atkins diet. This phase is designed to reduce the rate of BW loss to a half a pound per week until the goal weight is achieved. Personal carbohydrate tolerance is reached by slowly increasing daily carbohydrate intake by 10 g per week. Carbohydrate tolerance is achieved by gradually incorporating higher carbohydrate fruits, starchy vegetables, legumes and whole grains.
into the diet. If weight loss stalls, individuals must return to their previous carbohydrate intake. Once individuals can identify the g of carbohydrates they can consume to maintain their BW for a month, they can move into the final stage of the Atkins diet.

The final phase of the Atkins diet is referred to as “lifetime maintenance.” Once an individual has reached a “Carbohydrate Equilibrium,” the amount of carbohydrates that can be consumed without BW change, no more dietary adjustments are necessary. The focus of the lifetime diet remains on the consumption of unrestricted amounts of protein and fat for energy as they are considered “self-limiting,” meaning they are the types of foods that are difficult to overconsume. Whole food carbohydrates such as vegetables, berries, nuts and seeds are allowed in moderation but food of any kind that triggers overconsumption should be avoided completely.

Throughout all phases of the Atkins diet, individuals are allowed to consume broths, artificial sweeteners and sugar-free gelatin snacks, and non-calorie beverages such as tea, coffee and club soda. In addition, the diet plan recommends that individuals take an iron-free multi-vitamin that contains minerals as well as an omega-3 fatty acid supplement. The Atkins program encourages the diet’s followers to consume bars and shakes designed specifically for Atkins diet which are low in sugar and high in fiber, protein, fat, vitamins and minerals. These products were designed to provide the essential nutrients that can be lacking in a LC diet plan.

**Low-Fat Diets**

Another type of eating pattern is a LF diet. The Ornish diet is a commonly followed LF diet designed by a cardiologist, Dr. Dean Ornish, to help his patients reverse heart disease by clearing up coronary artery blockages while reducing BW (12). Though energy intake is not restricted, the diet plan places importance on the type of energy consumed. The Ornish diet is strictly vegetarian, making it high in fiber and very low in fat making it difficult for many people to follow. Because
the Ornish diet is also considered a total lifestyle approach, it recommends 30 minutes of exercise per day or 1 hour of exercise at least 3 days per week and stress management techniques such as yoga or meditation.

In the Ornish diet, fat intake is restricted to no more than 10% of energy. All meat, fish, poultry, oils, nuts, seeds, olives and avocado, sugar and sugar derivatives, full-fat dairy, and alcohol are discouraged and should be consumed sparingly. Non-fat dairy products and non-fat or low-fat (<2 g fat/serving) commercially available dairy products can be consumed in moderation. There is no restriction on high-fiber legumes, fruits, grains, or vegetables and they can be consumed freely until an individual is satiated.

While metabolism and energy needs typically decrease with BW loss, the Ornish diet claims to combat the plateau effect. Dr. Ornish believes that due to the “all-you-can eat” and “eat whenever you are hungry” message, metabolism will not decrease. Because of the high fiber content of the Ornish diet, food is slowly digested so that an individual feels satiated for a longer period of time and does not experience rapid fluctuation in blood glucose levels (17). Further, the addition of moderate physical activity can contribute to the maintenance of metabolism (18).

High-Protein Diets

Recently, HP diets have experienced a surge in popularity. Two commonly followed versions of HP diets are the Zone (13) and South Beach diets (14). The HP diet was originally developed as a dietary approach to reduce inflammation and correct hormonal imbalances that cause individuals to experience constant hunger and remain overweight. The diet minimizes carbohydrate intake to prevent an oversecretion of insulin and the promotion of excess fat deposition (19). The diet recommends the specific macronutrient distribution of 40% of energy from carbohydrate, 30% from fat and 30% from protein. To promote satiety, a small amount of protein (approximately 3
ounces) is included at each meal. Carbohydrates such as vegetables, legumes, whole grains and most fruits are defined as “favorable” in a HP diet and these are allowed in portions double the amount of the protein serving. Rice, pasta, high-sugar fruits, bread, cereal, bagels and carrots are considered “unfavorable” carbohydrates and are consumed only in very minimal amounts to stabilize insulin concentrations. Egg whites and egg substitutes are recommended over whole eggs as are low-fat and non-fat dairy products. To lessen inflammation, intake of saturated fat and omega-6 fatty acids are discouraged and olive and canola oil, nuts and avocados are allowed in small amounts (20). To further reduce inflammation, the diet plan recommends consuming a daily omega-3 fatty acid and polyphenol supplement (21).

In a HP diet plan, individuals are instructed to eat within one hour of waking, and at least every five hours during the day to reduce dramatic drops in glucose and insulin concentrations. Diet followers are instructed to eat three meals per day, each consisting of no more than 500 kcals and two snacks each consisting of 100 kcals or less. Individuals are encouraged to exercise and also to drink eight or more cups of water per day.

**Energy-Restricted Diets**

Energy-restricted diets (ERD) focus solely on reducing energy intake without a specific macronutrient recommendation for BW loss. The original Weight Watchers diet plan is a commercial program based on an ERD available all over the country and online. According to the Weight Watchers plan, every food is assigned a point value based on the calories, grams of total fat and grams of fiber in one serving (22). An individual’s energy needs are calculated using their BW, height, gender, age and level of activity which is then translated into points. Individuals are assigned the total number of points they are allowed to consume each day to induce BW loss. Individuals are allowed to consume any type of food they want to with their points; therefore, the diet can vary in
macronutrient composition. The point system allows the flexibility to incorporate a variety of foods into the diet as long as the points are counted. In the Weight Watchers program, physical activity is encouraged and extra points are awarded when exercised is performed. Individuals who participate in Watch Watchers attend weekly meetings to monitor BW and receive social support. In addition, they have access to advice and nutrition education online.

**Behavioral Weight Loss**

The LEARN (Lifestyle, Exercise, Attitudes, Relationships, and Nutrition) Program for Weight Management is the current standard in the cognitive-behavioral treatment for BW loss (23). The LEARN program is a step-by-step lesson plan with 16 lessons that build on one another. Lessons focus on eating modification, physical activity and thinking patterns. The LEARN manual contains a food record for regular monitoring of energy intake (24). Limiting energy intake, keeping a food record, increasing physical activity and practicing BW control behaviors such as distinguishing hunger from cravings are the key factors for inducing BW loss in the LEARN program.

The LEARN manual uses the 1992 USDA Food Guide Pyramid as the foundation for its nutrition recommendations (25). The ERD is based on a LF, high-carbohydrate dietary pattern with a macronutrient composition of 60%, 25% and 15% of energy coming from carbohydrate, fat and protein, respectively. Women are instructed to consume between 1200 and 1500 kcal per day and men are instructed to consume 1500 to 1800 kcal per day. The LEARN manual provides conventional food tables with the energy content of various foods and detailed instructions on how to track energy intake.

**Efficacy of Fad Diets for Weight Loss**
Before the advertisement or publication of many of these diet plans, there was little scientific evidence to substantiate their BW loss and health improvement statements. Because of this, several clinical studies have since been conducted to evaluate the safety, efficacy and feasibility of these diet plans in free-living overweight or obese individuals (15,26-33) Dietary interventions included in this review fulfill the following criteria: Medline accessible, conducted between 2000 and 2010, randomized controlled clinical trials that compared two or more weight-loss diets, followed participants for at least 6 months, included adult (18 years or older) study participants who were overweight or obese (BMI >25 kg/m²) at baseline, and had the same general clinical [BW, anthropometrics and blood pressure (BP)] and biochemical (lipids, glucose, and insulin) outcome measures. Interventions were excluded if they included women who were pregnant or patients with serious medical conditions. Clinical trials included all had the overall objective of comparing the fad diets to one another to examine their effect on BW and cardiometabolic markers in healthy individuals who are overweight or obese.

The first section of this review includes dietary interventions that compare BW loss when following a LF diet compared to a LC diet over a 6 to 12 month period. Obese adults following a LC diet had a significantly greater BW loss (7.0%) than those who followed LF diet (3.2%) after 6 months (27). Obese men and women who followed a LC diet demonstrated a significantly greater BW loss after 6 months than obese adults following a LF diet (12.9% vs. 6.7%) (29). Another study with obese men and women who had either diabetes mellitus or heart disease showed that while each diet group lost a significant amount of BW after 6 months, the LC group experienced significantly greater BW loss (32). These results are in contrast to a more recent study conducted in a group of obese men and women that demonstrated similar and significant BW loss in the LF and LC groups at 6 months (33). Further, BW loss was the same in both diet groups after 12 months (34).
Studies conducted solely in women also demonstrate greater BW loss with the LC diet over a 6 month period compared to the LF diet. A study conducted (26) with severely obese women (BMI >35 kg/m²) demonstrated that women on the LC diet lost 5.8 kg compared to those on the LF diet who lost 1.9 kg loss over a 6-month period. Parallel results were found in a separate study with moderately obese women (BMI range 29-36) in which women on the LC diet lost 8.5 kg after 6 months while the LF group lost only 3.9 kg (28).

In addition to studies comparing the classic LF and LC diets, there have been two large studies comparing multiple fad diets in a parallel-arm design. These clinical interventions include a HP diet and/or an ERD without a specific macronutrient distribution. When a LF, LC and HP diet were compared, McAuley et al. (30) found that adults following the LC and HP diets lost a similar and significantly greater amount of BW than those on the LF diet during the first 6 months of the diet. However, after 12 months, those on the LC diet had significant increases in BW while those on the LF and HP diets sustained more of their BW loss (15). In another study, Dansinger, et al. (31) compared the effects of a LF, LC, HP diet and an ERD in participants who were overweight or obese and had one additional risk factor for heart disease. After 6 months, BW loss was -3.2, -3.6, -3.4 and -3.5 kg for the LC, LF, HP diets and ERD, respectively. At 12 months, BW loss was -2.1, -3.3, -3.2 and -3.0 kg for the LC, LF, HP diets and ERD, respectively with no differences between diet groups after 6 or 12 months. The results from the preceding interventions indicate that LC, LF, HP diets or an ERD can induce BW loss as long as individuals adhere to each diet’s specific dietary restrictions. In the short-term, LC and HP diets may produce more BW loss, however, at long-term follow-up, BW losses appear to be comparable.

Efficacy of Fad Diets for the Improvement of Chronic Disease Risk Factors
This review has established that popular fad diets can be effective at inducing short-term BW loss over a 6 to 12 month period in overweight or obese but otherwise healthy adults. The next section examines the subsequent changes in BP, glucose, insulin and lipid concentrations that occur following the BW loss induced by fad diets. Foster et al. (27) found that individuals following a LF or a LC diet did not experience significant changes in BP, glucose or insulin concentrations after a 6-month weight-loss intervention. Those on the LF diet did experience greater decreases in total, low-density lipoprotein cholesterol (LDL) and high-density lipoprotein cholesterol (HDL) concentrations while those on the LC diet had greater decreases in triglyceride concentrations. Yancy et al. (29) found that after 6 months of following a LC or LF diet, both groups had similar and significant decreases in both systolic and diastolic BP. Compared to adults on the LF diet, those on the LC diet had greater decreases in serum triglyceride levels and greater increases in HDL levels while there was no difference between groups in LDL cholesterol changes. Another study by Shai et al. (32) demonstrated that women who followed the LC and the LF diet had similar decreases in BP and insulin levels with no change in fasting glucose concentrations. HDL cholesterol increased in both groups, but the increase was greater in those on the LC diet compared to the LF diet. Triglyceride concentration decreased significantly in individuals on the LC diet and LDL cholesterol levels did not change significantly within either group. Finally Tay et al. (33) showed that individuals on both the LF and LC diets had similar and significant decreases in BP, fasting glucose, and insulin concentrations following the 6-month intervention. Adults on the LC diet had a greater decrease in triglyceride concentration and increase in HDL cholesterol compared those on the LF diet. However, adults on the LF diet experienced a significant decrease in LDL cholesterol while it remained unchanged in the LC diet group. After 12 months of following the diets, individuals
following the LC diet experienced significantly greater increases in total, LDL and HDL cholesterol and reductions in triglycerides than those on the LF diet (34).

In BW loss interventions that included only women, Samaha et al. (26) found that the women on a LC diet had greater decreases in triglycerides, while BP and total, HDL, and LDL cholesterol concentrations did not change significantly for either diet group. Fasting glucose concentrations improved in both groups, though the improvement was greater in women on the LC diet because of the greater improvement in participants with diabetes. Insulin sensitivity did however, also improve more among the non-diabetic women on the LC diet compared to those on the LF diet. Finally Brehm et al. (28) found that BP, lipid levels, and fasting glucose and insulin concentrations improved for women on both the LC and LF diets over the course of the intervention, with no differences between the two diet groups at 6 months.

In the weight-loss intervention comparing LC, LF and HP diets, McAuley et al. (15,30) found that BP and fasting insulin concentrations decreased similarly in the three diet groups, while fasting glucose did not change among the groups. All diets induced a decrease in triglyceride concentration but those on the LC and HP diets experienced significantly greater reductions compared to individuals on the LF diet. LDL cholesterol significantly decreased in individuals on the LF and HP diets to the extent that the HP group had significantly lower LDL concentrations than individuals following the LC diet. In fact, 25% of participants on the LC diet had a 10% or greater increase in their LDL cholesterol concentrations after 6 months of following the diet. Between month 6 and 12, fasting triglycerides significantly increased in those on the LC diet negating the benefits originally acquired from baseline to 6 months. At month 12, HDL levels were significantly higher in individuals on the LC diet compared to those on the LF diet; triglyceride concentration was significantly lower in those following the HP diet compared to individuals on both the LF and LC.
diets; and LDL concentrations returned to baseline levels in all diet groups. There were no significant differences in BP, or fasting glucose and insulin concentrations between the diet groups at 12 months.

In the study conducted by Dansinger et al. (31), researchers found that after 6 months of following the diets, there were no significant changes in triglycerides, fasting glucose or insulin concentrations among individuals following a LC, LF, HP or ERD diet. After 6 months, those following the LC diet and ERD had reductions in systolic BP and those on the LF diet had reductions in diastolic BP. Individuals following the LF and HP diets and the ERD significantly reduced their total and LDL concentrations while those on the LC and HP diets had significant increases in HDL concentrations. At 12 months, there were no changes between or within diet groups in regards to BP, fasting glucose, or triglycerides concentrations. At 12 months, individuals following the LF diet and the ERD had a decrease in total cholesterol; those on the LF and HP diets and the ERD had a decrease in LDL and insulin concentrations and the LC, HP and ERD diet groups had an increase in HDL concentrations.

Together, the results from this section demonstrate that there is likely no significant effect of macronutrient composition on BP measurements, glucose or insulin concentrations. The benefits appear to result from weight loss alone. Conversely, there may be a differential effect of macronutrient composition on lipid concentration despite similar BW losses. LC diets may increase total and LDL cholesterol, yet induce reductions in triglyceride concentrations. LF diets appear to decrease total and LDL cholesterol but may also decrease beneficial HDL cholesterol (35). As for HP diets, the two dietary interventions in which they were included demonstrated that they have the potential to decrease both total and LDL concentrations while increasing HDL cholesterol concentrations, making them an ideal dietary pattern for improvement of the lipid profile. However,
more studies are needed to make a definitive statement about the effect of HP diets on cardiometabolic markers.

**Macronutrient Composition vs. Micronutrient Adequacy**

With BW losses and metabolic benefits being fairly similar, it is necessary to examine the nutrient adequacy of these dietary patterns before global recommendations can be made. The focus of popular fad diets (11-14) is mainly on macronutrient composition and providing quick BW loss, therefore, overall nutrient content of the diet is often disregarded. However, restricting a variety of foods or food groups (ie, carbohydrates, fat, or animal products) as recommended by such diets can cause insufficient intake of certain essential micronutrients that are found in the prohibited foods.

This section presents a research study which assessed the nutrient intake of free-living individuals following one of four popular dietary patterns (36). It is followed by a discussion of the possible areas of nutrient inadequacy for each of the included diets.

Gardner et al. (36) performed a dietary analysis of macronutrient and micronutrient intake in free-living overweight or obese women following 4 diet plans: the *Dr. Atkins New Diet Revolution* (11); *Eat More Weigh Less* by Dean Ornish (12); *Enter the Zone, A Dietary Roadmap* (13); and *The LEARN Program for Weight Management* (23). The length of the dietary intervention was 12 months, the final 10 months of which participants were on their own to follow the diets. For the first 8 weeks of the intervention, individuals in each diet group attended weekly classes, for which they were assigned to read approximately one-eighth of their respective books. After the 8 weekly classes were completed, participants continued to follow their assigned diet independently for the subsequent 10 months of the intervention. Baseline and week 8 dietary data were collected using 3 unannounced 24-hour dietary recalls via telephone which included 2 weekdays and 1 weekend day.
Multi-vitamin and mineral supplement use was negligible among women in the intervention; therefore, supplement use was not included in the nutrient analysis.

Approximately 70 women within each diet group completed the first 8 weeks of the intervention. Each group decreased their energy intake by approximately 500 kcals per day, with no difference in energy intake between groups. After 8 weeks, the percentage of energy by group was 17% carbohydrate, 28% protein and 55% fat for the Atkins group; 42% carbohydrate, 24% protein and 35% fat for the Zone group; 49% carbohydrate, 20% protein and 30% fat for the LEARN group and 63% carbohydrate, 17% protein and 20% fat for the Ornish group. Total dietary fiber and soluble fiber intake were lowest in the Atkins group and highest for Ornish group. Saturated fat intake was significantly highest in the Atkins diet and lowest in the Ornish diet while the intake pattern of mono- and polyunsaturated fats was also highest and lowest in the Atkins and Ornish groups, respectively.

When micronutrients were assessed, the Zone diet had the highest intake of vitamin A, niacin, vitamin B-6, vitamin C, vitamin E and vitamin K. The Zone and Atkins groups had significantly higher selenium and zinc intakes than the Ornish and LEARN groups. The Atkins diet group had significantly lower thiamine, folic acid, vitamin C and magnesium intakes than the other 3 diet groups and the Ornish diet group had the lowest selenium intake.

Researchers also examined the adequacy and inadequacy of several micronutrients to assess the risk of becoming deficient in certain nutrients if the dietary pattern was followed for an extended period of time. After 8 weeks of following the diet, they found that the Atkins group had intakes of thiamine, folic acid, vitamin C, iron and magnesium that were at risk for inadequacy. In addition, researchers found that the LEARN group had inadequate intakes of vitamin E, thiamine, calcium and magnesium; the Ornish group had inadequate intakes of vitamins E and B-12 and zinc. In contrast,
the Zone group had a decrease in the risk of inadequacies for vitamins A, E, K and C with no increase in risk of inadequacy for any micronutrient. The Atkins diet group also experienced a decrease in the risk of inadequacy for vitamin K. Neither the LEARN nor the Ornish diet groups had improvements in the intake of any vitamins or minerals.

After looking at the macronutrient intake and the dietary restrictions posed by each of the diet plans, many of these nutrient issues are not unexpected. A classic LC, Atkins-style dietary pattern has little consumption of dairy products; therefore, it is not unusual that the diet lacks calcium which is important for bone health. In addition, grains and fruit are restricted; therefore, it would be expected that the diet would be low in fiber, potassium, vitamin C and certain B vitamins such as thiamin and folate. With no restriction on high-fat meat or solid fat intake, the Atkins diet could potentially be high in saturated fat which can lead to an increased risk of cardiovascular disease (CVD) and myocardial infarction (37). Conversely, the Atkins diet can provide ample protein and unsaturated fatty acids which may be deficient in a LF dietary pattern. In addition, due to the high protein intake, the diet can be rich in nutrients found in animal products such as iron, vitamin B12 and zinc. Also, because green, leafy vegetables are encouraged in the Atkins diet plan, their consumption likely accounts for the adequate intake of vitamin K.

On the other end of the macronutrient spectrum is the Ornish diet. This diet plan is high in fruits, vegetables, legumes and whole grains which should provide adequate fiber, potassium, B vitamins and vitamin C. The Ornish diet is a vegetarian diet that recommends the avoidance of red meat, fish and poultry which can lead to an intake lacking in vitamin B12, zinc and iron. However, because low and non-fat dairy products such as cheese, milk and yogurt are allowed within the diet, adequate protein, vitamin B12, calcium and vitamin D can be obtained (38). The Ornish diet is also highly restrictive regarding fats. The diet simply restricts all fats and does not distinguish between
the heart-healthy monounsaturated and polyunsaturated fats and the less healthy saturated and \textit{trans} fats (39). Individuals who are vegetarian tend to demonstrate lower blood concentrations of polyunsaturated fatty acids (40) which puts them at greater risk for heart disease (41). In addition, with the restriction of fats and oils, there is the possibility of not being able to fully absorb the fat-soluble vitamins A, K and E (42).

The Zone diet is also high in fruits and vegetables; therefore, it is more likely to provide adequate fiber and essential vitamins and minerals such as vitamins A, E and C, potassium and phosphorus. In addition, the diet plan allows unrefined carbohydrate such as whole grain breads, pastas and rice which are high in B vitamins such as thiamin, riboflavin, niacin and folate and minerals such as magnesium and selenium. The Zone diet is also high in protein-containing foods so it has the potential to provide adequate iron, zinc and magnesium. While it limits overall fat intake, the Zone diet stresses the importance of healthier fat types. Moderate consumption of mono- and polyunsaturated fats is encouraged in the form of nuts, seeds, avocados and olives which can also provide ample vitamin E to the diet.

Interestingly, the LEARN diet plan, though based on the USDA dietary guidelines lacked vitamin E, thiamine, calcium and magnesium. On the other hand, the LEARN plan was based on the 1992 guidelines which recommended reducing overall fat intake to less than 30\% of energy without guidance on the type of fats to consume (25). This version of the guidelines considered nuts and seeds a high-fat protein source rather than a source of heart healthy monounsaturated and polyunsaturated fats; therefore, this may have contributed to the low vitamin E intake. The 1992 Food Guide Pyramid also recommended only 2-3 servings from both the dairy group and the meat, beans and nuts groups which may account for inadequate intake of calcium, thiamin and magnesium. In addition, the 1992 guidelines recommended that the majority of energy intake should come from
the breads, cereals and rice group giving it the potential to be high in thiamin, niacin, riboflavin and folic acid. However, the types of grain products to consume were not specified. Whole-grain breads, brown rice and oats tend to contain more fiber, magnesium and thiamin than their refined counterparts. Therefore, an update of the LEARN dietary recommendations may improve nutrient intakes.

**Overall Summary**

After examining BW loss and cardiometabolic outcomes from the fad diet plans, it appears that a LC diet may produce the greatest BW loss in the short-term (<6 months). However, the metabolic changes following a LC diet are similar and specifically in reference to lipid concentrations, may have negative consequences. After following a LC diet for 6 to 12 months, multiple studies showed an increase in total and/or LDL cholesterol whereas the LF and HP diets decreased these concentrations with similar or significantly less BW lost. Conversely, LC diets may have a greater potential to decrease triglycerides while LF diets may decrease cardioprotective HDL cholesterol concentrations following BW loss. Therefore, certain populations such as those with diabetes or hypertriglyceridemia may benefit from reducing carbohydrate intake and should therefore, consider following a LC diet for BW loss. Individuals with an overall high lipid profile and those at risk for heart disease should follow a LF or HP diet for BW loss to beneficially improve negative lipid parameters. When nutrient adequacy is examined, all of the diet plans have the potential to lack certain essential nutrients depending on the type of foods they restrict. To avoid any possible nutrient inadequacies with long-term consumption, the nutritional shortcomings of each diet plan should be considered when planning food intake to determine the need for vitamin or mineral supplementation.
Overall, the loss of BW is the strongest predictor of improvements in BP, lipid, glucose and insulin concentrations. These studies and others (43) show that ERDs of varying macronutrient compositions can be used to precipitate BW loss and metabolic benefits. Nutrition professionals should stay knowledgeable about the types of diets on the market so they can help individuals choose the one that works best for them. Choosing a diet on an individualized basis may improve adherence to the energy restriction and facilitate BW loss. The focus of a diet should not be macronutrient content but instead on energy restriction, nutrient adequacy to ensure long-term health benefits and reduce risk of future complications.

**Benefits of Physical Activity on Body Weight and Chronic Disease Risk Factors**

In addition to monitoring energy and nutrient intake, physical activity also can be an important component in a healthy lifestyle by helping maintain BW, improving body composition and reducing chronic disease risk factors. Higher levels of physical activity have been associated with lower BW and BMI in both children and adults (44,45). Physical activity contributes to a healthy BW by increasing energy expenditure and decreasing appetite and feelings of hunger, in some individuals (46). In addition, individuals who engage in regular physical activity are often better able to control appetite and regulate energy intake over the long-term (47). Physical activity is also related to better body composition and metabolic profile. Incorporating physical activity has been show to significantly reduce fat mass (FM) and improve waist circumference measurements (48). Time spent in physical activity is also associated with lower BP, serum glucose concentrations and greater levels of healthy HDL concentrations (45). Older adults who engage in long-term exercise training tend to have lower markers of inflammation (49) and oxidative stress (50). Finally, lean mass (LM) and bone mineral density (BMD) increase proportionally with physical activity (51).

**Physical Activity in Weight Loss Interventions**
When incorporated into an ERD, physical activity can provide additional benefits for body composition and BW. In a 12-month intervention conducted with overweight or obese individuals who were sedentary at baseline, 45 minutes of physical activity, 5 days a week plus dietary energy restriction resulted in significantly greater reductions in BW and body fat percentage (BF%) compared to energy restriction or exercise alone (52). Another intervention showed that there were greater decreases in BW, FM, waist and hip circumference in women who exercised for 3 days per week for 30 minutes compared to those who restricted energy alone (53). Despite losing a similar amount of BW, women who included daily aerobic exercise experienced a significant increase in their LM while those who remained sedentary experienced a significant decrease in LM (54). Similarly, a weight-loss intervention in which participants underwent a 25% energy restriction with or without exercise demonstrated that those who added exercise maintained their LM better following weight-loss than non-exercisers (55). Lastly, women who combined resistance training with energy restriction had greater reductions in FM and BF% compared to those women who were not physically active (56). Together, these studies illustrate the importance of exercise alone and in combination with energy restriction on maintaining a healthy BW and body composition and improving chronic disease profile.

**Skeletal Health**

**Body Weight and Bone Health**

While obesity and excess adiposity clearly have a negative effect on cardiovascular health and related risk factors, BW is considered to be protective for skeletal health (57). In individuals of all ages, genders and ethnicities, a higher BW is strongly associated with greater BMD (58-61). In clinical interventions, purposeful energy restriction and BW loss have been shown to reduce BMD (58,62,63). In longitudinal studies which included women of various ethnicities, researchers found
that those who entered their menopausal years with the lowest BWs experienced a more rapid loss of BMD at the hip and spine (59,61). Low BMD is a health concern because it increases an individual’s risk for fracture and the development of another chronic disease, osteoporosis (64). Of the 10 million individuals affected with osteoporosis, 80% are women making it a high priority women’s health issue. In the US alone, there are an estimated three million fractures per year attributed to osteoporosis, resulting in 25 billion dollars spent on the treatment of this disease (64). Many lifestyle factors can be modified to prevent negative changes in BMD including dietary intake, physical activity (54), body composition (65), and inflammatory status (66), even following BW loss (67,68).

**Bone Remodeling**

To have observable effects on BMD, changes begin at the cellular level with the bone remodeling process. Bone remodeling is a continuous process occurring throughout the lifespan which contributes to growth and development (69). Bone remodeling also has an integral role in maintaining total body mineral homeostasis of calcium and phosphorus (70). Bone remodeling is a complex balance between bone breakdown and bone formation (69) orchestrated by two cells that have opposing effects on bone. Osteoclasts secrete enzymes to break down old bone and osteoblasts secrete collagen, non-collagenous proteins and hydroxyapatite crystals necessary to create new bone. The process of bone remodeling is initiated by osteoclast precursor cells attaching to the bone surface where they differentiate into mature osteoclasts. Osteoclasts have a ruffled border that is used to form a tight seal against the surface of the bone creating a resorption lacuna (71). The activated osteoclasts then begin to secrete acid hydrolases that breakdown the inorganic bone matrix into calcium and other minerals (72). Once bone erosion is complete, osteoclasts undergo apoptosis (73).
The remodeling process then continues with osteoblast maturation and bone formation. Multiple proteins and growth factors are released from the resorption pit which leads to the maturation of preosteoblast cells and stimulation of bone formation (74). Calcium (Ca++) from the extracellular environment enters through Ca+ channels on the surface of the mature osteoblast where it is combined with inorganic phosphorus. The osteoblasts then create a membrane-bound vesicle that contains proteins, acidic phospholipids and a nucleation core on which hydroxyapatite crystals can form from the newly created calcium phosphate clusters (75). When bone formation is stimulated, osteoblasts settle into the lacuna pit that was formerly occupied by osteoclasts and secrete collagen type I and the vesicles containing the hydroxyapatite crystals (76). The vesicles are deposited between cavities in the collagen fibrils on the surface of the bone with the help of non-collagenous proteins which are also secreted by the osteoblast (74). After this point, the process of mineralization within the collagen matrix is not well-defined. It is purported that other minerals are incorporated into the collagen matrix with the help of non-collagenous proteins and other growth factors leading the mineralization of new bone (77). After bone formation is complete, the majority of osteoblasts undergo apoptosis or turn into osteocytes which are cells that line the bone surface (69). The bone remodeling process takes approximately 4 to 6 months to complete and its overall purpose is to maintain bone strength by replacing older, damaged bone.

**Triad of Bone Formation**

There are many growth factors and proteins that regulate the bone remodeling process; however, this review will focus on the signaling pathway that consists of the proteins: osteoprotegerin (OPG), receptor activator of nuclear factor-κB (RANK), and receptor activator of nuclear factor-κB ligand (RANKL) (78). RANK is found on the surface of osteoclast precursor cells and acts as the receptor for RANKL while OPG is decoy receptor for RANKL secreted by the
osteoblasts. The bone remodeling process is initiated by the stimulation of RANKL secretion and the binding of RANKL to RANK on immature osteoclast cells. This binding leads to osteoclast maturation and the activation of bone resorption (79). When osteoclastogenesis on a certain part of the bone surface nears completion, OPG is secreted by nearby osteoblasts or cells of osteoblastic lineage in response to hormones, cytokines or other growth factors (80,81). OPG acts as a decoy receptor for RANKL to prevent its binding to and activation of RANK. The deactivation of RANK signals for osteoclastogenesis to stop and formation to begin. Through infancy, adolescence and young adulthood, bone resorption and formation remain in balance so that no net bone loss occurs. However, if the remodeling process becomes uncoupled with bone resorption exceeding bone formation, bone loss occurs (82). Uncoupling can occur due to a reduction in estrogen as seen during menopause or BW loss (83), other hormonal fluctuations, (84), poor dietary intake (85) or the presence of inflammation (86).

Weight Loss and Bone Mineral Density

Though some weight-loss interventions have documented negative changes in BMD, results are equivocal. Studies that demonstrated the most significant decreases in BMD were conducted with postmenopausal women (58, 87,88), a population that lacks the bone-protective effects of estrogen (61,89) and who likely use their excess FM as a surrogate source of estrogen (90). Additional studies that showed significant BMD losses were conducted in individuals who drastically reduced their energy consumption and subsequent calcium and vitamin D intake (87) or in bariatric surgery patients who lost a substantial amount of BW over a short period of time while also experiencing multiple micronutrient deficiencies (91).

Weight-loss interventions in postmenopausal women have shown reductions in BMD at the total body (88) as well as at the hip and spine, (58,87) two high risk fracture areas. However, upon
assessment of the nutrient content of the ERD in those who lost BMD (88), it was revealed that calcium and vitamin D intakes were below the 1997 Institution of Medicine recommendations (92). Further, weight-loss interventions in which postmenopausal women received calcium supplementation in addition to their regular dietary intake demonstrated an amelioration of BMD losses (58,87).

Studies conducted in gastric bypass patients who lost 25-60% of their BW over a 12-month period also demonstrated significant reductions in BMD of the hip (83) and spine (91). The authors speculate that the change in BMD is attributed to a reduction in skeletal loading (59), low intake of calcium and vitamin D and probable nutrient malabsorption (93). When patients are given calcium and vitamin D supplements post-operatively, bone loss is lessened though not completely ameliorated (94). Researchers pose the argument that BMD decreases demonstrated by obese individuals following dramatic weight loss are the physiological return to a BMD state appropriate for the new BW (63). This homeostatic process is not necessarily detrimental except in cases of extreme malabsorption and malnutrition.

Conversely, weight-loss interventions in premenopausal women typically result in little if any change in BMD (67,68,95,96,97). In studies where BMD was reduced, it can be attributed to decreases in calcium intake (98), or the BMD change is considered clinically insignificant (99). Similar to the interventions conducted in postmenopausal women, studies in which premenopausal women were supplemented with calcium and vitamin D demonstrate that BMD typically remains unchanged while in some studies, it increased (67,68). Cumulatively, these interventions suggest that if nutrient intake is adequate during BW loss, BMD loss can be partially attenuated or prevented.

Physical Activity and Bone Mineral Density
Physical activity also affects BMD. Physical activity increases whole body blood flow providing bone with adequate nutrients, growth hormones and oxygen for proper growth (100). The dynamic nature of movement provides intermittent mechanical loading on involved bones which increases bone strength and BMD. Weight-bearing exercises such as walking or running in particular are beneficial to bone (101), whereas static loading has little effect on bone cell proliferation (102). Children and adolescents who are physically active tend to have greater bone mass than those who are sedentary (103,104). Further, the benefits of exercise persist into adulthood as those adults who were very physically active in their young adulthood have greater BMD at the whole body, spine and hip (105). In older individuals, physical activity has been shown to protect against age-related bone loss (106) and reduce the risk of fracture (107).

Because of the beneficial effects of physical activity on bone mass, researchers have tested its effectiveness in maintaining BMD during a weight-loss intervention. Because many weight-loss interventions are often too short in duration to detect significant changes in BMD, researchers examine changes in serum measures of bone turnover as surrogate markers for current bone status. In a 6-week study in which overweight men and women underwent energy restriction with or without the inclusion of weight-bearing exercise, those who added walking or light jogging 5 days per week had an increase in the markers of bone formation, osteocalcin (OC) and bone alkaline phosphatase (BAP) with no change in markers of bone resorption (108). In a study conducted in premenopausal women that lasted for 3 months, women who engaged in aerobic exercise had significant increases in BMD of the hip and spine compared to the weight-loss only group who experienced significant reductions in BMD at both sites (54). In postmenopausal women, the group that incorporated 45-60 minutes of walking on 3 days of the week during a 6-month weight-loss intervention had significant increases in BMD of the hip compared to the weight-loss only group.
who had a reduction in hip BMD (109). The addition of gymnasium-based resistance training maintained BMD of the hip and spine in postmenopausal women after a 12-month compared to women in the weight-loss only group who experienced significant decreases in BMD at these sites (110). Another intervention in postmenopausal women showed that those who included treadmill exercise 3 times per week for 6 months maintained hip and spine BMD following weight-loss compared to the weight-loss only group who had a reduction in BMD of the hip (111).

In contrast to these results, there are studies that found no additional benefit of incorporating exercise into a weight-loss intervention. When investigators examined the effect of either weight-bearing (jogging) or non-weight bearing exercise (cycling) versus diet alone on markers of bone formation and resorption over a 6-week period in premenopausal women, they found that OC and C-terminal telopeptide of type I collagen (CTX) increased similarly within all 3 weight loss groups after weight loss (112). A weight-loss intervention that tested weight-bearing or non-weight bearing exercise versus diet alone found no greater benefit of either exercise on bone turnover markers or BMD after 12 weeks (113). And in a cohort of premenopausal Japanese women, the addition of three 90 minute weight training sessions per week for 14-weeks provided no additional benefit on BMD of the spine or forearm compared to group who did not include resistance training during the weight-loss intervention (56). While incorporating exercise into a weight-loss intervention for a short period of time (~6 weeks) may not provide a bone benefits, it appears that engaging in exercise for period of 6 months or more can be protective against BMD loss during energy restriction. Therefore, maintaining nutrient adequacy of the ERD and incorporating physical activity should be recommended for individuals who are undergoing weight-loss to protect bone strength and integrity.

**Inflammatory Regulation of Bone Remodeling**
Beside weight loss and lifestyle factors such as dietary intake and physical activity, stress and inflammatory status also have the potential to influence bone remodeling through regulation of the OPG and RANKL pathway (78). Oxidative stress produces reactive oxygen species (ROS). ROS increase the expression and secretion of RANKL (114) stimulating osteoclast differentiation and the activation of bone resorption (115). In addition, ROS can inhibit osteoblast differentiation thus concurrently decreasing bone formation (116). The pro-inflammatory cytokines interleukin (IL)-1β (IL-1β), IL-7 (117) and tumor-necrosis factor-α (TNF-α) have also been shown increase bone resorption through multiple pathways (118). IL-7 stimulates the induction of RANKL and increases secretion of TNF-α (119). Both TNF-α and IL-1β stimulate osteoclast differentiation and upregulate osteoclastic activity. These pro-inflammatory cytokines can also decrease the action of OPG thus protecting osteoclast survival. Conversely, interferon-γ reduces the expression of RANKL, which works to increase secretion of OPG and suppress osteoclastogenesis (117). Together, oxidative stress and inflammation can stimulate bone resorption while inhibiting bone formation leading to an uncoupling of the bone remodeling process in favor of bone breakdown. Over time, chronic stimulation of bone resorption by oxidative stress and inflammation could lead to bone loss and increased risk of fracture.

**Relationship between Inflammation and Osteoporosis**

Researchers have recently found evidence to link bone loss and reduced BMD to oxidative stress and inflammation. In a population-based study of men and women, researchers observed that the oxidative stress marker, 8-epi-Prostaglandin F_{2α} (8-epi-PGF_{2α}), was negatively associated with total body BMD (120). Researchers also established a negative association between BMD at the spine and total hip and the oxidative stress marker, 8-hydroxy-20-deoxyguanosine, in a cross-sectional study of postmenopausal women. In this same study, women with higher 8-hydroxy-20-
deoxyguanosine levels had a higher concentration of bone resorption markers and were more likely to have osteoporosis (114).

Pro-inflammatory cytokines have also been linked to poor bone mass. Crohn’s disease, arthritis, periodontitis, metabolic syndrome, diabetes and heart disease are inflammatory diseases that are characterized by increased circulating concentrations of pro-inflammatory cytokines (121-124). Individuals who have these conditions also tend to demonstrate lower BMD and have a higher risk of fracture and an increased incidence of osteoporosis (123,125). In a cohort of healthy women over age 30, those with highest levels of C-reactive protein (CRP) had lower total alkaline phosphatase concentrations, a marker of bone formation (126). This result is in agreement with other studies in which women in the highest tertiles of CRP had the lowest levels of serum osteocalcin, a marker of bone formation (127) and the highest levels of N-telopeptide of bone type I collagen (NTx), a marker of bone resorption (128). Greater CRP concentrations have also been linked to lower BMD in elderly women (129) and an increase incidence of fracture (127,130). Finally, premenopausal and postmenopausal women with the highest CRP concentrations had an increase in osteoporosis risk of 35% and 54%, respectively (126). These results consistently suggest a negative association between higher levels of oxidative stress and inflammation and bone health even in healthy individuals.

**Obesity as an Inflammatory Disease**

Interestingly, persistently high levels of oxidative stress and circulating inflammatory proteins are not exclusive to disease states. Once thought to be inert, adipose tissue is now recognized as a metabolically active endocrine organ that secretes multiple hormones, proteins and cytokines (131). Specifically, adipose tissue secretes the pro-inflammatory cytokines, TNF-α, IL-1, IL-6, and CRP (132,133). As adipose tissue expands to accommodate excess energy storage, as in obesity, there is an increase in secretion of cytokines and a release of ROS. As of result, obese
individuals exhibit greater oxidative stress (134) and markers of inflammation (131,135). This hypoxic biological environment (135) has been purported as a causative factor in the development of many co-morbidities associated with obesity including hypertension, hyperlipidemia and cancer (136,137). Because of its putative role in the development of multiple inflammatory-related conditions, researchers have begun examining the potentially detrimental effect of obesity-induced inflammation on bone health.

**Fat Mass and BMD**

BW exerts a strong protective effect on bone for multiple reasons (138) and moderate BW loss may induce a slight reduction in BMD. However, new evidence indicates that there is a relationship between carrying extra BW as excess FM and having a low BMD. A study comparing women of a healthy BMI to women who were obese found those who were obese had lower serum markers of bone formation in conjunction with higher serum and urinary markers of bone resorption (139). This indicates an uncoupling of the bone remodeling process with a shift towards bone breakdown which if persists can lead to bone loss (140). In another population study conducted by Zhao et al. (141), bone mass was negatively related to FM percentage even after controlling for the effects of BW. The consistent negative effect of FM is also evident in adolescent females and children as young as 10 years old. In adolescent females, body fat percentage was inversely related to radial cortical bone mineral content and the strength-strain index, a measure of bone strength (142). Rocher et al. (143), found that obese children had lower total body BMD than age-matched children of a normal BW. In addition, multiple researchers have linked greater abdominal adiposity in premenopausal women to a lower BMD (144,145). To that end, in a cross-sectional study of pre- and postmenopausal women, researchers found that individuals with a higher FM percentage had
lower bone mineral content and had a greater incidence of fracture, osteopenia and osteoporosis compared to women with lower percentage FM (146).

The relationship between excess FM and inflammation and inflammatory stimulation of bone resorption has prompted researchers to explore the relationship between obesity-induced inflammation and BMD. A study by Jeon et al. (124) found that postmenopausal women with metabolic syndrome had reduced BMD at the lumbar spine and femur neck. In these women they also found that CRP levels predicted lower BMD at the femur neck (124). In this study, researchers were only able to establish a relationship between greater waist circumference, not obesity, and a lower BMD. However, in another study, researchers found that visceral adiposity was negatively related to total body and lumbar spine BMD in obese, adolescent girls. Through statistical means, researchers postulated that this relationship was likely mediated through the adipokines, E-selecting and adiponectin (147). In sum, these studies challenge the protective effect of FM on bone health. In fact, they point to a potential detrimental effect of excess adiposity, particularly abdominal adiposity, on BMD due to an increase in circulating pro-inflammatory cytokine concentrations. Excess adiposity may be a problem particularly for individuals who become obese at a young age as they will experience chronic inflammation for a longer period of time, leading to a constant stimulation of bone resorption and possible bone loss.

Dietary Components and Inflammation

There are many lifestyle factors that can also affect circulating pro-inflammatory concentrations. Components of an individual’s diet can both positively and negatively affect their inflammatory status. Regular consumption of plant foods such as fruits (148), vegetables (149,150), whole grains (151) and nuts (152) have been associated with reduced levels of inflammation. In addition, diets rich in deeply colored foods and drinks such as grapes, wine (153) and cocoa products
(154) have also been linked to lower circulating concentrations of inflammatory proteins while a diet high in *trans* fat is associated with higher pro-inflammatory concentrations (155). The common denominator in these foods is their high antioxidant content which has been purported as the reason for their anti-inflammatory effects (156).

**Weight Loss reduces Inflammation**

In addition to anti-inflammatory components of the diet, BW and FM loss can also help reduce oxidative stress (157) and circulating inflammatory proteins (34,131,158-160). 8-epi-PGF$_{2\alpha}$, a marker of oxidative stress, was significantly reduced in men following a BW loss of 14% (157). In a weight-loss intervention conducted in overweight yet otherwise healthy, young women, CRP concentrations were reduced by 43% following a BW loss of only 3.5% (158). Individuals with abdominal obesity and one attribute of metabolic syndrome reduced their CRP concentrations by 50% after losing 12.5 kg of BW, 10 kg of which was FM (34). Obese, postmenopausal women who lost 15.6% of their BW and 25% of their FM had a 32% decrease in CRP concentrations (161).

Researchers have found that BW loss can reduce other inflammatory markers as well. After losing 3 kg of adipose tissue, women who were obese had a decrease of 17% in their IL-6 concentrations (131). With a loss of 5.7 kg of BW and 4.5% of body fat, breast cancer survivors had a significant 9% decrease in TNF-$\alpha$ concentrations (160). The reduction in inflammatory markers becomes greater as the magnitude of BW loss increases. A 26 kg weight loss in severely obese individuals following bariatric surgery precipitated a 23% decrease in IL-6 concentrations (159). These same individuals also experienced a significant increase in the expression of the anti-inflammatory protein adiponectin (159). These studies demonstrate that BW loss can decrease pro-inflammatory protein concentrations while simultaneously increasing the anti-inflammatory protein,
adiponectin. Therefore, consumption of a diet containing foods that are high in antioxidants in combination with BW loss may have an additive effect on reducing inflammatory status.

None of these interventions in which inflammatory concentrations were reduced through dietary means or BW loss reported the subsequent changes in bone markers or BMD. Therefore, to date there is a lack of causal evidence linking obesity-induced inflammation to a reduced BMD. However, it is a plausible theory that reducing circulating inflammatory proteins may decrease inflammation-induced activation of bone resorption and in turn protect BMD. To further explore the relationship between adiposity-induced inflammation and bone, research studies are needed that measure the change in pro- and anti-inflammatory proteins, bone markers and BMD before and after significant BW loss.

**Health Potential of Cocoa**

**History of Cocoa as Medicine**

Various bioactive compounds found in food are now being acknowledged for their health-promoting properties with cocoa specifically recognized for its anti-inflammatory potential. Cocoa and chocolate products have been around for centuries and are one of the most extensively studied foods of the modern age. Historical writings dating back to the early Americas document that Europeans settlers integrated cocoa powders and pastes and unsweetened chocolate into many of their medicinal remedies. In the 18th and 19th centuries, chocolate was used to cure ailments ranging from asthma to cholera. It was even included as part of a weight gain diet for patients with smallpox and yellow fever (162). While the usage of chocolate in our current society has shifted from homeopathic treatment to sweet treat. Interest in the health benefits of chocolate had seen a resurgence particularly in the area of inflammation and cardiovascular health (163,164).

**Structure and Function of Cocoa Flavanols**
Cocoa contains a variety of plant-derived phenolic compounds called flavonoids that act as antioxidants (165). Flavonoids are a family of antioxidants containing compounds of similar chemical structure and divided into several subclasses. Flavan-3-ols or flavanols are the most prevalent flavanoids found in cocoa. Flavanols consist of two aromatics rings that each contain an unsubstituted hydroxyl group at their third position (166). This particular chemical ring structure gives them the ability to scavenge free radicals (167) and act as a redox-active metal chelator (168). The most biologically active chemical species of flavanols present in cocoa products are non-esterified monomers called (-)-epicatechin and (+)-catechin (169). These flavanol monomers act as antioxidants by donating free electrons to damaging oxidative species thereby reducing them. Scavenging free radicals prevents lipid peroxidation and tissue breakdown which are responsible for the development of several chronic diseases.

The average American consumes approximately 18-50 mg of flavanols from food products each day (170). Flavanols are found in fruits, vegetables, tea and red wine (171), but cocoa products contain the highest concentration and demonstrate the greatest antioxidant capacity (172). Dark chocolate has 950 mg of phenolic compounds per 40 g serving (173), compared to 30 mg found in a 5 ounce glass of red wine. Numerous longitudinal studies have linked a greater consumption of cocoa and chocolate products to improved cardiovascular health (164,173,174,175). For example, the Kuna Indians in Panama regularly consume a regional cocoa drink containing approximately 900 mg of flavanols which has been hypothesized as the contributor factor to their lower incidences of hypertension and CVD related mortality (174). A 15-year long study that followed older men, who were free of CVD at baseline, found that those in the highest tertile of cocoa intake (~4.5 g/day) had lower systolic and diastolic BP, and half the relative risk (RR) of cardiovascular and all-cause mortality when compared to those with the lowest cocoa intake (<0.5g/day) (174). Similar results
were observed in the Iowa’s Women’s Health study. As cocoa consumption increased, the risk of
CVD mortality decreased over a 16 year period (175). A meta-analysis of multiple prospective food
intake studies showed that individuals with the highest chocolate intake had a reduced risk of
coronary heart disease mortality (RR = 0.81) compared to individuals with the lowest chocolate
intake (173).

The cardiovascular benefits and decreased mortality rate have been attributed to cocoa’s
effects on nitric oxide homeostasis, endothelial function and BP (176). To examine the reason
behind these benefits, multiple clinical interventions have been conducted to assess cocoa’s effect on
the risk factors for the development of CVD. To induce a biologically significant effect, many of the
interventions used flavanol doses that are significantly higher than usual flavanol intake. In the
following clinical studies, researchers have found that the acute and chronic supplementation with
high-flavanol (HF) cocoa beverages and chocolate products ranging in dosage amount from 250 to
1,000 mg can improve vascular reactivity and reduce BP, oxidative stress, lipid levels and glucose
and insulin concentrations.

**Cocoa’s Effect on Vascular Reactivity and Blood Pressure**

The effect of consuming cocoa flavanols has been most thoroughly studied in regards to its
effect on nitric oxide (NO) levels and BP. Flow mediated dilation (FMD), a measure of endothelial
function, can be increased, and systolic and diastolic BP can be decreased by the consumption of HF
chocolate or cocoa beverages (177-182). Reduced FMD is a sign of endothelial dysfunction and is
likely the precipitating event in the progression of hypertension, atherosclerosis and insulin
insensitivity (183). Therefore, a flavanol-induced increase in arterial blood flow can potentially
improve BP and glucose and insulin utilization.
In a cross-over study, participants who consumed either a high-fat meal that contained 918 mg of flavanols or the same high-fat meal without the additional flavanols demonstrated an improvement in their FMD for several hours following the meal that contained flavanols (152). In another study conducted in women with hyperlipidemia, participants who consumed a HF cocoa beverage daily (446 mg/d) for six weeks saw an improvement of 76% in brachial artery blood flow compared to those who consumed a low-flavanol beverage daily (43 mg/d) (154). In smokers, a group known to have reduced blood flow, researchers measured both acute and chronic ingestion of a HF cocoa drink. The acute consumption of cocoa drinks ranging in concentration from 28 to 918 mg of flavanols led to significant dose-dependent increases in FMD. Daily consumption of the HF cocoa drink (900 mg/d) over a 7-day period resulted in continual increases in FMD from baseline and sustained improvement in FMD for up to 2 hours after ingestion (181). In healthy adults, those who consumed a HF chocolate bar (260 mg flavanols/d) for 2 weeks experienced a significantly greater increase in FMD compared to the group who consumed the low-flavanol chocolate bar (46 mg flavanols/d) (184).

An increase FMD is clinically significant when its effects are evident on BP measurements. A cross-over study in elderly individuals who had been recently diagnosed with stage 1 isolated systolic hypertension, had reductions in systolic and diastolic BP of 5.1 mmHg and 1.8 mmHg, respectively during the 2 weeks in which they consumed a HF chocolate bar compared to the period in which they consumed a flvanol-free chocolate bar (185). In another cross-over study conducted in healthy soccer players, consuming a dark chocolate bar (168 mg of flavanols/d) for 14 days reduced diastolic BP by 5.0 mmHg compared to no change following the 14-day consumption of a flavanol-free white chocolate bar (168). In a cross-over study in individuals with normal BP levels, individuals demonstrated significantly lower systolic BP following the 15-day period in which they
consumed the HF dark chocolate bar (500 mg of flavanols/d) compared to the period in which they consumed a flavanol-free white chocolate bar (178). In a follow-up intervention with individuals who were hypertensive, researchers again found that FMD and diastolic and systolic BP were significantly lower after the 15-day period in which individuals consumed the HF dark chocolate compared to no change after the flavanol-free chocolate period (179). Women with pre-hypertension or stage 1 hypertension who consumed dark chocolate with 30 mg of flavanols daily showed a significant reduction in systolic and diastolic BP after 18 weeks compared to the women who consumed flavanol-free white chocolate (180). Together, these results indicate that consumption of a biological active amount of HF cocoa or chocolate can improve FMD and induce clinically significant reductions in BP levels.

Cocoa’s Effect on Lipid Concentrations

Because it is another important risk factor in the development of CVD, cocoa consumption has been examined for its effect on lipid concentrations. Unlike the FMD and BP results, cocoa’s effects on lipids have been contradictory. Soccer players who consumed 168 mg of flavanols in the form of chocolate candy reduced total and LDL cholesterol concentrations by 11% and 15%, respectively after 14 days (177). In individuals with hypertension, researchers found that total and LDL cholesterol were reduced in the 15-day period after consuming a HF dark chocolate bar with 88 mg of flavanols compared to a 15-day period of consuming flavanol-free white chocolate bar (179). Oxidation of LDL cholesterol was reduced and HDL cholesterol increased following a 4-week consumption of dark chocolate and cocoa powder (466 mg flavanols/day) in healthy subjects (186). Another study in healthy subjects showed an increase in HDL cholesterol concentration following the consumption of dark chocolate containing 275 mg flavanols for 3 weeks (187). In contrast to these results, there have been several studies that found no change in cholesterol concentrations with
regular consumption of HF chocolate in dosages ranging from 30 mg/d to 500 mg/d compared to their flavanol-free control groups (154,178,180,184). A meta-analysis of randomized clinical interventions using cocoa further substantiate these result as researchers concluded that cocoa and chocolate products do not exert a significant effect on LDL or HDL cholesterol concentrations (188).

**Cocoa’s Effect on Glucose and Insulin Homeostasis**

While there is extensive research examining the more well-established risk factors for CVD, more recent research has focused on the newly established risk factors for CVD which include insulin sensitivity, oxidative stress and inflammation. Two studies conducted by Grassi et al. (178,179) have demonstrated promising effects of cocoa consumption on glucose and insulin concentrations. The first cross-over study conducted in healthy subjects showed a significant increase in insulin sensitivity and decrease in insulin resistance after consuming a dark chocolate bar containing 500 mg of flavanols for 15 days (178). The follow-up study conducted in individuals with hypertension demonstrated similar results (179). In contrast, a study in individuals with pre-hypertension, showed no change in fasting glucose concentrations with daily consumption of a HF chocolate bar containing 30 mg of flavanols (180). HF cocoa consumption in individuals with diabetes has also resulted in no evident effect on glucose and insulin homeostasis. Older adults with type 2 diabetes had no change in fasting glucose concentrations after a 30 day ingestion of HF chocolate providing 963 mg flavanols (189). Another study conducted in diabetic adults found no change in insulin resistance or glycemic control after 8 weeks of daily HF chocolate consumption (190). With so few studies, further research is necessary before a conclusive statement can be made about the effect of cocoa consumption on glucose and insulin metabolism.

**Cocoa’s Effect on Markers of Inflammation and Oxidative Stress**
In vitro studies have demonstrated that cocoa flavanols can reduce secretion of the pro-inflammatory cytokines TNF-α, IL-6, IL-1β and IL-12 (191-193) and protect human cells against oxidative stress-induced free radical formation (194). Additionally, in a rat model of periodontitis, cocoa flavanols ameliorated oxidative stress-induced inflammation (195). Due to its ability to reduce oxidative stress and inflammation in cell culture and animal models, researchers began to explore the effect of cocoa consumption on pro-inflammatory cytokines and oxidative stress in humans. Daily consumption of a HF cocoa beverage and chocolate bar providing 650 mg flavanols for 6 weeks resulted in no change in serum IL-1β, IL-6, TNF-α or CRP levels or in urinary F2 isoprostane concentrations (196). Two additional studies found similar results. Following the consumption of dark chocolate containing 365 mg of flavanols per day for three weeks and 466 mg of flavanols per day for 4 weeks, individuals demonstrated no significant change in urinary F2 isoprostane concentrations (186,187). Results from these studies indicate that there is likely no effect of flavanol supplementation on oxidative stress and inflammation in vivo.

Additive Effect of Cocoa and Weight Loss

The multiple cardiovascular benefits of cocoa and chocolate consumption are evident even when these products are added isocalorically to usual intake. Interestingly, these effects mirror the health benefits that occur following BW loss. BW loss of as little as 10% can increase insulin sensitivity and reduce BP, LDL cholesterol concentrations and markers of inflammation (167,168). While cocoa and chocolate are consumed regularly by many people in the US (199,200), they are often avoided during BW loss. The rigidness of most ERDs do not accommodate the inclusion of cocoa and chocolate products on a daily basis. This is unfortunate as the incorporation of cocoa products into an ERD may increase the beneficial effects of BW loss on certain chronic disease risk factors. In addition, the complete restriction of one’s favorite foods can decrease adherence to an
ERD and reduce the ability to lose BW (201). Further, a sweet snack can be incorporated daily within an ERD as long as energy from the snack is controlled (202). Therefore, in a country where snacks often replace meals (203), it would be ideal to find a snack that provides health benefits as well as promotes adherence to an ERD and facilitates BW loss.

**Additional Benefits of Cocoa**

Cocoa products have additional characteristics that may be helpful when following an ERD. Chocolate products contain essential micronutrients that may be lacking during an ERD (36), may help induce satiety (204-207) and may enhance energy levels and mood (208). Chocolate contains magnesium, copper and manganese which are nutrients that are integral for the bone-building process (209) and healthy muscle function as well as glucose metabolism (210). Chocolate also provides potassium, a nutrient that has been identified as a nutrient of concern in the US population (212). Potassium is necessary for maintaining whole body calcium homeostasis by regulating urinary conservation and excretion of calcium (213). As a positive anion, potassium neutralizes acidic byproducts that are a result of protein metabolism and produces a slightly alkaline urine for waste excretion. Chronic consumption of diet that produces a high acid ash has been associated with an increase in urinary calcium excretion which over time can potentially increase bone resorption (214). Consequently, a higher intake of potassium is associated with greater BMD (214) and reduced risk of osteoporosis (215). In addition, greater potassium intake is related to lower risk of hypertension, stroke, cardiac dysfunction, renal damage, and kidney stones (216,217).

**Chocolate and Satiety**

Chocolate products have a unique fatty acid composition that may aid in inducing satiety while following an ERD. Chocolate contains cocoa butter which is rich in stearic, palmitic and oleic fatty acids (218). When these types of long-chain fatty acids are consumed, they proceed to the
intestine where they are broken down into free fatty acids and monoglycerides before entering circulation (219). Free fatty acids trigger receptors in the gut to release cholecystokinin (CCK), an appetite-suppressing hormone (204). CCK delays gastric emptying thereby increasing the feeling of stomach fullness and stimulating the termination of food intake (205-207). When the effect of chocolate consumption was tested in clinical studies, the results support its satiating effects. In a crossover study in which women consumed 50 g of chocolate, an apple or water, participants reported the greatest reduction in hunger and a greatest increase in energy after consuming the chocolate (208). The consumption of a high-fat snack of chocolate produced a greater reduction in postprandial ghrelin levels and induced higher satiety scores compared to other lower fat snacks in both men and women (220). In addition, the act of simply smelling dark chocolate is associated with a reduction in ghrelin levels and suppressed self-reported appetite (221). Therefore, consuming a snack of chocolate with its satiating fatty acid composition may induce earlier feelings of fullness thus aiding in the reduction of energy intake while following an ERD (222).

Cumulatively, cocoa products can improve the intake of some essential micronutrients that may be inadequate during an ERD (36), they can induce satiety and reduce appetite due to their fatty acid composition (204-207) and they may increase energy levels (208). These positive attributes in combination with the metabolic and inflammatory benefits make cocoa and chocolate products the ideal snacks to consume while following an ERD. Further, in combination with BW loss, cocoa products could potentially provide additional improvements in metabolic and inflammatory parameters.

Conclusions

Obesity, characterized by excess adiposity, has become an increasing health concern in our society. Excess adiposity is associated with increased oxidative stress and systemic inflammation
which are the main precursors in the development of insulin resistance, hypertension, atherosclerosis and cancer. Reduction of adiposity through BW loss can decrease markers of oxidative stress and inflammation and risk factors for chronic health conditions. However, adherence to an ERD is necessary to induce BW loss and receive the associated health benefits. Multiple clinical trials testing various fad diets have demonstrated that an overall decrease in energy intake is more predictive of BW loss than the specific macronutrient composition of the ERD. In addition, the beneficial changes in metabolic parameters such as BP, and lipid, glucose and insulin concentrations are more influenced by the amount of BW lost than dietary constituents. However, micronutrient content of the ERD may also play a part in risk reduction for other chronic diseases such as osteoporosis. An excessive reduction in energy without adequate nutrient intake can negatively impact bone integrity. Fortunately, if micronutrient intake is sufficient and physical activity is incorporated while following an ERD, BMD can be maintained or even improved.

Individuals with obesity and other chronic diseases have high levels of oxidative stress and circulating concentrations of systemic pro-inflammatory cytokines which can stimulate excess bone resorption and may lead to bone loss over time. While BW loss can decrease ROS and pro-inflammatory cytokines, other bioactive components of an ERD may work to further decrease oxidative stress and inflammation and also potentially maintain BMD. Cocoa and chocolate products contain flavanols which are potent antioxidants. Consumption of cocoa flavanols has been associated with a reduced risk of CVD morbidity and mortality through their ability to improve vascular function, FMD and BP and reduce lipid, glucose and insulin concentrations. As an antioxidant, cocoa flavanols may be able to reduce oxidative stress and inflammation. Flavanols also have the potential to benefit bone by reducing inflammation-induced bone resorption. While they are a universally consumed snack, chocolate products are usually avoided when following an
ERD. This is unfortunate as cocoa products may provide nutrients that are essential for bone and muscle health yet may be lacking in an ERD. They may also aid in inducing satiety and increasing energy levels when consumed as a snack food. In combination with BW loss, cocoa products may provide additional health benefits that are greater than each factor alone.

In conclusion, BW loss can improve long-term health. It should be accomplished by following an ERD that provides adequate nutrients and has a macronutrient composition that will promote the greatest dietary adherence and incorporating regular physical activity. Consuming a well-balanced ERD that is rich in foods with anti-inflammatory properties may further ameliorate risk factors for chronic diseases while maintaining short-term bone health.
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CHAPTER 3

A REDUCED-CALORIE DIETARY PATTERN INCLUDING A DAILY SWEET SNACK PROMOTES BODY WEIGHT REDUCTION AND BODY COMPOSITION IMPROVEMENTS IN PREMENOPAUSAL WOMEN WHO ARE OVERWEIGHT AND OBESE: A PILOT STUDY

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ABSTRACT

Reduced-calorie diets (RCD) are difficult to follow, because they often require elimination of certain foods, leading to poor compliance and limited success. However, a low-calorie nutrient-dense diet has the potential to accommodate a daily snack without exceeding energy requirements, even during weight loss. This pilot study evaluated the effects of a reduced-calorie diet including either a daily dark chocolate snack (DCS) or a non-chocolate snack (NCS) on anthropometric and body composition measurements. In a randomized clinical trial, 26 overweight and obese (body mass index ≥25 to ≤43 kg/m²) premenopausal women were assigned to a RCD that included either a daily DCS or NCS (n=13/group) for 18 weeks. At baseline and end of study, body weight (BW, kg) and waist and hip circumferences (cm) were measured, along with fat mass (FM, kg), lean mass (LM, kg), and body fat percentage (BF%) by dual-energy X-ray absorptiometry. Energy and macronutrient intakes were estimated from four-day food records. Within and between group changes from baseline were analyzed using paired t-tests and independent t-tests, respectively. Women in both snack groups reduced estimated daily energy intake (p<0.001). Women in both the DCS and NCS groups, respectively, experienced decreases (p<0.001) in BW (-5.1 vs. -5.1 kg), hip (-5.8 vs. -5.4 cm) and waist circumferences (-5.7 vs. -3.5 cm), FM (-3.9 vs. -3.6 kg) and BF% (-3.4 vs. -3.1 %), with no change in LM. Improvements in anthropometric and body composition measurements among overweight and obese premenopausal women can be achieved with a RCD including either a daily DCS or NCS.
INTRODUCTION

Based on cross-sectional observations of adults in the United States, it is estimated that one in three individuals is trying to lose weight (1). In spite of this, sixty-eight percent of American adults are overweight or obese (2) suggesting that current weight-loss interventions are only modestly effective at helping individuals achieve and sustain a healthy weight status (3). The most commonly cited cause of unsuccessful weight loss during active intervention and long-term maintenance of weight loss is poor dietary compliance (4,5). Specifically, diets that promote rapid weight loss are challenging, because they typically require dramatic alterations in usual dietary intake leading to confusion and/or an inability or unwillingness to fully comply in the short-term (6,7). Long-term maintenance of restrictive diets is tenuous, and many individuals regain weight over time (8,9) and/or enter into patterns of weight-cycling (10,11) which present additional pathways for adverse health outcomes (12,13). Nutrition interventions that emphasize healthy lifestyle choices, include flexible dietary patterns, and encourage moderate weight reduction may be more compatible with short-term weight loss, successful long-term weight loss maintenance and reduction in risk of chronic disease (5).

Individuals who consume a nutrient-dense dietary pattern (rich in high-fiber whole grains, vegetables and fruits, and non-fat fluid milk, moderate in lean proteins) can meet their daily nutrient intake needs with additional energy remaining within their daily caloric recommendations (14). Using these “extra” kilocalories (kcals) to incorporate a favorite food or snack into an energy-controlled dietary pattern could improve diet satisfaction, thereby lowering the perception of restriction, and increasing the likelihood of long-term adherence (15). Dietary compliance over a
longer period of time can lead to greater weight loss (4,16) and weight stability (17) contributing to sustained improvements in health status (5,17,18).

Incorporating snacks between meals can be appropriate for both appetite and weight control when the energy content of the snack is moderated (19). When asked about their favorite snack, approximately 40% of women in the United States responded that chocolate is their sweet treat of choice (20). Chocolate is favored because of its pleasant sensory properties and ability to temporarily elevate mood (21). The overconsumption of any calorically-dense sweet treat can potentially lead to weight gain; therefore, women often abstain from chocolate and other sweet snacks when trying to lose weight. Unfortunately, restriction of a particular food can induce cravings and may lead to an undesirable rebound effect of increased or uncontrollable consumption when the particular food is reintroduced (22,23). Because chocolate is a highly desired snack, examining whether women can include a moderate amount of chocolate into a daily reduced-calorie diet (RCD) is important. Further, understanding whether chocolate elicits unique effects when incorporated into a RCD requires comparison to a sweet non-chocolate snack.

Therefore, this randomized clinical trial aimed to test the feasibility of purposefully including two different types of energy-controlled daily sweet snacks into a RCD on selected outcomes. The objective of this pilot study was to examine whether an energy-controlled dark chocolate snack (DCS) versus a non-chocolate snack (NCS) produced differential changes in anthropometric and body composition measurements when incorporated into a RCD over 18 weeks. It was hypothesized that premenopausal women who were overweight or obese and who followed a RCD with either a DCS or NCS incorporated into daily intake could maintain an energy deficit and experience significant positive changes in anthropometric and body composition measurements, despite the type of sweet snack consumed.
METHODS

Participants and Recruitment

The research was advertised as an 18-week weight loss study using posted flyers and electronic-mail notices. One hundred thirty-seven women responded to advertisements, of which 33 were eligible and completed baseline measurements (DCS, n=17; NCS, n=16). Remaining women did not meet eligibility criteria, return required forms or maintain interest in participating in the study. This was a pilot sample of women to test feasibility for a future, larger trial.

Enrolled participants were premenopausal women, aged 25 to 45 years, who were overweight or obese (body mass index ≥25 to ≤43 kg/m²). Women were eumenorrheic, engaged in <5 hours of physical activity per week and were weight stable during the 6 months before the study. Exclusion criteria included presence of metabolic disorders or chronic diseases, such as cardiovascular, renal, liver, and bone diseases. This study was approved by the Institutional Review Board for Research Involving Human Subjects at The Pennsylvania State University. Each participant provided written informed consent before entry into the study.

Data were collected before the dietary intervention (baseline) and after 18 weeks (February-July 2009) of the RCD (week 18). Women were compensated $80 dollars at the end of the study for their participation.

Dietary Intervention

After stratification by baseline age, body mass index and physical activity, participants were randomly assigned to either the weight loss with DCS or weight loss with NCS group. Participants in both groups followed a RCD designed to induce a 2-pound weight loss per week with a
macronutrient composition of approximately 50% carbohydrate, 30% fat, and 20% protein. After baseline testing, energy intake levels were set at 1500, 1600, 1700, or 1800 kcal/day for each woman, using the Harris-Benedict equation (24).

Participants in the DCS group consumed one dark chocolate tasting square (Hershey’s® Extra Dark, 60% cacao, The Hershey Company, Hershey, PA) at two intervals each day (90 kcal/day) and one, 8-ounce sugar-free cocoa beverage (The Hershey Company) at the first meal of the day (65 kcal/day) as part of the RCD. Participants in the NCS group consumed a non-chocolate sweet snack of fruit-flavored licorice (The Hershey Company) at two intervals each day (90 kcal/day) along with one, 8-ounce sugar-free non-cocoa beverage (The Hershey Company) at the first meal of the day (65 kcal/day) as part of the RCD. Throughout the study, women refrained from consuming any cocoa or chocolate products unless part of the RCD. Snacks and beverages, as noted above, were provided to participants as part of the study protocol.

Participants were instructed on a food exchange system and portion sizes that represented exchanges from each of six exchange groups. Handouts with food choices, dietary patterns and menu plans were provided. For 18 weeks, women in both groups attended weekly nutrition education sessions that included lessons on basic nutrition knowledge, food purchasing and preparation, portion size moderation, recipe modification, and eating away from home. Compliance with assigned snack intervention was assessed using forms on which women self-recorded weekly intake of snacks and beverages and concurrent confirmation by an investigator via product counts. Compliance was defined as intake of ≥85% of weekly snacks and beverages.

Dietary Measurements

To assess change in food and nutrient intakes, participants completed 4-day food records at baseline and week 18. Participants recorded food and beverage intake on 3 weekdays and 1
weekend day in the week before data collection sessions. Food records were analyzed with the Food Processor® dietary analysis software (version 10.6.0, 2010, *esha* Research, Salem, OR) to estimate average daily intake of total energy (kcal), and carbohydrate, protein, and fat (% of total kcal).

**Physical Activity Record**

At baseline and week 18, participants completed the Stanford 7-day Physical Activity Recall Scale (25). For each day of the week before data collection sessions, participants reported the approximate number of hours they slept, worked on the computer, watched television and spent in moderate, hard, and very hard activity from which kcals/day expended were estimated. Women were asked to maintain baseline level of physical activity throughout the 18-week intervention.

**Anthropometric Measurements**

At baseline and week 18, standing height was measured with a stadiometer (Seca 700, Seca North America East, Hanover, MD) to the nearest 0.1 cm, and fasting (overnight) body weight (BW) was measured to the nearest 0.1 kg using an electronic scale (TBF-410GS, Tanita Corporation, Arlington Heights, IL). Two measurements of the waist and hip were taken to the nearest 0.1 cm using an adjustable tape measure and then averaged. Waist circumference was measured at the narrowest point of the waist approximately 1 inch above the belly button and the hips were measured at the widest part of the buttocks. Participants wore lightweight clothing and were shoeless during measurements.

**Soft Tissue Mass Measurements**

Dual-energy X-ray absorptiometry (QDR 4500A, Hologic, Inc., Bedford, MA) was used to measure body composition at baseline and week 18. Total body scans were analyzed to determine fat mass (kg), lean mass (LM; kg) and body fat percentage. Participants were scanned in a supine position using standard protocols. All scans were completed by one technician to eliminate inter-
tester variation. Quality control scans were completed daily, and the coefficients of variation for LM and fat mass were 1.02%, and 1.87%, respectively.

Statistical Analyses

Study participants were characterized using descriptive statistics (means ± SD). One-way analysis of variance was used to assess differences in all characteristics between snack groups at baseline. Statistical analyses were conducted with only those women who completed both baseline and week 18 measurements.

Changes within and between snack groups from baseline to week 18 were analyzed using paired \( t \)-tests, and independent \( t \)-tests, respectively, for anthropometric and body composition measurements, estimated dietary intake, snack and beverage compliance, and physical activity. Statistical tests were two-sided with significance set at \( p < 0.05 \). Data analyses were conducted using the Statistical Package for the Social Sciences (version 17.0, 2008, SPSS Inc., Chicago, IL).

RESULTS AND DISCUSSION

Subject Characteristics

Of the 33 women who began the study, a total of 26 women (DCS, \( n = 13 \); NCS, \( n = 13 \)), with a mean age of 36.5 ± 4.9 years, completed the 18-week intervention. There were no differences in age, body mass index, or activity level between the study-completers and dropouts or between diet groups at baseline (Table).

Snack Compliance and Educational Session Attendance

Compliance with snack and beverage intakes, respectively, was 93.2% and 93.0% for the DCS group and 94.7% and 94.9% for the NCS group. Class attendance for the DCS group was 78.0% and for the NCS group was 73.1%. Compliance and class attendance did not significantly differ between groups.
**Estimated Dietary Intake Data**

Mean estimated energy intake at baseline was 1939 kcals and 2092 kcals for the DCS and NCS group, respectively (Table). Macronutrient composition of intakes in the DCS and NCS groups, respectively, were 48.5% carbohydrate, 34.3% fat, and 15.8% protein and 52.0% carbohydrate, 31.1% fat, and 14.9% protein (not significantly different). At week 18, the DCS group significantly reduced estimated daily energy intake by 444 kcals ($p<0.01$). The NCS group significantly reduced estimated daily energy intake by 631 kcals ($p<0.01$) and significantly increased estimated dietary protein intake by 2.3% ($p<0.05$). The assigned snack and beverage mix accounted for 6.1% and 3.0% of estimated daily kcals, respectively, for a total of 9.1%, within the 5-15% range of kcals reported as available for non-essential or “extra” kcals by the 2010 Dietary Guidelines Advisory Committee (14). Estimated energy and macronutrient intake did not differ significantly between groups at baseline or week 18.

**Physical Activity**

Estimated physical activity did not significantly differ between DCS or NCS groups at baseline or week 18. Estimated energy expended during daily tasks and physical activity significantly decreased ($p<0.01$) from baseline to week 18 for all women and within each group. Change in physical activity may largely be explained by the seasonal change from the beginning of the study (summer) to week 18 (winter). This change also may partially explain the discrepancy between the estimated reduction in energy intake (~445 to ~630 kcals/day) and the magnitude of weight loss over the 18-week intervention (~5.1 kg). While the decrease in energy intake should have precipitated greater weight loss, women did not maintain energy expenditure from physical activity, potentially resulting in less weight loss than expected. This further illustrates the importance of energy restriction and exercise for weight loss and maintenance (26).
**Body Weight and Composition Changes**

After 18 weeks, mean weight loss for each snack group was 5.1 kg ($p<0.001$) (Table). For the DCS group, waist and hip circumferences decreased by 5.7 and 5.8 cm, respectively (Figure). Fat mass decreased by 3.9 kg ($p<0.001$) and body fat percentage decreased by 3.4% ($p<0.001$). For the NCS group, waist and hip circumference decreased by 3.5 and 5.4 cm, respectively. Fat mass decreased by 3.6 kg ($p<0.01$) and body fat percentage decreased by 3.1% ($p<0.01$). Neither the DCS nor NCS group experienced a change in LM (Figure). Significant differences in BW and composition measurements between groups were not found at baseline or week 18.

Results from this randomized clinical trial demonstrated that premenopausal women who were overweight or obese and who included either daily sweet snack while following a RCD were able to maintain an energy deficit, lose a significant amount of BW, and improve specific anthropometric and body composition measurements over an 18-week period. In addition, women in both intervention groups reduced BW and fat mass without negatively impacting LM which was likely the result of slow, steady BW loss (27).

The most salient finding from this study is that while women were required to consume a daily sweet snack, they did not exceed the energy requirements of the RCD. The anticipated consumption of the daily sweet snack may have alleviated some of the cravings commonly experienced during food restriction (22,23). Further, either sweet snack when included as a part of an overall RCD did not inhibit positive changes in BW and body composition. This research finding suggests that any energy-controlled sweet snack of choice could be incorporated into a RCD as long as overall energy deficit remains.

Other dietary intervention studies have explored the effectiveness of “popular” diets (4,17) on weight loss. Diets such as the Atkin’s (28), Zone (29), and Ornish diet (30) have the ability to
precipitate weight loss if followed strictly. However, such interventions differ from the current study in that the aforementioned diets have rigid macronutrient composition prescriptions which can be difficult to follow for longer periods of time particularly for women who prefer more unstructured eating patterns (31). As the length and intensity of these more complex interventions increase, a steady decline in dietary compliance is commonly observed (4,16,17,32). As demonstrated in previous studies, poor compliance prohibits additional weight loss (4,16,17,32).

Women in the current study were able to reduce energy intake by over 400 kcal per day and consume snacks without substantially altering the macronutrient composition of their pre-intervention intake, unlike other, more restrictive weight loss approaches (28-30). Drastic changes in usual intake of macronutrients were not required, potentially facilitating dietary compliance. Dietary compliance was demonstrated by the clinically significant weight loss and change in body composition experienced by the women in the current study. Therefore, this intervention technique may provide an effective weight-loss strategy for women who struggle with other more restrictive diet plans.

While this study has novel findings, limitations exist. This was a pilot study; hence, the sample size was small. Only premenopausal women were included, thus limiting generalizability to women in other stages of the lifespan and to men. The DCS was compared to only a NCS, and snacks were included as part of the RCD. Further comparison of sweet snacks as part of a daily RCD to other non-sweet snacks and/or to a RCD without snacks is warranted. Participants were not able to choose their sweet snack (i.e., randomized to group)—a choice that if given, may have led to overconsumption. Conversely, chocolate is considered the most highly craved snack in North America (33) and is recognized as the favorite food of 40% of women (20) making it an appropriate choice for this study.
CONCLUSIONS

This study tested the feasibility of purposefully incorporating a highly desirable sweet snack such as chocolate compared to a non-chocolate snack into a RCD. All women who followed this RCD with either a DCS or NCS were able to maintain an energy deficit, lose a significant amount of BW and improve body composition regardless of the type of snack consumed. When prescribing a dietary plan for weight loss, an individual’s food preferences and usual intake pattern, which also includes snacks, should be considered. Education about the importance of controlling sweet snack portion size and its contribution to overall daily energy intake is critical.
REFERENCES


Table 3.1 Characteristics of study participants at baseline and week 18 by dark chocolate and non-chocolate snack group

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Dark Chocolate Snack (DCS)</th>
<th>Non-Chocolate Snack (NCS)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline n=13</td>
<td>Week 18 n=13</td>
</tr>
<tr>
<td>Age (years)</td>
<td>36.3 ± 4.9</td>
<td>37.3 ± 4.8</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>163.3 ± 2.8</td>
<td>164.7 ± 6.9</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>84.3 ± 16.2</td>
<td>79.2 ± 17.9</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>31.5 ± 5.4</td>
<td>29.6 ± 6.0</td>
</tr>
<tr>
<td>Estimated daily energy intake (kcal/d)</td>
<td>1939 ± 334</td>
<td>1496 ± 339</td>
</tr>
<tr>
<td>Carbohydrate (% of total energy)</td>
<td>48.5 ± 8.3</td>
<td>49.2 ± 3.6</td>
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<tr>
<td>Fat (% of total energy)</td>
<td>34.3 ± 7.6</td>
<td>34.0 ± 6.4</td>
</tr>
<tr>
<td>Protein (% of total energy)</td>
<td>15.8 ± 2.2</td>
<td>15.5 ± 2.4</td>
</tr>
</tbody>
</table>

*p<0.05, **p<0.01, ***p<0.001

*p-value analyzed using paired t-tests for within snack group changes from baseline. There were no significant differences between snack group in changes in anthropometric measurements or estimated dietary intakes.
Figure 3.1 Changes in anthropometric and body composition measurements from baseline to week 18 by snack group

*p<0.05, **p<0.01, *** p<0.001; p-value analyzed using paired t-tests for within snack group changes from baseline. There were no significant differences between snack group in changes in anthropometric or body composition measurements.
CHAPTER 4

CHANGES IN BODY WEIGHT, BLOOD PRESSURE AND SELECTED METABOLIC MARKERS WITH AN ENERGY-RESTRICTED DIET INCLUDING A SWEET SNACK
ABSTRACT

Background: Body weight (BW) loss can be achieved when incorporating sweet snacks into an energy-restricted diet (ERD). The type of sweet snack included in the ERD may produce differential effects on the metabolic improvements associated with BW loss.

Objective: To determine the effects of incorporating either two daily energy-controlled dark chocolate (DC) or non-chocolate (NC) snacks into an ERD on BW loss and metabolic outcomes.

Design: Randomized clinical trial.

Participants/Setting: Sixty overweight or obese premenopausal women completed an 18-week university-based weight-loss intervention.

Intervention: Women were randomly assigned to consume a sugar-free cocoa beverage and two DC snacks (n=30) or sugar-free vanilla beverage and two NC snacks (n=30) daily while following an ERD.

Main Outcome Measures: Dietary intake was measured at baseline and week 18 and BW, anthropometrics, BP and serum glucose, insulin and lipid concentrations were measured at baseline, and weeks 6, 12 and 18.

Statistical Analysis: Data were analyzed using repeated measures analysis of variance.

Results: Using the intention-to-treat analysis, women in the DC and NC groups significantly reduced energy intake (both \( p<0.001 \)) and lost 4.4±3.3 kg (mean±SD) (\( p<0.001 \)) and 5.0±4.9 kg (\( p <0.001 \)), respectively. Both groups lowered systolic and diastolic BP, respectively [DC=2.7 (\( p<0.05 \)), 2.7 (\( p <0.01 \)); NC=3.4 (\( p<0.01 \)), 4.2 (\( p <0.01 \)) mmHg]. Glucose and insulin concentrations, respectively, decreased by 13.0 mg/dL (\( p<0.001 \)) and 1.9 µU/mL (\( p<0.01 \)) in the DC group and by 14.9 mg/dL (\( p<0.001 \)) and 1.9 µU/mL (\( p<0.01 \)) in the NC group. Total cholesterol
increased in the NC group ($p<0.05$) with no significant changes in lipid concentrations for the DC group. There were no significant differences in main outcome measures between snack groups.

**Conclusions:** Overweight or obese, but otherwise healthy, premenopausal women following an 18-week ERD with either DC or NC sweet snacks lost equivalent amounts of BW and improved BP measurements and glucose and insulin concentrations.
INTRODUCTION

Overweight and obesity have become major public health concerns as 68% of adults in the United States (U.S.) are now affected (1). Individuals with excess body weight (BW) spend 30% more for healthcare than those of normal BW due to the increased incidence of co-morbidities such as diabetes, hypertension and heart disease (2). To improve current health status and prevent future complications, the primary treatment for these individuals is reduction of excess adiposity through moderate BW loss (3). The key dietary objective for inducing BW loss is a reduction in total daily energy intake below energy needs (4). Several dietary programs for BW loss, such as the Atkins (5), Ornish (6), and Zone plans (7), focus on specific macronutrient combinations to lower BW. Clinical trials have shown that these energy-restricted diet (ERD) programs result in significant short-term weight loss (8-10); however, most are ineffective at maintaining the BW that was initially lost (11-13).

The lack of sustainability of fad diets may be due to the numerous food restrictions and strict macronutrient recommendations (14-15). Gradual relaxation in dietary adherence to such diet programs can lead to weight regain (4,16). Habitual weight-cycling can lead to a proportionally greater BW as fat mass (17) which produces a hypoxic environment in adipose tissue, leading to elevated biomarkers of inflammation (18). Chronic systemic inflammation has been linked to the development of insulin resistance, atherosclerosis and cancer (19,20). A loss of 10 to 15% of initial BW is generally accepted as the level required to maximize health benefits and minimize the potential for weight regain and its associated adverse inflammatory responses (21).

Dietary interventions should include approaches compatible with long-term healthy lifestyles, rather than the induction of temporary weight-loss (16,21,22). Tailoring nutrition advice to current eating patterns and preferred food intakes has been shown to promote initial BW loss of
10% and sustain long-term weight loss (3). Many fad diet programs restrict all high-fat and/or high-sugar snack foods. Allowing individuals to consume snacks that they normally enjoy in energy- and portion-controlled amounts as part of an ERD may make it easier to adhere to long-term reductions in energy intake because of the less dramatic alteration in food choices. Incorporating a usual snack food or treat into an ERD can easily be accomplished by purposefully allotting a limited number of daily kilocalories (kcals) from the overall energy allowance to a particular snack food or treat. In a pilot study conducted with overweight or obese women, participants incorporating either high-fat dark chocolate (DC) or high-carbohydrate non-chocolate (NC) snacks into an ERD significantly reduced initial BW by approximately 6% in 18 weeks (23). Therefore, including preferred snacks in an ERD may be more sustainable long-term without appreciable effects on BW loss, regardless of the ERD’s macronutrient composition.

Chocolate is one of the most commonly consumed sweet snacks among women in the U.S. and around the world (24-26), with approximately one-half of women reporting weekly consumption (27). In addition, U.S. women are more likely than men to consume sweet foods such as ice cream, pastries and NC candy on a regular basis (26). Complete elimination of sweet snacks from an ERD may be unnecessary for BW loss. The aim of this 18-week randomized intervention was to examine the effects of incorporating two daily DC snacks or two daily NC snacks into an ERD in premenopausal, community-living women who were overweight or obese. Outcome measures included estimated energy intake, BW, anthropometric and blood pressure (BP) measurements and serum glucose, insulin and lipid concentrations. It was hypothesized that women could significantly decrease energy intake, reduce BW and improve metabolic indicators of health while incorporating either two daily DC or two daily NC sweet snacks into an ERD.
METHODS

Participants

Participants were recruited from the central Pennsylvania area by word-of-mouth, newsletter and newspaper advertisements, electronic-mail notices and flyers posted in the local community. Two-hundred and three women provided verbal consent for an initial telephone screening and were assessed for study eligibility. Of these 203 women, 118 were excluded because they did not meet inclusion criteria, complete or return required forms or were no longer interested after hearing study details. After screening, 85 women were enrolled in the study, but due to the recruitment interval, 25 women withdrew before baseline testing was completed (Figure 1).

This dietary intervention included women aged 25 to 45 years with a body mass index (BMI; kg/m²) of ≥ 25.0 and < 43.0. Women were moderately physically active (≤ 5 hours of planned exercise per week), eumenorrheic (≥ 8 menstrual cycles/year) and BW-stable for at least 6 months prior to study participation based on self-report. Further inclusion criteria included a score of less than 50 on the Zung Self-Rating Depression Scale/Status Inventory and an absence of a chocolate intolerance, aversion or allergy. Exclusion criteria included women who currently smoked, were pregnant or attempting to become pregnant, had a hysterectomy and/or ovariectomy without hormone replacement therapy and those who used an oral contraceptive for < 2 years in duration (if used). Women who used medications, including steroid or thyroid hormones, bisphosphonates, anticonvulsants and glucocorticoids, or consumed ≥ 40 grams of chocolate per day (i.e., equivalent of one standard chocolate bar or more per day) were also excluded. All study participants underwent medical examinations by their personal healthcare providers to ensure that they were overweight but otherwise healthy.
Written informed consent was provided by all participants before entry into the study. The Institutional Review Board for Research Involving Human Subjects at The Pennsylvania State University approved the study protocol.

Study Design

This was an 18-week parallel-arm dietary intervention in which participants were enrolled in two cohorts, with the first cohort of women starting in July 2009 and the second cohort beginning in March 2010. After enrollment of each cohort, women were stratified by baseline age, BMI and physical activity and then randomly assigned to consume either two daily DC snacks (n=30) or two daily NC (n=30) sweet snacks while following an ERD.

Dietary Intervention

Participants in both snack groups followed an ERD with a macronutrient composition of 50% carbohydrate, 30% fat, and 20% protein designed to induce a 2-pound BW loss per week. Baseline energy levels were set between 1,300 and 1,800 kcal per day as determined using the Harris-Benedict equation which takes each woman’s age, body height and weight into consideration.²⁸

Women in the DC snack group consumed one, 8-ounce sugar-free natural cocoa beverage (The Hershey Company, Hershey, PA, USA) in the morning (65 kcal/day) and one DC tasting square (Hershey’s® Extra Dark, The Hershey Company) after lunch and dinner each day (90 kcals/day). Women in the NC snack group drank one, 8-ounce sugar-free cocoa-free vanilla beverage (The Hershey Company; 65 kcal/day) in the morning and consumed one NC sweet snack (fruit-flavored licorice; The Hershey Company) at the same daily intervals as the DC group (90 kcals/day). Participants in both snack groups were instructed not consume any cocoa or chocolate products throughout the 18-week intervention unless part of the snack or beverage assignment.
A registered dietitian educated participants on their ERD, which was based on a food exchange system. Women were assigned a certain number of servings from each of the exchange groups and were provided with handouts that contained food options, dietary patterns and individualized meal plans based on their energy level. Women attended weekly nutrition education classes to learn about nutrition information, dining in restaurants, food selection and preparation, and recipe modification. Trouble-shooting and motivational concerns were also discussed and addressed. Education sessions were specific to snack groups, though topics were the same for both groups. One registered dietitian led all of the education sessions for both snack groups to maintain consistency between groups. Snacks and beverage mixes were dispensed, and compliance with snack and beverage intervention was assessed by participant self-report and concurrent investigator-conducted snack counts. For completing the dietary intervention, participants received monetary compensation of $80.

Outcome Measures

Dietary intake was evaluated at baseline and week 18. Anthropometric, physical activity and BP measurements were completed and whole blood samples were collected at baseline, week 6, week 12 and week 18.

Dietary Intake Assessment

Dietary intake was estimated using 4-day food records. Women recorded all foods and beverages that they consumed on 3 weekdays and 1 weekend day in the week before measurement sessions at baseline and week 18. Handouts containing pictures of standard serving sizes of different foods and beverages were provided to aid in recording intake. Food records were evaluated using Food Processor® dietary analysis software (version 10.6.0, 2010, esha Research, Salem, OR, USA)
for estimated average daily intake of total energy (kcals); carbohydrate, fat, protein and alcohol (% of total kcal); total sugar (g), fiber (g), saturated fat (g) and cholesterol (g); and sodium (mg).

*Anthropometric Measurements*

Height (cm) was measured using a stadiometer (Seca 700, Hamburg, Germany), and BW (kg) and body fat percentage (BF%) was indirectly measured using a calibrated electronic scale (410GS, Tanita Corporation, Arlington Heights, IL, USA). Investigators calculated BMI (kg/m$^2$) from height and BW measurements for each participant. Using a spring-calibrated measuring tape (Gulik II, Country Technology, Gay Mills, WI, USA), two measurements each of the waist at the narrowest point above the belly button and hips at the widest part of the buttocks were taken to the nearest 0.1 cm and averaged. For all measurements, women were dressed in lightweight clothing without shoes.

*Physical Activity*

Physical activity was estimated using the Stanford 7-day Physical Activity Recall Scale (29). For 7 days before a measurement session, participants recorded the estimated number of hours they slept, spent in front of a television or computer screen and participated in moderate, hard and very hard activity. Total hours of moderate, hard and very hard activity were summed from the recall scale and divided by 7 to estimate hours of physical activity per day.

*Blood Pressure*

Seated systolic and diastolic BP (mmHg) was measured by a Registered Nurse using a standard sphygmomanometer (Baumanometer® Desk Model, Copiague, NY, USA). Two BP measurements were recorded with a 2- to 3-minute rest period between readings; values were averaged.
Sample Collection

Venous blood samples were obtained by a registered nurse between the hours 0700–0930 after a 12-hour fast. Samples were centrifuged at $810 \times p$ for 12 minutes after being allowed to clot for 30 minutes. Serum was pipetted into cryovials where it was stored at -80°C until completion of bioassays.

Metabolic Profile Including Serum Glucose, Insulin and Lipids

Serum glucose (Kit #1070, Stanbio Labs, Boerne, TX, USA) was measured (mg/dL) using ultra-violet (UV) spectrophotometry (version 3.0, Simple Reads Software, Varian, Santa Clara, CA, USA), and serum insulin (Catalog #IS130D, CalBiotech, Spring Valley, CA, USA) was measured ($\mu U/mL$) using enzyme-linked immunosorbent assay (GEN5 version 1.10, Epoch, BioTek, Winooski, VT, USA). Insulin resistance was estimated by homeostasis model assessment of insulin resistance (HOMA-IR) using the formula: fasting insulin concentration ($\mu U/mL$) x fasting glucose concentration (mg/dL) x 0.0555 /22.5 (30).

Serum total cholesterol (mg/dL), high-density lipoprotein (HDL)-cholesterol (mg/dL) and triglyceride (mg/dL) concentrations (Kits #1010, #0599, and #2100, respectively, Stanbio Labs) were measured using UV spectrophotometry (Varian). The equation: low-density lipoprotein (LDL)-cholesterol = total cholesterol – HDL-cholesterol – (triglyceride/5) was used to calculate LDL-cholesterol concentration (mg/dL).

All serum samples were analyzed in duplicate. Intra-assay coefficients of variation (CVs) for glucose and insulin were 7.4 and 6.0%, respectively. Intra-assay CVs for serum total cholesterol, HDL-cholesterol and triglyceride were 6.0, 5.9 and 7.9%, respectively.

Statistical Analyses
Using BW change from baseline to week 18 as the primary outcome, 21 participants per group were required to detect a treatment difference with 80% power when using \( t \)-tests and a 2-sided type I error of 5%. Using data from the 60 women who completed baseline measurements, data were first analyzed using the intention-to-treat model. The 9 women who did not complete the study (i.e., non-completers) were included in intention-to-treat analyses by replacing missing data with the last available measurement value. A secondary efficacy analysis was conducted by including only the 51 women who completed the 18-week intervention.

Data are presented as means ± SD unless otherwise indicated. Differences between the two cohorts were analyzed using independent \( t \)-tests. Differences in characteristics at baseline between snack groups and between study-completers and those who withdrew also were analyzed using independent \( t \)-tests.

Using intention-to-treat data, a 2 x 4 analysis of variance (ANOVA) with repeated measures on the time factor was performed to assess differences in anthropometrics and BP measurements and metabolic indicators between snack groups over four intervals. The interaction of snack group (Treatment) by interval (Time) was also assessed. Data were analyzed using the Statistical Package for the Social Sciences (version 17.0, 2008, SPSS Inc., Chicago, IL, USA). All tests were two-sided with significance set at \( p<0.05 \).

**RESULTS**

There were no statistically significant differences between the two cohorts of women in baseline characteristics or estimated dietary intakes, with the exception of self-reported physical activity (see below); therefore, data from both cohorts were combined and used in analyses. Sixty women, with a mean ± SD age of 35.9 ± 5.8 years and BMI of 31.0 ± 4.4 kg/m\(^2\) began the intervention (Table 1). Fifty-one of the women (85%) completed the intervention with no significant
difference between snack groups in discontinuation rate. Statistically significant differences in age, height, BW, BMI, waist and hip circumferences and physical activity between the DC (n=30) and NC (n=30) group at baseline were not detected (Table 1). One Native-American woman, two African-American and 57 Caucasian women participated in the study with no differences in race between groups. There were no statistically significant differences in these same characteristics at baseline for women randomly assigned to snack groups who completed (n=51) the study compared to those who withdrew (n=9).

**Snack Compliance and Class Attendance**

Respectively, snack and beverage compliance was 90 and 90% for the DC group and 92 and 94% for the NC group. Attendance at nutrition education classes was 74 and 75% for the DC and NC group, respectively. Neither snack and beverage compliance nor class attendance significantly differed between snack groups.

**Intention-to-Treat Analysis**

**Dietary Intake Assessment**

Fifty-nine women completed 4-day food records at baseline. Estimated total daily energy intake was 2025 and 2105 kcals for the DC and NC group, respectively (Table 2). At baseline, percent of kcals from carbohydrate, fat and protein, respectively, was 49.1, 34.7 and 15.8% for the DC group and 50.5, 33.1 and 14.9% for the NC group. Percent of kcals from alcohol was 1.3% for the DC group and 1.5% for the NC group. There were no significant differences between groups at baseline in estimated energy and macronutrient intakes.

At week 18, the DC and NC groups, respectively, reduced their average daily energy intake by 379 kcals and 437 kcals (both $p<0.001$). Percent of kcals from protein increased ($p<0.01$) in the
NC group but not in the DC group. There were no significant differences in estimated energy or macronutrient intakes between groups at week 18.

At baseline and week 18, there were no significant differences between groups in estimated intakes of total sugar, fiber, saturated fat, cholesterol and sodium (Table 2). At week 18, the DC group significantly reduced dietary intakes of total sugar, saturated fat and cholesterol and sodium, while the NC group significantly decreased total sugar, saturated fat and cholesterol intakes. There were no significant differences between groups in changes in nutrient intakes over time.

*Anthropometric Measurements*

Women in the DC group lost 5.3% ($p<0.001$) of BW, while women in the NC group lost 5.9% ($p<0.001$) of BW from baseline to week 18 (Table 3). The rate of BW change over time for women in the DC compared to NC group was not statistically significantly different (Figure 2). Women within both snack groups significantly reduced BMI, waist and hip circumferences and BF% over time (Table 3). Changes in these anthropometric measurements did not differ significantly between snack groups at any time interval, and the changes over time did not differ significantly between groups.

*Physical Activity*

Self-reported physical activity (hr/day) was significantly greater in the first cohort of women compared to the second cohort ($p<0.01$) at baseline. Therefore, a physical activity change variable was created for each group (DC and NC) by cohort (1 and 2). The change variable was then compared among these four groups using ANOVA. There was no significant difference in the change in physical activity from baseline to week 18 among snack by cohort groups. The effect of time on physical activity was assessed within each snack group using repeated measures ANOVA.
There was no significant difference in self-reported physical activity within the four groups or between snack groups over time.

**Blood Pressure**

From baseline to week 18, women in both snack groups experienced significant reductions in systolic and diastolic BP (Table 4). For women within the DC group, the significant change in diastolic BP occurred by week 12. For women within the NC group, significant changes in systolic and diastolic BP occurred by week 12. Significant differences in BP measurements between groups were not found, and the changes within snack groups over time were not significantly different between snack groups.

**Metabolic Profile**

Women in the DC group had decreases in serum glucose concentration by week 6 (7.5% ↓, \( p<0.001 \)), and at week 12 (5.3% ↓, \( p<0.01 \)) and week 18 (12.9% ↓, \( p<0.001 \)) compared to baseline (Table 4). Women in the NC group also experienced decreases in serum glucose by week 6 (4.9% ↓, \( p<0.05 \)), and at week 12 (4.0% ↓, \( p<0.05 \)) and week 18 (15.2% ↓, \( p<0.001 \)) compared to baseline. Serum glucose concentrations did not significantly differ between groups at any time interval, and the change over time within groups did not significantly differ between snack groups (Figure 3).

Serum insulin concentration decreased in the DC group (26.4% ↓, \( p<0.01 \)) and within the NC group (26.8% ↓, \( p<0.01 \)) from baseline to week 18 (Table 4). Differences between groups at any time interval or for the change over time between snack groups in serum insulin were not statistically significant. HOMA-IR followed a similar pattern as serum insulin for both snack groups (Figure 3).

Serum lipid concentrations did not differ significantly between snack groups at any time interval (Table 4). In the DC group, serum total and LDL cholesterol increased significantly by week 12 but returned to baseline levels at week 18. HDL-cholesterol decreased by week 6 but
returned to baseline level at week 18 for women in the DC group. Women in the NC group had an increase in serum total cholesterol from baseline that persisted to week 18 (8.5% \( \uparrow \), \( p<0.05 \)). Women in the NC group also had a decrease in HDL-cholesterol from baseline to week 6 (9.5% \( \downarrow \), \( p<0.001 \)) that returned to baseline levels at week 18. Changes over time in serum lipids were not significantly different between snack groups.

**Efficacy Analysis**

**Dietary Intake Assessment**

For those participants who completed the study (n=51), women in the DC (n=26) and NC group (n=25) reduced estimated energy intake by 454 kcals/day (\( p<0.001 \)) and 529 kcals/day (\( p<0.001 \)), respectively. Macronutrient intake did not significantly change within the DC group, while percent of kcals from protein increased for women within the NC group (\( p<0.01 \)). Changes in total sugar, fiber, saturated fat, cholesterol and sodium intakes within and between groups over time were similar to results previously reported using the intention-to-treat analysis.

**Anthropometric and Blood Pressure Measurements and Metabolic Profile**

Due to the low number of dropouts from each snack group, changes in anthropometric (Table 5) and BP measurements (Table 6) and glucose, insulin, HOMA-IR and lipid concentrations (Table 6) in the efficacy analysis were greater but had similar statistical significance to changes observed in the intention-to-treat analysis.

**DISCUSSION**

Overweight and obese premenopausal women who followed an 18-week ERD that included two daily sweet snacks were able to achieve an energy deficit, reduce BW and significantly improve BP and glucose and insulin concentrations. Women in both groups had significant BW loss and
improvements in metabolic parameters without appreciably altering the macronutrient composition of their usual diet. In addition, consumption of either high-fat DC or high-carbohydrate NC snacks as part of an ERD produced the same beneficial health outcomes. This indicates that for the purposes of losing BW and improving related metabolic parameters, reducing overall energy is likely more important than consuming a specific macronutrient profile. The current study did not include a group that did not receive two daily portion-controlled sweet snacks, which would provide information about whether the inclusion of sweet snacks in an ERD facilitates BW loss. Although this needs to be evaluated further, this study does show that inclusion of two portion-controlled sweet snacks does not hinder BW loss. Further, the low attrition rates in this intervention compared to those conducted using fad diets suggest that an ERD in which sweet snacks are purposefully incorporated two times daily may be an effective option for inducing moderate weight loss and reducing chronic disease risk factors over an 18-week period.

The effect of dietary intake and macronutrient content of the diet during BW loss has been debated in recent years. Several fad diet programs stress the importance of manipulating macronutrient content to induce more weight loss and improve health outcomes (5-7,32). The contemporary low-carbohydrate diet reduces carbohydrates to 20/g per day and discourages high-carbohydrate fruits, nuts and legumes (5). The classic low-fat, higher carbohydrate diet stresses a decreased consumption of any type of fat and most animal products (6). The more modern focus on macronutrient consumption is present in the form of the Zone and South Beach diets which suggest that individuals should consume a special ratio of macronutrients (40% carbohydrate, 40% protein and 30% fat) to induce weight loss and maintain health (7,32). Randomized clinical trials show that over a 3- to 6-month intervention period, these diets can induce a 5 to 7% loss of BW and produce modest improvements in metabolic markers (11,13-15,33). However, these programs require strict
dietary regimens that dictate the types of food that can be consumed as well as when they can be eaten. Following individuals for 12 to 24 months demonstrates that long-term adherence to these dietary patterns is poor and initial weight loss is not maintained (14). It is likely that drastic changes in dietary intake may be unsustainable for many people. Dietary approaches that can be maintained for longer periods of time to prevent BW regain may be necessary to sustain metabolic health benefits that were initially achieved (31).

Despite consuming 33% and 50% of energy from fat and carbohydrate respectively, which is outside the recommendations of the fad diet regimens, women in the current study were able to reduce total energy intake. Not only did women in both groups maintain an energy deficit, but they also significantly reduced total sugar, saturated fat, cholesterol and sodium intakes. Women in this intervention lost a similar amount of BW as those following the fad diets without dramatically altering their usual macronutrient intake and while consuming a sweet snack twice a day. BW loss and anthropometric improvements were consistent over the 18-week period and did not stop after the first 12 weeks. These results indicate that a variety of macronutrient compositions can facilitate BW loss and improvements in anthropometric measurements as long as an energy reduction is achieved. In addition, dietary composition during an ERD should be individualized according to personal preference and does not have to be a one-size-fits all approach.

Metabolic benefits also were achieved by women in the current study without the manipulation of macronutrient composition. Despite beginning the intervention with normal BP levels, the moderate 6.5% weight loss coincided with a significant reduction in BP over an 18-week period. The magnitude of the decreases in systolic and diastolic BP observed in this study is similar to the degree of reductions demonstrated by other lifestyle interventions (34) and those interventions using the fad diet patterns (11,15,33). The degree of BP changes in women in the current study
would be associated with a 9% relative risk reduction in non-fatal heart attack and stroke on a population level as demonstrated in other studies (35). Further, this reduction in BP is only slightly less than more severely energy-restricted weight-loss plans that show decreases in BP of 6 to 8% after a 7 to 12% BW loss (36), a 6 to 8% reduction in BP with the use of diuretics or beta-blockers (37), or a 7% reduction in BP following an exercise intervention, all of which were conducted in individuals with pre-established hypertension (38). The current study shows that only modest weight loss can further reduce BP even in normotensive women who are overweight or obese.

Other dietary interventions of a similar duration and magnitude of weight loss showed non-significant decreases or no change in fasting glucose or insulin concentrations unless women were randomized to low-carbohydrate diet groups (11,15,33). The current study demonstrated that reducing carbohydrate intake was not necessary to reduce fasting glucose and insulin concentrations. Instead, fasting glucose concentrations appeared to decrease steadily throughout the intervention concurrently with weight loss. This could be explained by the already moderate level of carbohydrate (48-50% of kcals) and fat (31-34% of kcals) composition of the diet consumed by women in this study. Research also has shown that those who consumed a dietary pattern, rich in high-fiber, complex carbohydrates and low glycemic index foods, had improvements in fasting glucose and insulin levels (15,39,40). In this intervention, women decreased total energy intake but did not change their total intake of fiber thus increasing percentage of carbohydrate attributed to fiber from 7.8 to 9.5%. Therefore, displacing higher glycemic foods with higher fiber foods would be expected to increase insulin sensitivity (40). Interestingly, the complete restriction of sweet snacks was not necessary to produce beneficial effects in glucose and insulin concentrations during weight loss even in healthy individuals.
Maintenance of cholesterol concentrations within the healthy range (41) is likely a reflection of the age and overall health of the women at baseline. Studies with similar weight loss that also demonstrated reductions in total and LDL-cholesterol or triglycerides were usually conducted in individuals with elevated levels at baseline (11,13,22,33). Interestingly, dark chocolate is composed of a high percentage of stearic acid, the saturated fatty acid not associated with increased cholesterol concentrations (40). Women in the DC group did not experience any change in cholesterol concentrations with twice daily consumption of DC demonstrating the lipid-neutral effects of stearic acid (42,43). Further, the inclusion of snacks that were high in saturated fat did not significantly increase saturated fat intake in overall dietary intake, likely due to their portion-controlled nature. These results indicate that either high-fat or high-carbohydrate sweet snacks can be consumed on a daily basis without negatively affecting cholesterol concentrations.

A limitation of this intervention is that it was conducted in a population of premenopausal women of a specific age range thus limiting its applicability to other women or to men. While this intervention was conducted with community-dwelling women, it did include nutrition education classes. It would be hard to separate the positive outcomes that may have been attributed to weekly visits with a registered dietitian from the benefits of the diet. Our study was not a metabolic feeding trial; therefore, this study relied on self-reported dietary intake which can be subjective and sometimes underreported in overweight and obese individuals. Nonetheless, conducting this intervention in a community-dwelling setting mimics a real world scenario. Importantly, the positive changes in BW, FM and metabolic markers demonstrate compliance with the dietary intervention. Finally, this study would benefit from a long-term follow-up period to determine whether women continued to consume the calorie-controlled snack, remained adherent with the ERD and maintained metabolic benefits. This is an area for further research.
CONCLUSION

In conclusion, women who are overweight and obese can reduce energy intake and lose BW while incorporating two daily energy- and portion-controlled sweet snacks into an ERD. This dietary intervention demonstrates that women without established hyperglycemia or hypertension can experience clinically significant decreases in glucose and insulin concentrations and BP with possible reduction in chronic disease risk by losing only 6.5% of BW. By reducing energy and BW, premenopausal women with excess adiposity who are otherwise healthy may reduce their risk of developing chronic disease before risk factors have reached elevated levels. To achieve significant energy restriction and subsequent BW loss and improvement in metabolic profile, daily snacks may be included to make an ERD more closely resemble an individual’s current eating pattern. Future research should be conducted to determine whether the inclusion of snacks in an ERD can increase long-term adherence to energy reduction and the subsequent effects on metabolic and clinical markers of disease risk.
REFERENCES


Figure 4.1 Study flow diagram of participants

203 Assessed for Eligibility

118 Excluded
- Did not meet inclusion criteria
- Did not return the required forms
- Lost interest

85 Enrolled in Study

25 Withdraw Before Baseline
  2 Moved from area
  2 Pregnancy
  2 Personal or family illness
  7 Time commitment
  12 Lost contact

30 Randomized to Dark-Chocolate Group

4 Withdraw Before Study Completion
  3 Family issues
  1 Time commitment

26 Completed 18-Week Intervention

30 Included in Intention-to-Treat Analysis
26 Included in Secondary Completers Analysis

30 Randomized to Non-Chocolate Group

5 Withdraw Before Study Completion
  2 Personal or family illness
  2 Time commitment

25 Completed 18-Week Intervention

30 Included in Intention-to-Treat Analysis
25 Included in Secondary Completers Analysis
Table 4.1 Baseline characteristics of participants by snack group

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Dark Chocolate Snack Group (n=30) Mean ± SD</th>
<th>Non-Chocolate Snack Group (n=30) Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yrs)</td>
<td>36.0 ± 5.8</td>
<td>35.9 ± 6.0</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>164.9 ± 4.6</td>
<td>164.9 ± 6.5</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>83.7 ± 13.6</td>
<td>85.1 ± 12.7</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>30.8 ± 4.9</td>
<td>31.2 ± 3.8</td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>89.4 ± 10.2</td>
<td>92.0 ± 10.6</td>
</tr>
<tr>
<td>Hip circumference (cm)</td>
<td>115.4 ± 10.0</td>
<td>116.4 ± 8.8</td>
</tr>
<tr>
<td>Body fat (%)</td>
<td>40.4 ± 5.7</td>
<td>41.2 ± 5.5</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>118.8 ± 7.7</td>
<td>119.7 ± 9.2</td>
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<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>72.8 ± 7.6</td>
<td>74.9 ± 7.5</td>
</tr>
<tr>
<td>Glucose (mg/dL)</td>
<td>100.6 ± 10.9</td>
<td>98.1 ± 10.3</td>
</tr>
<tr>
<td>Insulin (µU/mL)</td>
<td>7.2 ± 4.2</td>
<td>7.1 ± 4.1</td>
</tr>
<tr>
<td>Homeostasis model assessment of insulin resistance</td>
<td>1.8 ± 1.1</td>
<td>1.7 ± 1.0</td>
</tr>
<tr>
<td>Total cholesterol (mg/dL)</td>
<td>158.3 ± 45.1</td>
<td>160.3 ± 45.1</td>
</tr>
<tr>
<td>High-density lipoprotein-cholesterol (mg/dL)</td>
<td>51.5 ± 10.2</td>
<td>49.7 ± 10.8</td>
</tr>
<tr>
<td>Low-density lipoprotein-cholesterol (mg/dL)</td>
<td>94.0 ± 24.0</td>
<td>96.1 ± 43.4</td>
</tr>
<tr>
<td>Triglycerides (mg/dL)</td>
<td>64.4 ± 20.5</td>
<td>72.4 ± 32.8</td>
</tr>
</tbody>
</table>

Significant differences in baseline characteristics did not exist between snack groups.
Table 4.2 Estimated dietary intakes by snack group and changes over time, including baseline values carried forward for cases of missing data

<table>
<thead>
<tr>
<th>Dietary Variable</th>
<th>Dark-Chocolate Snack Group (n=29)</th>
<th>Non-Chocolate Snack Group (n=30)</th>
<th>Dark-Chocolate vs. Non-Chocolate Group, P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Daily energy intake (kcal)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>2025 ± 447</td>
<td>2105 ± 531</td>
<td>NS</td>
</tr>
<tr>
<td>Week 18</td>
<td>1646 ± 354</td>
<td>1668 ± 395</td>
<td>NS</td>
</tr>
<tr>
<td>Change</td>
<td>-379 ± 447***</td>
<td>-437 ± 471***</td>
<td>NS</td>
</tr>
<tr>
<td>Carbohydrate distribution (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>49.1 ± 9.0</td>
<td>50.5 ± 7.6</td>
<td>NS</td>
</tr>
<tr>
<td>Week 18</td>
<td>48.5 ± 4.4</td>
<td>50.7 ± 7.8</td>
<td>NS</td>
</tr>
<tr>
<td>Change</td>
<td>-0.6 ± 9.4</td>
<td>+0.2 ± 6.1</td>
<td>NS</td>
</tr>
<tr>
<td>Fat distribution (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>34.7 ± 6.1</td>
<td>33.1 ± 6.6</td>
<td>NS</td>
</tr>
<tr>
<td>Week 18</td>
<td>33.8 ± 5.5</td>
<td>31.5 ± 7.7</td>
<td>NS</td>
</tr>
<tr>
<td>Change</td>
<td>-0.9 ± 5.8</td>
<td>-1.6 ± 6.3</td>
<td>NS</td>
</tr>
<tr>
<td>Protein distribution (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>15.8 ± 2.5</td>
<td>14.9 ± 2.3</td>
<td>NS</td>
</tr>
<tr>
<td>Week 18</td>
<td>16.2 ± 2.7</td>
<td>16.5 ± 3.1</td>
<td>NS</td>
</tr>
<tr>
<td>Change</td>
<td>+0.4 ± 2.4</td>
<td>+1.6 ± 3.0**</td>
<td>NS</td>
</tr>
<tr>
<td>Alcohol distribution (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>1.3 ± 2.2</td>
<td>1.5 ± 3.6</td>
<td>NS</td>
</tr>
<tr>
<td>Week 18</td>
<td>1.5 ± 3.2</td>
<td>1.2 ± 2.7</td>
<td>NS</td>
</tr>
<tr>
<td>Change</td>
<td>+0.2 ± 2.6</td>
<td>-0.3 ± 3.6</td>
<td>NS</td>
</tr>
<tr>
<td>Total sugar (g)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>84.4 ± 33.0</td>
<td>103.9 ± 45.0</td>
<td>NS</td>
</tr>
<tr>
<td>Week 18</td>
<td>70.9 ± 21.3</td>
<td>75.4 ± 26.3</td>
<td>NS</td>
</tr>
<tr>
<td>Change</td>
<td>-13.5 ± 31.8*</td>
<td>-28.5 ± 45.5*</td>
<td>NS</td>
</tr>
<tr>
<td>Total fiber (g)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Baseline</td>
<td>21.2 ± 10.2</td>
<td>18.9 ± 6.9</td>
<td>NS</td>
</tr>
<tr>
<td>Week 18</td>
<td>19.6 ± 7.1</td>
<td>18.3 ± 5.2</td>
<td>NS</td>
</tr>
<tr>
<td>Change</td>
<td>-1.8 ± 12.4</td>
<td>-0.6 ± 7.1</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>Baseline</td>
<td>Week 18</td>
<td>Change</td>
</tr>
<tr>
<td>---------------------</td>
<td>----------</td>
<td>---------</td>
<td>------------</td>
</tr>
<tr>
<td>Saturated fat (g)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>25.2 ± 8.2</td>
<td>27.3 ± 10.8</td>
<td>NS</td>
</tr>
<tr>
<td>Week 18</td>
<td>19.2 ± 7.5</td>
<td>20.3 ± 10.1</td>
<td>NS</td>
</tr>
<tr>
<td>Change</td>
<td>-6.0 ± 8.9****</td>
<td>-7.0 ± 11.4**</td>
<td>NS</td>
</tr>
<tr>
<td>Cholesterol (mg)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>257 ± 112</td>
<td>235 ± 99</td>
<td>NS</td>
</tr>
<tr>
<td>Week 18</td>
<td>181 ± 79</td>
<td>184 ± 85</td>
<td>NS</td>
</tr>
<tr>
<td>Change</td>
<td>-76 ± 126**</td>
<td>-51 ± 96**</td>
<td>NS</td>
</tr>
<tr>
<td>Sodium (mg)</td>
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<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>3407 ± 934</td>
<td>3520 ± 1289</td>
<td>NS</td>
</tr>
<tr>
<td>Week 18</td>
<td>2928 ± 817</td>
<td>3174 ± 934</td>
<td>NS</td>
</tr>
<tr>
<td>Change</td>
<td>-479 ± 976*</td>
<td>-346 ± 1180</td>
<td>NS</td>
</tr>
</tbody>
</table>

Values are means ± SD.

*P<0.05, **P<0.01, ***P<0.001 within group change from baseline.
Table 4.3 Anthropometric measurements by snack group and changes from baseline, including baseline values carried forward for cases of missing data

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Dark Chocolate Snack Group (n=30)</th>
<th>Non-Chocolate Snack Group (n=30)</th>
<th>Dark Chocolate vs. Non-Chocolate Group, P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight (kg)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>83.7 ± 13.6</td>
<td>85.1 ± 12.7</td>
<td>NS</td>
</tr>
<tr>
<td>Week 6</td>
<td>-2.7 ± 1.9***</td>
<td>-2.6 ± 2.4***</td>
<td>NS</td>
</tr>
<tr>
<td>Week 12</td>
<td>-4.2 ± 2.7***</td>
<td>-4.0 ± 3.6***</td>
<td>NS</td>
</tr>
<tr>
<td>Week 18</td>
<td>-4.4 ± 3.3***</td>
<td>-5.0 ± 4.9***</td>
<td>NS</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>30.8 ± 4.9</td>
<td>31.2 ± 3.8</td>
<td>NS</td>
</tr>
<tr>
<td>Week 6</td>
<td>-1.0 ± 0.7***</td>
<td>-1.0 ± 0.9***</td>
<td>NS</td>
</tr>
<tr>
<td>Week 12</td>
<td>-1.6 ± 1.0***</td>
<td>-1.5 ± 1.3***</td>
<td>NS</td>
</tr>
<tr>
<td>Week 18</td>
<td>-1.6 ± 1.2***</td>
<td>-1.8 ± 1.8**</td>
<td>NS</td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>89.4 ± 10.2</td>
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<td>NS</td>
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<tr>
<td>Week 6</td>
<td>-3.0 ± 2.1***</td>
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<td>Week 12</td>
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<td>Week 18</td>
<td>-5.0 ± 5.1***</td>
<td>-5.1 ± 5.3***</td>
<td>NS</td>
</tr>
<tr>
<td>Hip circumference (cm)</td>
<td></td>
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<tr>
<td>Baseline</td>
<td>115.4 ± 10.0</td>
<td>116.4 ± 8.8</td>
<td>NS</td>
</tr>
<tr>
<td>Week 6</td>
<td>-3.9 ± 2.6***</td>
<td>-3.6 ± 3.1***</td>
<td>NS</td>
</tr>
<tr>
<td>Week 12</td>
<td>-4.7 ± 3.8***</td>
<td>-4.7 ± 3.9***</td>
<td>NS</td>
</tr>
<tr>
<td>Week 18</td>
<td>-5.2 ± 4.1***</td>
<td>-4.8 ± 4.1***</td>
<td>NS</td>
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<tr>
<td>Body fat (%)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Baseline</td>
<td>40.4 ± 5.7</td>
<td>41.2 ± 5.5</td>
<td>NS</td>
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<tr>
<td>Week 6</td>
<td>-1.4 ± 1.2***</td>
<td>-1.0 ± 1.7***</td>
<td>NS</td>
</tr>
<tr>
<td>Week 12</td>
<td>-2.7 ± 2.1***</td>
<td>-2.1 ± 2.4***</td>
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</tr>
<tr>
<td>Week 18</td>
<td>-3.2 ± 2.6***</td>
<td>-2.7 ± 3.3***</td>
<td>NS</td>
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</table>
Values are means ± SD for baseline values and mean ± SD for change from baseline values.

*P<0.05, **P<0.01, ***P<0.001 within group changes from baseline.
Figure 4.2 Body weight changes over time by snack group

Values are mean ± SEM.

*** P<0.001 for within group changes from baseline.

There were no significant differences in body weight between snack groups at any interval or for the change over time between snack groups.
Table 4.4 Blood pressure measurements and metabolic profile by snack group and changes from baseline, including baseline values carried forward for cases of missing data

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Dark Chocolate Snack Group (n=30)</th>
<th>Non-Chocolate Snack Group (n=30)</th>
<th>Dark Chocolate vs. Non-Chocolate Group, P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Systolic blood pressure (mmHg)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>118.8 ± 7.7</td>
<td>119.7 ± 9.2</td>
<td>NS</td>
</tr>
<tr>
<td>Week 6</td>
<td>-1.7 ± 5.5</td>
<td>-2.0 ± 6.5</td>
<td>NS</td>
</tr>
<tr>
<td>Week 12</td>
<td>-0.6 ± 5.6</td>
<td>-1.8 ± 4.2*</td>
<td>NS</td>
</tr>
<tr>
<td>Week 18</td>
<td>-2.7 ± 6.6*</td>
<td>-3.4 ± 5.5**</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Diastolic blood pressure (mmHg)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>72.8 ± 7.6</td>
<td>74.9 ± 7.5</td>
<td>NS</td>
</tr>
<tr>
<td>Week 6</td>
<td>-1.6 ± 4.7</td>
<td>-1.6 ± 7.2</td>
<td>NS</td>
</tr>
<tr>
<td>Week 12</td>
<td>-2.2 ± 5.5*</td>
<td>-2.7 ± 6.5*</td>
<td>NS</td>
</tr>
<tr>
<td>Week 18</td>
<td>-2.7 ± 5.3**</td>
<td>-4.2 ± 7.0**</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Glucose (mg/dL)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>100.6 ± 10.9</td>
<td>98.1 ± 10.3</td>
<td>NS</td>
</tr>
<tr>
<td>Week 6</td>
<td>-7.5 ± 11.3***</td>
<td>-4.8 ± 11.1*</td>
<td>NS</td>
</tr>
<tr>
<td>Week 12</td>
<td>-5.3 ± 7.8**</td>
<td>-3.9 ± 8.9*</td>
<td>NS</td>
</tr>
<tr>
<td>Week 18</td>
<td>-13.0 ± 13.2***</td>
<td>-14.9 ± 11.3***</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Insulin (IU/mL)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>7.5 ± 4.2</td>
<td>7.1 ± 4.1</td>
<td>NS</td>
</tr>
<tr>
<td>Week 6</td>
<td>+0.4 ± 6.9</td>
<td>-0.2 ± 2.6</td>
<td>NS</td>
</tr>
<tr>
<td>Week 12</td>
<td>-1.0 ± 3.0</td>
<td>-0.4 ± 3.2</td>
<td>NS</td>
</tr>
<tr>
<td>Week 18</td>
<td>-1.9 ± 3.2**</td>
<td>-1.9 ± 3.6**</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Homeostasis model assessment of insulin resistance</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>1.87 ± 1.14</td>
<td>1.64 ± 0.99</td>
<td>NS</td>
</tr>
<tr>
<td>Week 6</td>
<td>+0.04 ± 2.06</td>
<td>-0.08 ± 0.72</td>
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</tr>
<tr>
<td>Week 12</td>
<td>-0.33 ± 0.86</td>
<td>-0.13 ± 0.78</td>
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</tr>
<tr>
<td>Week 18</td>
<td>-0.63 ± 0.81***</td>
<td>-0.62 ± 0.88***</td>
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</tr>
</tbody>
</table>
Total Cholesterol (mg/dL)

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Week 6</th>
<th>Week 12</th>
<th>Week 18</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>158.3 ± 45.1</td>
<td>160.3 ± 45.1</td>
<td>-6.1 ± 17.9</td>
<td>-1.9 ± 21.7</td>
</tr>
<tr>
<td></td>
<td>160.3 ± 45.1</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-7.5 ± 17.8*</td>
<td>+1.4 ± 23.6</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+4.6 ± 20.0</td>
<td>+13.7 ± 22.6*</td>
<td>NS</td>
</tr>
</tbody>
</table>

High-density lipoprotein-cholesterol (mg/dL)

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Week 6</th>
<th>Week 12</th>
<th>Week 18</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>51.5 ± 10.2</td>
<td>49.7 ± 10.8</td>
<td>-3.1 ± 6.2*</td>
<td>-4.7 ± 5.9***</td>
</tr>
<tr>
<td></td>
<td>49.7 ± 10.8</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>-1.6 ± 4.0*</td>
<td>-1.8 ± 6.1</td>
<td>+1.6 ± 7.2</td>
<td>+2.1 ± 6.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>NS</td>
</tr>
</tbody>
</table>

Low-density lipoprotein-cholesterol (mg/dL)

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Week 6</th>
<th>Week 12</th>
<th>Week 18</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>94.0 ± 24.0</td>
<td>96.1 ± 43.4</td>
<td>-4.1 ± 17.4</td>
<td>+3.0 ± 23.4</td>
</tr>
<tr>
<td></td>
<td>96.1 ± 43.4</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>+8.1 ± 16.0*</td>
<td>+2.0 ± 22.8</td>
<td>+1.4 ± 20.3</td>
<td>+9.0 ± 24.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>NS</td>
</tr>
</tbody>
</table>

Triglycerides (mg/dL)

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Week 6</th>
<th>Week 12</th>
<th>Week 18</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>64.4 ± 20.5</td>
<td>72.4 ± 32.8</td>
<td>+5.6 ± 33.3</td>
<td>-1.8 ± 24.7</td>
</tr>
<tr>
<td></td>
<td>72.4 ± 32.8</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>+5.6 ± 25.9</td>
<td>+5.6 ± 22.7</td>
<td>+4.5 ± 31.9</td>
<td>+5.6 ± 45.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>NS</td>
</tr>
</tbody>
</table>

Values are means ± SD for baseline values and mean ± SD for change from baseline values.

*P<0.05, **P<0.01, ***P<0.001 within group changes from baseline.

Homeostasis model assessment-estimated insulin resistance calculated by fasting insulin concentration ($\mu$U/mL) x fasting glucose concentration (mg/dL) x 0.0555 /22.5.
Figure 4.3 Serum glucose and insulin concentrations and HOMA-IR changes over time by snack group

Dark-chocolate snack group (♦) (n=30) and non-chocolate snack group (□) (n=30).

Values are mean ± SEM.

*P<0.05, **P<0.01, *** P<0.001 for within group changes from baseline.
There were no significant differences in glucose, insulin or HOMA-IR between snack groups at any interval or for the change over time between snack groups.
Table 4.5 Anthropometric measurements by snack group and changes from baseline, for women who completed the study

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Dark Chocolate Snack Group (n=26)</th>
<th>Non-Chocolate Snack Group (n=25)</th>
<th>Dark Chocolate vs. Non-Chocolate Group, P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight (kg)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>83.1 ± 13.6</td>
<td>84.9 ± 13.7</td>
<td>NS</td>
</tr>
<tr>
<td>Week 6</td>
<td>-3.1 ± 1.7***</td>
<td>-3.1 ± 2.3***</td>
<td>NS</td>
</tr>
<tr>
<td>Week 12</td>
<td>-4.9 ± 2.3***</td>
<td>-4.8 ± 3.4***</td>
<td>NS</td>
</tr>
<tr>
<td>Week 18</td>
<td>-5.1 ± 17.8***</td>
<td>-5.9 ± 4.8***</td>
<td>NS</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>30.8 ± 4.9</td>
<td>31.0 ± 4.0</td>
<td>NS</td>
</tr>
<tr>
<td>Week 6</td>
<td>-1.1 ± 0.6***</td>
<td>-1.1 ± 0.9***</td>
<td>NS</td>
</tr>
<tr>
<td>Week 12</td>
<td>-1.8 ± 0.6***</td>
<td>-1.8 ± 1.3***</td>
<td>NS</td>
</tr>
<tr>
<td>Week 18</td>
<td>-1.9 ± 1.1***</td>
<td>-2.2 ± 1.8***</td>
<td>NS</td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>88.8 ± 10.5</td>
<td>92.1 ± 11.1</td>
<td>NS</td>
</tr>
<tr>
<td>Week 6</td>
<td>-3.5 ± 1.9***</td>
<td>-4.0 ± 3.6***</td>
<td>NS</td>
</tr>
<tr>
<td>Week 12</td>
<td>-4.9 ± 2.5***</td>
<td>-5.7 ± 3.7***</td>
<td>NS</td>
</tr>
<tr>
<td>Week 18</td>
<td>-5.8 ± 5.1***</td>
<td>-6.1 ± 5.3***</td>
<td>NS</td>
</tr>
<tr>
<td>Hip circumference (cm)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>115.7 ± 10.1</td>
<td>116.3 ± 9.5</td>
<td>NS</td>
</tr>
<tr>
<td>Week 6</td>
<td>-4.5 ± 2.3***</td>
<td>-4.3 ± 3.0***</td>
<td>NS</td>
</tr>
<tr>
<td>Week 12</td>
<td>-5.5 ± 3.6***</td>
<td>-5.6 ± 3.7***</td>
<td>NS</td>
</tr>
<tr>
<td>Week 18</td>
<td>-6.0 ± 3.9***</td>
<td>-5.8 ± 3.8***</td>
<td>NS</td>
</tr>
<tr>
<td>Body fat (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>40.2 ± 5.7</td>
<td>41.0 ± 5.8</td>
<td>NS</td>
</tr>
<tr>
<td>Week 6</td>
<td>-1.6 ± 1.1***</td>
<td>-1.2 ± 1.7**</td>
<td>NS</td>
</tr>
<tr>
<td>Week 12</td>
<td>-3.1 ± 1.9***</td>
<td>-2.5 ± 2.4**</td>
<td>NS</td>
</tr>
<tr>
<td>Week 18</td>
<td>-3.7 ± 2.4***</td>
<td>-3.3 ± 3.3***</td>
<td>NS</td>
</tr>
</tbody>
</table>
Values are means ± SD for baseline values and mean ± SD for change from baseline values. *P<0.05,
**P<0.01, ***P<0.001 within group changes from baseline.
Table 4.6 Blood pressure measurements and metabolic profile by snack group and changes from baseline, for women who completed the study

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Dark Chocolate Snack Group (n=26)</th>
<th>Non-Chocolate Snack Group (n=25)</th>
<th>Dark Chocolate vs. Non-Chocolate Group, P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>118.3 ± 8.0</td>
<td>120.7 ± 8.7</td>
<td>NS</td>
</tr>
<tr>
<td>Week 6</td>
<td>-2.0 ± 5.9</td>
<td>-2.6 ± 6.9</td>
<td>NS</td>
</tr>
<tr>
<td>Week 12</td>
<td>-0.3 ± 6.2</td>
<td>-2.4 ± 4.4*</td>
<td>NS</td>
</tr>
<tr>
<td>Week 18</td>
<td>-3.6 ± 7.4*</td>
<td>-4.3 ± 5.9**</td>
<td>NS</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>71.8 ± 7.5</td>
<td>75.6 ± 7.8</td>
<td>NS</td>
</tr>
<tr>
<td>Week 6</td>
<td>-1.8 ± 5.0</td>
<td>-2.0 ± 7.8</td>
<td>NS</td>
</tr>
<tr>
<td>Week 12</td>
<td>-2.5 ± 6.1</td>
<td>-3.3 ± 6.9*</td>
<td>NS</td>
</tr>
<tr>
<td>Week 18</td>
<td>-3.5 ± 5.9*</td>
<td>-5.3 ± 7.5**</td>
<td>NS</td>
</tr>
<tr>
<td>Glucose (mg/dL)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>101.7 ± 10.9</td>
<td>97.3 ± 10.3</td>
<td>NS</td>
</tr>
<tr>
<td>Week 6</td>
<td>-8.7 ± 11.8***</td>
<td>-5.3 ± 11.9*</td>
<td>NS</td>
</tr>
<tr>
<td>Week 12</td>
<td>-6.3 ± 8.1**</td>
<td>-4.6 ± 9.5*</td>
<td>NS</td>
</tr>
<tr>
<td>Week 18</td>
<td>-15.0 ± 13.1***</td>
<td>-17.4 ± 10.3***</td>
<td>NS</td>
</tr>
<tr>
<td>Insulin (µU/mL)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>7.2 ± 4.6</td>
<td>7.1 ± 4.3</td>
<td>NS</td>
</tr>
<tr>
<td>Week 6</td>
<td>+0.4 ± 7.5</td>
<td>+0.1 ± 2.6</td>
<td>NS</td>
</tr>
<tr>
<td>Week 12</td>
<td>-1.2 ± 3.2</td>
<td>-0.2 ± 4.2</td>
<td>NS</td>
</tr>
<tr>
<td>Week 18</td>
<td>-2.2 ± 3.4**</td>
<td>-2.0 ± 3.9*</td>
<td>NS</td>
</tr>
<tr>
<td>Homeostasis model assessment of insulin resistance</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>1.79 ± 1.19</td>
<td>1.69 ± 1.03</td>
<td>NS</td>
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<tr>
<td>Week 6</td>
<td>+0.05 ± 2.24</td>
<td>-0.04 ± 0.73</td>
<td>NS</td>
</tr>
<tr>
<td>Week 12</td>
<td>-0.40 ± 0.92*</td>
<td>-0.10 ± 0.80</td>
<td>NS</td>
</tr>
<tr>
<td>Week 18</td>
<td>-0.72 ± 0.83***</td>
<td>-0.67 ± 0.91***</td>
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</table>
Total Cholesterol (mg/dL)

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Week 6</th>
<th>Week 12</th>
<th>Week 18</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>158.7 ± 26.1</td>
<td>-7.1 ± 19.2</td>
<td>+8.9 ± 19.1*</td>
<td>+2.7 ± 20.1</td>
</tr>
<tr>
<td></td>
<td>163.3 ± 45.0</td>
<td>-3.0 ± 23.0</td>
<td>+1.6 ± 25.6</td>
<td>+15.9 ± 23.7**</td>
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</table>

High-density lipoprotein-cholesterol (mg/dL)

<table>
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<tr>
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<th>Baseline</th>
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<th>Week 12</th>
<th>Week 18</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>51.9 ± 10.7</td>
<td>-3.6 ± 6.5*</td>
<td>-1.9 ± 4.3*</td>
<td>+1.8 ± 7.8</td>
</tr>
<tr>
<td></td>
<td>49.1 ± 11.1</td>
<td>-5.3 ± 6.2***</td>
<td>-2.1 ± 6.6</td>
<td>+2.4 ± 6.4</td>
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Low-density lipoprotein-cholesterol (mg/dL)

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Week 6</th>
<th>Week 12</th>
<th>Week 18</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>93.5 ± 25.9</td>
<td>-4.7 ± 18.7</td>
<td>+9.6 ± 17.0*</td>
<td>+4.6 ± 26.2</td>
</tr>
<tr>
<td></td>
<td>99.2 ± 43.4</td>
<td>+2.6 ± 25.1</td>
<td>+2.4 ± 24.7</td>
<td>+12.0 ± 26.3*</td>
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</tbody>
</table>

Triglycerides (mg/dL)

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Week 6</th>
<th>Week 12</th>
<th>Week 18</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>66.4 ± 21.3</td>
<td>+6.5 ± 35.9</td>
<td>+6.0 ± 28.2</td>
<td>+5.2 ± 34.4</td>
</tr>
<tr>
<td></td>
<td>75.5 ± 33.9</td>
<td>-2.9 ± 29.4</td>
<td>+6.6 ± 24.4</td>
<td>+6.7 ± 48.9</td>
</tr>
<tr>
<td></td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

Values are means ± SD for baseline values and mean ± SD for change from baseline values.

*P<0.05, **P<0.01, ***P<0.001 within group changes from baseline.

Homeostasis model assessment-estimated insulin resistance calculated by fasting insulin concentration (µU/mL) x fasting glucose concentration (mg/dL) x 0.0555 /22.5.
CHAPTER 5

EFFECTS OF INCORPORATING COCOA FLAVANOLS INTO A WEIGHT-LOSS DIET
ON MARKERS OF OXIDATIVE STRESS, INFLAMMATION AND BONE TURNOVER
ABSTRACT

Background: Reducing body weight (BW) and consuming cocoa flavanols can potentially lower adiposity-induced oxidative stress and inflammation. The combination may provide additive effects that benefit bone mineral density (BMD).

Objective: This study explored the effect of BW loss combined with daily consumption of high flavanol (HF) cocoa products on markers of inflammation and bone health.

Design: Women [(Mean ± SD) age: 36.2 ± 5.8; body mass index 30.9 ± 4.4 kg/m²] were randomized to consume a HF cocoa beverage and two dark chocolate snacks daily (AF; n=25) or a flavanol-free beverage and two licorice snacks daily (NF; n=25) while following an energy-restricted diet (ERD) for 18 weeks. BW and markers of oxidative stress, inflammation and bone turnover were measured at baseline, week 6, 12 and 18. BMD was measured at baseline and week 18. Repeated measures ANOVA were used to examine within and between group changes over time.

Results: After significant BW loss in the AF and NF group (both P < 0.001), interleukin (IL)-6 (both P < 0.001) and IL-1β (both P < 0.001) decreased in both snack groups. The AF group also had reductions in tumor necrosis factor-α (P < 0.01). Both groups had increases in osteoprotegerin (AF: P < 0.01; NF: P < 0.001) and in forearm BMD (AF: P < 0.01; NF: P < 0.001). The AF group also had an increase in hip BMD (P < 0.01). Differences in biomarker changes were not statistically significant between AF and NF groups.

Conclusions: Consuming additional flavanols did not provide greater benefits to biomarkers of oxidative stress, inflammation and bone turnover or BMD following weight loss.
INTRODUCTION

Individuals with excess body weight (BW) attributed to fat mass (FM) are at increased risk for developing multiple co-morbidities (1). Once thought to be relatively inert (2), adipose tissue is now known to secrete a multitude of chemical mediators that regulate body weight (BW), hunger and satiety, food consumption and fat and carbohydrate metabolism (3,4). Adipose cells also secrete the pro-inflammatory cytokines tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), C-reactive protein (CRP) and IL-1β (5,6). Expansion of adipose tissue due to the accumulation of energy evidenced in obesity induces an inflammatory response and creates a hypoxic physiological environment (7). As a result, individuals who are obese often have higher circulating concentrations of pro-inflammatory cytokines (8) and are considered to be under greater oxidative stress. This milieu of adipose-derived oxidative stress and inflammation has been purported as the precipitating factor in the development of many chronic conditions associated with obesity (9-11).

Reducing adipose tissue through BW loss can decrease oxidative stress (12) and inflammation (13) and in turn, decrease the risk of developing various chronic diseases (14,15). Despite the health benefits of weight loss, reducing BW may negatively impact bone health in certain populations (16-18). In males and females of all ages and ethnicities, a greater BW is robustly associated with a higher BMD (19-22). Greater BW is associated with higher levels of FM and fat-free mass (FFM) both of which contribute to load-bearing on the skeleton and promote BMD accretion (23). Intentional weight loss can result in the reduction of BMD (24,25), and longitudinal data demonstrate that women entering their postmenopausal years with a low BW tend to lose BMD at a higher rate than their higher BW counterparts (20,22). Low BMD increases an individual’s risk of developing osteoporosis and experiencing fractures (23).
Besides BW, multiple factors influence BMD including dietary intake, physical activity, body composition and presence of oxidative stress and inflammation. Specifically, oxidative stress and inflammation can be potent regulators of the bone remodeling process. Oxidative stress produces reactive oxygen species (ROS) which have been shown to induce bone resorption by upregulating osteoclast activity and decreasing osteoblast activity (26,27). Pro-inflammatory cytokines also have been shown to increase osteoclast differentiation and bone resorption while inhibiting the formative action of osteoblasts (28,29). Consequently, bone loss (30), reduced BMD and increased incidence of osteoporosis all have been linked to conditions characterized by oxidative stress (31) and inflammation (32). Because individuals who are overweight or obese tend to have chronically higher levels of oxidative stress markers and circulating pro-inflammatory cytokine concentrations (9,14), they may also have a continuous stimulation of bone resorption with a reciprocal inhibition of bone formation (33). Therefore, while a greater BW is normally protective, having excess BW attributed to FM, may put individuals at risk for reduced BMD. To that end, researchers have recently linked higher levels of body fat to lower BMD (34,35) and increased incidence of fracture, osteopenia and osteoporosis (36).

In addition to BW loss, certain bioactive components found naturally in food may also decrease oxidative stress and improve inflammatory status (37). Flavanols, the antioxidants found in cocoa, have demonstrated the capacity to reduce oxidative stress (38) and inflammation (39,40) in vitro. Further, epidemiologic and intervention research has linked a greater consumption of antioxidants to a higher BMD (41-46). Because of their antioxidant potential, the consumption of cocoa flavanols may reduce obesity-induced oxidative stress and inflammation. This reduction in chronic inflammation may attenuate excess stimulation of bone resorption and lead to the maintenance of BMD.
Because BW loss and consumption of flavanols have the potential to independently decrease oxidative stress and inflammation, the combination of these two techniques may produce an additive effect. To explore the effect of flavanol consumption and BW loss on markers of oxidative stress, inflammation, bone resorption and formation and BMD, an 18-week intervention was conducted in which women consumed a weight-loss diet that incorporated either a daily high-flavanol (HF) cocoa beverage and twice daily dark chocolate (DC) snack or a daily flavanol-free (FF) vanilla beverage and twice daily FF snack.

SUBJECTS AND METHODS

Subjects

A total of 60 healthy, overweight or obese premenopausal women were enrolled in the primary study (47). For this secondary analysis of data collected during the primary study, only women who completed all testing sessions and provided blood and urine samples for complete evaluation were included (n=50).

Originally, subjects were recruited in two cohorts between February 2009 and February 2010. Subjects had to be weight stable for the previous 6 month period, engage in less than 5 hours of physical activity per week and be free of bone disorders, impaired renal function, or cardiovascular or metabolic disease. Exclusion criteria included the use of anti-inflammatory medications or medication known to affect bone metabolism, current smoking, or women who had undergone a hysterectomy or ovariectomy without hormone replacement therapy. This study was approved by the Institutional Review Board at The Pennsylvania State University. Written informed consent was provided by each subject before entry into the study.

Experimental Design
Subjects were randomized (41) into one of two intervention groups: weight loss with additional flavanols (AF; n=25) or weight loss with no additional flavanols (NF; n=25). Both groups followed a self-selected ERD that recommended 50% of energy from carbohydrate, 30% of energy from fat and 20% of energy from protein. Baseline total daily energy levels for BW maintenance were determined for each subject using the Harris-Benedict equation (48) and 500 kcals per day were subtracted to induce a moderate weekly BW loss.

The AF group consumed a HF cocoa beverage that provided 280 mg flavanols/d (65 kcal/d) and two DC snacks providing 240 mg flavanols/d (Hershey®’s Extra Dark, 60% cacao; 90 kcals/d). The NF group consumed a FF vanilla beverage (65 kcal/d) and two FF licorice snacks (90 kcals/d). Each day for 18 weeks, subjects consumed their assigned beverage with breakfast and consumed one snack after lunch and one snack after dinner. Throughout the intervention, subjects in both groups refrained from consuming additional cocoa or chocolate products. All beverages and snacks were provided by The Hershey Company (Hershey, PA, USA).

Procedures and Sample Collection

A registered dietitian instructed subjects on how to follow the individualized ERD using a food exchange system. Subjects attended weekly nutrition education classes for the purposes of facilitating dietary adherence and BW loss. At weekly classes, beverage mix and snacks were distributed and compliance with the assigned intervention was assessed through subject self-report and investigator verified snack counts.

Study Protocol

Subjects attended measurement (i.e., testing) sessions at baseline and at week 6, 12 and 18, consisting of anthropometric measurements, a blood draw and 24-hour urine collection. In addition,
women completed food records and underwent dual-energy X-ray absorptiometry (DXA) scans at baseline and week 18.

*Dietary Intake*

Subjects were provided with 4-day food record forms on which they recorded all the foods and beverages consumed on 3 weekdays and 1 weekend day, in the week prior to the measurement sessions. Food records were analyzed with Food Processor® dietary analysis software (version 10.6.0; esha Research, Salem, OR, USA) to estimate average daily intake of total energy (kcals), carbohydrate, protein, and fat intake (% of total kcal), and calcium (mg) and vitamin D (IU) intake.

*Physical Activity*

Due to the influence of physical activity on BMD and to establish the independent effects of incorporating flavanols during weight loss on BMD, subjects were instructed to maintain baseline physical activity level. To monitor physical activity, subjects completed the Stanford 7-day Physical Activity Recall Scale (PAR) (49). The approximate number of hours spent resting, working on the computer and watching television was recorded. Time spent in moderate, hard and very hard activity was also recorded and summed. The average hours spent in daily physical activity was calculated for each subject.

*Anthropometric Measurements*

Height was measured to the nearest 0.1 cm with a stadiometer (Seca 700, Seca, Hamburg, Germany) and BW was measured to the nearest 0.1 kg using an electronic scale (TBF-410, Tanita Corporation, Arlington Heights, IL, USA). Using a spring-calibrated measuring tape (Gulik II, Country Technology, Gays Mills, WI, USA), two waist measurements at the narrowest part above the belly button and two hip measurements at the widest part of lower body just below the buttocks
were taken to the nearest 0.1 cm. For all measurements, subjects wore light clothing and had shoes removed.

**Body Composition and Bone Mineral Density**

Fat mass (FM; kg), fat-free mass (FFM; kg) and abdominal FM (abFM; kg) were measured using DXA (QDR 4500A, Hologic, Inc., Bedford, MA, USA; version 12.7.3.1). DXA was also used to measure total body (TB) BMD (g/cm²) and BMD of the lumbar spine (LS, L1-L4), non-dominant total proximal femur (hip) and non-dominant total forearm (FA). Women were scanned in a supine position for total body scans and as per standard protocol for the separate body sites. Scans were conducted and analyzed by one technician to eliminate inter-tester variation; quality control scans were completed daily. During analysis of TB scans, a 50 cm region of interest box was placed at the top of the ileac crest in the abdominal region. Within this 50 cm area, FM was analyzed and labeled as abFM. Test-retest reliability for TB BMD, FM, FFM and abFM were 0.97, 1.87, 1.02 and 3.32%, respectively, and for LS, hip and FA BMD were 0.88, 1.56, and 1.03%, respectively, with 26 premenopausal women who were overweight and obese.

**Sample Collection**

Fasting venous blood samples (~30 mL) were collected after an overnight fast. Blood samples were allowed to clot at room temperature for 30 minutes before they were spun in a 4°C centrifuge at 810 x g for 12 minutes. For the 24-hour urine sample, subjects were given a 4-L container in which they collected all voided urine for the 24-hour period before their scheduled measurement appointment. Serum and urine were aliquotted into cryovials and stored at -80°C for later analysis.

**Marker of Oxidative Stress**
Urinary 8-epi-Prostaglandin F\(_2\alpha\) (8-epi-PGF\(_2\alpha\)) was measured by enzyme-linked immunosorbent assay (ELISA; GEN5 version 1.10 for Epoch, BioTek, Winooski, VT, USA), using a commercial ELISA kit (Oxford Biomedical Research, Oxford, MI, USA). Samples were prepared according to manufacturer protocol and analyzed in duplicate. The intra-assay coefficient of variation (CV%) for 8-epi-PGF\(_2\alpha\) was 5.0%.

**Markers of Inflammation**

Serum TNF-\(\alpha\), IL-6, CRP, and IL-1\(\beta\) were analyzed (GEN5 version 1.10 for Epoch, BioTek) using commercial ELISA kits (R&D System Inc., Minneapolis, MN, USA). Samples were prepared according to manufacturer protocol and analyzed in duplicate. For the IL-1\(\beta\) assay, there were samples in week 12 and week 18 that fell below the detection of the kit. For these samples, 0.1 pg/mL was imputed as the value which was half of the mean of the samples from week 6. Intra-assay CV% was 7.5, 5.5, 4.4, 6.6% for TNF-\(\alpha\), IL-1\(\beta\), IL-6, and CRP, respectively.

**Markers of Bone Turnover**

Serum osteocalcin (OC; Human Osteocalcin ELISA 1-43/49, ALPCO Diagnostics, Salem, NH, USA), osteoprotegrin (OPG; ALPCO Diagnostics), N-telopeptide of bone type I collagen (NTx; Osteomark NTx, Inverness Medical Professional Diagnostics, Princeton, NJ, USA) and receptor activator of nuclear factor (NF)-\(\kappa\)B ligand (RANKL; ALPCO Diagnostics) were analyzed (GEN5 version 1.10 for Epoch, BioTek) using commercial ELISA kits. All serum samples were prepared according to manufacturer protocol and analyzed in duplicate using their respective commercial kits. Intra-assay CV% for OC, OPG, NTx and RANKL was 8.7, 7.0, 9.3 and 11.9%, respectively.

**Markers of Mineral Metabolism**

Serum and urinary calcium and phosphorus concentrations were measured using quantitative ultra-violet spectrophotometry (Simple Reads Software version 3.0 for Cary 50, Varian
spectrophotometer, Santa Clara, CA, USA). Samples were prepared according to manufacturer protocols and analyzed in duplicate using Calcium LiquiColor® kits and phosphorus Liqui-UV® kits from Stanbio Labs (Boerne, TX, USA). Intra-assay CV% for serum calcium and phosphorus were 7.2 and 7.6%, respectively, and 6.9 and 8.4% for urinary calcium and phosphorus, respectively.

Statistical Analyses

Subject characteristics for each interval are reported as means ± standard deviations. Differences in baseline characteristics between cohorts and between AF and NF groups were analyzed using independent t-tests. Changes in dietary intake, body composition and BMD within and between groups from baseline to week 18 were analyzed using paired and independent t-tests, respectively. A 2 x 2 x 2 analysis of variance (ANOVA) and a 2 x 2 x 4 ANOVA with repeated measures on the time factor was performed to assess main effects of cohort (cohort 1 and 2) and group (AF and NF) over two intervals for BMD and over four intervals for BW and biomarkers of oxidative stress, inflammation, bone turnover and mineral metabolism, respectively. If cohort x time or group x time interactions were detected, follow-up 2 x 2 or 2 x 4 ANOVAs were conducted to identify the intervals at which the significant interaction effect occurred. Significance tests were two-sided with significance set at P < 0.05. Data analyses were conducted using the Statistical Package for the Social Sciences version 17.0 (2008; SPSS Inc., Chicago, IL, USA).

RESULTS

Sixty women began the intervention and 50 women completed all testing sessions (AF n=25, NF n=25). Baseline characteristics for these 50 subjects are presented in Table 1 by AF and NF group. At baseline, there were significant differences between cohorts in physical activity (P < 0.05) and NTx concentration (P < 0.05). However, there were no significant differences detected between flavanol groups at baseline in any variable. Racial/ethnic categorization of subjects who completed
the intervention was 94% Caucasian, 4% black and 2% Native American, with no significant
difference in race/ethnicity between study completers and non-completers or between the AF and NF
groups.

Snack Compliance

Compliance with the daily beverages and twice daily snacks were greater than 90% for both
groups. Neither beverage nor snack compliance differed significantly between groups.

Dietary Intake

At baseline, mean daily energy intake was 2023 ± 363 kcals and 2106 ± 525 kcals for the AF
and NF groups, respectively. After 18 weeks, the AF group reduced estimated daily energy intake
by 416 kcals (P < 0.001) and the NF group reduced energy intake by 529 kcals (P < 0.001). The
macronutrient composition of the ERD did not significantly change for the AF group; however,
percent of energy from protein increased in the NF group (P < 0.01) over time. Estimated calcium
and vitamin D intakes did not significantly change in either group over time. Estimated energy,
macronutrient, calcium and vitamin D intakes were not significantly different between the AF and
NF groups at baseline or week 18 or in the changes over time (Table 2).

Physical Activity

At baseline, physical activity (hr/day) was significantly greater in the first cohort compared
to the second cohort of subjects (P < 0.05) according to the PAR. Therefore, each group (AF and
NF) within each cohort (1 and 2) were treated as a separate group, creating four groups. For each
flavanol by cohort group (AF-1, AF-2, NF-1, NF-2), a physical activity change score was calculated
and then compared among groups using ANOVA. Change from baseline to week 18 for total
physical activity was not significantly different between groups. A repeated measures ANOVA was
used to analyze the effect of time on physical activity within each of the four cohort groups. There were no significant differences within any group at any interval.

**Anthropometric Measurements and Body Composition**

The ERD resulted in a mean weight loss for all women (n=50) of 5.5 kg (P < 0.001), with the AF and NF group reducing BW by 5.1 kg (P < 0.001) and 5.9 kg (P < 0.001), respectively, from baseline to week 18 (**Table 3**). Changes in BW in the AF and NF group were significantly different compared to baseline at each subsequent interval. The AF group had significant decreases in waist and hip circumferences (**Table 3**) and FM and abFM (**Table 4**) (all P < 0.001 for changes from baseline to week 18). The NF group had significant decreases in waist and hip circumferences (**Table 3**) (both P < 0.001 from baseline to week 18) and FM (P < 0.001), FFM (P < 0.05) and abFM (**Table 4**) (P < 0.01 for changes from baseline to week 18). There were no significant differences in BW, waist or hip circumferences, FM, FFM or abFM between cohorts or between the AF and NF groups at any interval throughout the intervention or in the change over time.

**Bone Mineral Density**

There was a significant effect of cohort on hip and FA BMD (**Figure 1**). At week 18, FA BMD was significantly greater in cohort 1 (P < 0.01). Changes over time for hip (P < 0.01) and FA BMD (P < 0.001) were significantly different between cohorts and the increases in hip and FA BMD (both P < 0.001) from baseline to week 18 were significant in cohort 1 while cohort 2 had no significant changes in BMD measurements.

There was a significant effect of time on BMD within the AF and NF groups. After 18 weeks, the AF group had significant increases in hip and FA BMD (both P < 0.01) with no change in TB or LS BMD (**Table 4**). The NF group had a significant increase in FA BMD (P < 0.001) after 18 weeks with no change in TB, LS or hip BMD (**Table 4**). There were no significant differences in
TB, LS, hip and FA BMD between the AF and NF groups at baseline or week 18 or in the change over time.

**Oxidative Stress**

After 18 weeks, neither the AF nor the NF groups had statistically significant changes in 8-epi-PGF$_{2\alpha}$ concentration (Table 5). There was no significant difference in this marker of oxidative stress between cohorts or between the AF and NF groups at any interval throughout the intervention or in the change over time.

**Inflammation**

A significant effect of cohort was evident for IL-1β and TNF-α concentrations. TNF-α was significantly greater in cohort 1 at weeks 6 and 12 (both P < 0.001) (Figure 2). Change in TNF-α concentration over time was not significantly different between cohorts (P < 0.001) but the change from baseline to week 18 within cohort 1 was significant (P < 0.01) while it was not significant for cohort 2. IL-1β was significantly greater in cohort 2 at week 6 (P < 0.05). Change over time was not significantly different between cohorts and the change from baseline to week 18 was significant for both cohort 1 and 2 (P < 0.001) (Figure 3).

There was a significant effect of time on inflammatory markers between the AF and NF groups. From baseline to week 6, the AF group had a significant reduction in TNF-α (P < 0.05) that continued to week 18 (P < 0.01) (Table 5). The AF group also had reductions in IL-6 and IL-1β concentrations from baseline to week 6 (both P < 0.001) that persisted to week 18 (both P < 0.001) (Table 5). The NF group had significant reductions in IL-6 (P < 0.001) and IL-1β (P < 0.001) concentrations from baseline to week 6 that continued to week 18 (both P < 0.001) (Table 5). As previously noted, IL-1β concentrations were non-detectable in 80% of serum samples for both groups at weeks 12 and 18. To conduct statistical analyses, the value 0.1 pg/mL was entered for all
samples with non-detectable levels of IL-1β. The AF group had a reduction in CRP concentration (P < 0.01) from baseline to week 12 that returned to baseline levels by week 18. The NF group also experienced a significant reduction in CRP concentration (P < 0.05) at week 6 that returned to baseline levels by week 18 (Table 5). Changes in CRP concentrations from baseline to week 18 were not significant within the AF or NF groups. There was not a significant effect of cohort on IL-6 or CRP concentrations at any interval throughout the intervention or in the change over time. There were no significant differences in inflammatory markers between the AF and NF groups at any interval throughout the intervention or in the change over time.

**Bone Turnover and Mineral Metabolism**

OPG, NTx and RANKL concentrations also demonstrated significant cohort effects. OPG concentration was significantly greater in cohort 2 at weeks 6 (P < 0.001) and 12 (P < 0.01). Change in OPG concentration over time was significantly different between cohorts (P < 0.001) and the increase from baseline to week 18 within cohort 2 was significant (P < 0.001) while it was not significant for cohort 1 (Figure 4). NTx concentration was significantly higher in cohort 1 at baseline (P < 0.001) and at weeks 6 (P < 0.01) and 12 (P < 0.05). Change in NTx concentrations over time was significantly different (P < 0.05) between cohorts (Figure 5) and the change from baseline to week 18 was significant within cohort 1 (P < 0.01) while it was not significant for cohort 2. RANKL concentration was significantly greater in cohort 2 at week 6 (P < 0.001) and significantly greater in cohort 1 at week 12 (P < 0.01). Change in RANKL from over time was not significant within either cohort and the change from baseline to week 18 was not significantly different between cohorts (Figure 6).

Within the AF and NF groups, there was a significant effect of time on markers of bone turnover. The AF (P < 0.01) and NF (P < 0.05) groups had decreases in serum OPG concentrations
from baseline to week 6; however, by week 18, OPG had significantly increased in the AF (P < 0.01) and NF (P < 0.001) groups (Table 6). Serum OC, NTx and RANKL did not significantly change in either the AF or NF group over time, and there were no significant differences in any marker of bone turnover between the AF and NF groups at any interval throughout the intervention or in the change over time.

The effect of time was significant for mineral metabolism within the AF and NF groups. The AF group had a significant increase in serum calcium concentration from baseline to week 6 (P < 0.01) that returned to baseline levels by week 18 (Table 6). The AF group also had a significant increase in serum phosphorus concentration at week 6 (P < 0.001) that returned to baseline levels by week 18. The NF group had a significant increase in serum phosphorus from baseline to week 6 (P < 0.001), which persisted to week 18 (P < 0.001). Neither group had significant changes in serum or urinary calcium or urinary phosphorus concentrations over time. There were no significant differences in any marker of mineral metabolism between cohorts or between the AF and NF groups at any interval throughout the intervention or in the change over time.

**DISCUSSION**

A weight-loss dietary intervention in which women consumed additional flavanols in the form of a daily HF cocoa beverage and twice daily DC snack versus a daily vanilla beverage and twice daily licorice snack with no additional flavanols was conducted to examine the effect of a combined weight-loss and flavanol intervention on oxidative stress, inflammation and bone health. This intervention demonstrated that following BW loss, overweight and obese women in both groups had similar and significant reductions in IL-6 and IL-1β while neither group had a change in 8-epi-PGF$_2$α concentration. Women who consumed additional flavanols also had a significant decrease in TNF-α concentration, though the overall reductions in inflammatory markers in the AF group were
not greater than those experienced by the group that did not consume the additional flavanols. Despite evidence that cocoa flavanols can reduce ROS and pro-inflammatory cytokines \textit{in vitro} (38-40), results from this study indicated that cocoa flavanol consumption at a level of 520 mg/day did not change markers of oxidative stress or inflammation \textit{in vivo} (50-52). Furthermore, this study demonstrated that the combination of BW loss and consumption of a HF cocoa beverage and DC twice daily did not induce a greater decrease in oxidative stress or inflammation in overweight or obese women.

The present intervention also demonstrated that overweight or obese premenopausal women following a nutritionally adequate ERD for 18 weeks were able to significantly reduce BW without experiencing a negative effect in bone turnover, mineral metabolism or BMD. Following the intervention, individuals in both groups experienced an increase in their OPG concentrations without substantial changes in markers of bone resorption. An increase in OPG concentration, a decoy receptor that inhibits osteoclast activity, indicates a potential shift in the skeletal balance towards bone formation. Both groups also experienced gains in FA BMD with the AF group also demonstrating a significant gain in BMD at the hip. While the magnitude of increases in FA and hip BMD demonstrated in this study do not translate into significant reductions in fracture risk (53,54) and therefore, may not be clinically significant, women did not experience reductions in BMD following short-term BW loss. Similar to inflammation results, women who consumed additional flavanols did not experience greater reductions in markers of bone resorption, greater increases in bone formation or greater increases in BMD following the 18-week intervention. The current study shows that BW loss in overweight or obese premenopausal women does not have to result in negative changes in bone turnover or BMD, at least in the short-term.
In vitro research has suggested a potential role for flavanols in the reduction of oxidative stress and inflammation (38,55); the current study, however, found no effect of consuming 520 mg of additional flavanols during BW loss. The lack of additional change in markers of inflammation in the AF group may be attributed to multiple factors. Compared to other antioxidants such as vitamin C and E, absorption of flavanols is very low and excretion rates are rapid (56). The flavanols in this intervention were presented within a food matrix; therefore, absorption may have been further reduced due to the interference of other sugars and fats present in the DC (57). In addition, flavanol levels peak in human serum approximately two hours after consumption and typically return to baseline concentrations within four hours (58). The biological effect of long-term flavanol consumption is also limited as flavanols do not accumulate in the plasma (59). Cumulatively, their brief presence in serum, rapid excretion rate and inability to accumulate reduces the ability of flavanols to exert an antioxidant effect in vivo (60). Constant DC consumption throughout the day may be needed to maintain serum concentrations at levels high enough to observe a significant anti-inflammatory effect. It is also possible that the dose of daily flavanols used in the current study was not enough to produce an anti-inflammatory effect in a healthy sample of women. Though previous research has demonstrated a promising effect of cocoa flavanols in vitro, the current study suggests that the anti-inflammatory effects do not translate into decreases in systemic inflammation in a human model when additional flavanols are consumed in the form of cocoa products.

While there were no differences between the AF and NF groups in inflammatory markers, a significant cohort effect was evident in TNF-α and IL-1β concentrations. TNF-α concentration was different between cohorts at week 6 only, a point at which cohort 1 experienced a significant spike in TNF-α concentration. Though the change over time was only significant for cohort 1, both cohorts exhibited a reduction in TNF-α concentration from baseline levels. In addition, while IL-1β
concentration appeared to decrease at a faster rate for cohort 1, both cohorts experienced similar and significant decreases in IL-1β concentrations by week 18. Because TNF-α and IL-1β tended to decline in all women for both cohorts 1 and 2 over time, it is probable that BW loss was the precipitating factor in the decrease of inflammatory proteins and cohort did not differentially affect inflammatory outcomes in this study.

Chronic health conditions in which individuals exhibit greater levels of oxidative stress (30,31) and inflammation (32,36,61,62) are often associated with a lower BMD. Greater levels of circulating pro-inflammatory cytokines have also been linked to increased risk of fracture (63) and higher incidence of osteoporosis (32). This may be due to the influence oxidative stress and pro-inflammatory cytokines has on the bone remodeling process. ROS produced under conditions of oxidative stress can increase the expression and secretion of RANKL, a ligand that stimulates osteoclast differentiation and bone resorption *in vitro* (26). In addition, ROS can decrease osteoblast activity (27). Pro-inflammatory cytokines have been shown to increase bone resorption *in vitro* as well. IL-17, a pro-inflammatory cytokine, can increase expression of RANKL and stimulate the secretion of TNF-α and IL-1β (64). TNF-α and IL-1β secretion further induces RANKL and promotes osteoclast differentiation (28) while simultaneously inhibiting the action of OPG (29). These actions can uncouple the bone remodeling process, leading to an upregulation of resorption and an inhibition of formation which over time can result in net bone loss. Therefore, conditions associated with oxidative stress and chronically elevated levels of pro-inflammatory cytokines, such as obesity, have the potential to continuously induce bone resorption and reduce BMD over time (65). Despite the purported beneficial contribution of FM on skeletal loading (21), the negative effect of inflammation induced by excess adiposity may be greater than the protection it exerts on BMD over time (34-36).
Previous work has shown a possible benefit of antioxidant consumption in maintaining bone health. In cross-sectional and longitudinal studies, consuming foods high in flavanols (41) and both consumption of or supplementation with isoflavones (42,43) and vitamin C (44,45) have been associated with higher BMD and a reduced risk of fracture. Experimental studies have also provided promising evidence linking reductions in oxidative stress and inflammation to improvements in bone. Ascorbate supplementation was shown to suppress oxidative stress and inhibit osteoclast differentiation in mice (66,67). A study in a rat model of periodontitis demonstrated that a diet high in cocoa flavanols was associated with a reduction in oxidative stress and a subsequent decrease in bone loss after four weeks (55). In a clinical intervention study, subjects demonstrated reductions in both oxidative stress and bone resorption after 4 months of supplementation with lycopene (46).

Because of the potential for antioxidants to protect bone health, it was theorized that women who consumed additional flavanols would have a greater decrease in oxidative stress and inflammation which would translate into a larger reduction in bone resorption, a greater increase in bone formation and maintenance of BMD following BW loss. Instead, women in both groups experienced increases in OPG and BMD, regardless of flavanol consumption.

The maintenance of bone mass demonstrated by the women in this weight-loss intervention could be due to a variety of reasons. Other weight-loss interventions can attribute bone loss to a reduction in estrogen concentrations as seen in postmenopausal women (17,19,68), rapid BW loss due to highly restrictive energy intake (69), decreased calcium and vitamin D intake (18,68,70) or bariatric surgery (71-73). Estrogen, a hormone that moderates osteoclastic activity, decreases with BW loss (74). This predisposes postmenopausal women who have reduced estrogen concentrations to bone loss following BW reduction (18,19,68). This was likely not a factor in the current study as all women were premenopausal. Inadequate macro- or micronutrient consumption due to a
substantial reduction in energy intake may also lead to bone loss. In weight-loss interventions that involve energy restrictions without supplementation, a reduction in BMD is often associated with inadequate vitamin or mineral consumption (18,19). Conversely, interventions with ERDs that provide adequate calcium and vitamin D or supplementation of these nutrients during BW loss often demonstrate maintenance or improvement in BMD (18,19,73,75,76). The current study found similar results. Despite a moderate energy deficit, women in the current weight-loss intervention were able to maintain protein, calcium and vitamin D intake, all of which have been shown to positively affect BMD and markers of bone formation (77).

Many interventions in which BMD is reduced during weight loss failed to address physical activity (17,18,19,68,71,72,73). In studies where supplementation of calcium or vitamin D or additional energy did not fully ameliorate bone loss, researchers attributed the loss of bone mass to a reduction in load-bearing on the skeleton (18). The incorporation of weight-bearing exercise such as walking or resistance training has been shown to counteract the reduction in mechanical stress due to a loss of BW and lead to maintenance or improvement in BMD (78-80). The current intervention, however, did not include an exercise component; instead women were asked to continue their existing physical activity regimen. According to the PAR, all women within each cohort and each group maintained their physical activity level, yet their BMD was maintained or improved at certain sites. This may be explained by several factors. First of all, the tool used in the current study to measure physical activity may not have been sensitive enough to detect a significant change in activity and it is also based on subject self-report which can be unreliable (81). As the women lost BW they may have unknowingly increased their daily activities and the inclusion of an accelerometer or pedometer may have provided a more accurate measure of activity (82). In addition, eligible participants were allowed to engage in up to five hours of physical activity per
week, therefore, maintaining only a few hours of physical activity each week may have been adequate to protect BMD during weight loss.

Lastly, this intervention demonstrated significant cohort effects associated with changes in markers of bone turnover and BMD which was may have been attributed to seasonality. Women in the first cohort began the study in July (i.e., summer) and finished in early December, whereas the second cohort began in early spring and ended in July. Women in cohort 1 demonstrated a decrease in NTx, a marker of bone resorption, had no changes in OPG or RANKL concentrations yet experienced significant increases in hip and FA BMD. Cohort 2, on the other hand, had no change in both NTx and RANKL concentrations and despite an increase in OPG also had non-significant changes in BMD. Results from this intervention are consistent with other studies that demonstrate hip, spine and total body BMD peak in the summer months, gradually decline during the winter months and reach their lowest point between January and March (83-87). This study and others also show that declines in BMD are typically accompanied by an increase in markers of bone resorption (84,85,87,88) but not necessarily a decrease in markers of bone formation. Interestingly, markers of bone formation tend to either increase or remain unchanged in conjunction with a decrease in BMD (84,85,87). The decrease in NTx concentration in cohort 1 may have been sufficient to significantly increase BMD of the hip and FA without the necessity of a concomitant increase in bone formation. Conversely, the absence of change in BMD for cohort 2 may have been attributed to the stability of their bone resorption markers.

These results indicate that cohort 1 and 2 were likely at two different stages in the bone formation cycle. Women in cohort 1 were tested in July as they were attaining their peak yearly BMD, results supported by the significant decrease in NTx concentration and increases in hip and FA BMD. Conversely, cohort 2 began the intervention in March when they were likely at the trough
of their yearly BMD. Results do suggest, however, that cohort 2 may have been experiencing a shift
towards bone formation as demonstrated by their significant increase in OPG and stable resorption
markers at week 18. Since this intervention only lasted 18 weeks, it is possible that if followed for
an additional 2-3 months, a significant increase in BMD may have become evident in cohort 2 while
the opposite effect may have occurred in cohort 1. Because all women within their respective cohort
experienced a similar pattern in terms of bone turnover and BMD, the changes observed in the
current intervention appear to be an effect of seasonality rather than the result of BW loss, flavanol
consumption or physical activity.

Cumulatively, results from the current intervention suggest three key points. First, BW loss
was likely responsible for the reduction in inflammation. Secondly, women in both flavanol groups
experienced decreases in markers of inflammation and increases in BMD indicating that a potential
relationship between a reduction of inflammation and improvements in bone health cannot be ruled
out. Because it typically takes 6 to 12 months to observe clinical changes in BMD, a targeted
intervention aimed at reducing inflammation may produce a more significant effect on BMD if it
lasts for 6 months or more. Finally, in overweight or obese premenopausal women without
underlying metabolic or bone diseases, there was no greater health benefit of consuming
approximately 520 mg of flavanols during BW loss. Therefore, this study indicates that with only a
moderate restriction of energy, adequate nutrient intake and maintenance of physical activity, bone
markers and BMD can remain unaltered in premenopausal women following short-term BW loss.

Sample characteristics could be a potential limitation in the current study. This study was
conducted in premenopausal women who were in general good health with oxidative stress and
inflammatory profiles within the normal range at baseline. There may have been greater reductions
in markers of oxidative stress or inflammation if conducted in a sample with higher inflammatory
status at baseline. In addition, this study was part of a larger study which was originally powered to
detect changes in BW and BMD. Because only 25 women in each group completed the study, the
ability to detect a differential effect of flavanol consumption on markers such as CRP, 8-epi-PGF$_{2\alpha}$
and bone resorption markers may have been limited due to their high variability. The significant
effect of cohort seasonality may also have limited the ability to detect changes in BMD that may
have been associated with weight loss or flavanol consumption.

In conclusion, the current study demonstrated that overweight and obese women who lost
BW had significant reductions in markers of inflammation without appreciably affecting bone
resorption markers, mineral metabolism or BMD. Further, consuming additional flavanols did not
provide significantly greater reductions in markers of oxidative stress, inflammation or bone
resorption following BW loss. Finally, women were able to enjoy two different types of sweet snack
twice daily while following an ERD without inhibiting beneficial changes in inflammation or
negatively affecting indicators of bone health. Therefore, healthy, overweight and obese
premenopausal women who follow an 18-week ERD can reduce markers of inflammation and the
risk of developing chronic diseases through moderate BW loss without detrimentally affecting bone
mass in the short-term.
REFERENCES


Table 5.1 Baseline characteristics by flavanol group

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>AF Group (n=25)</th>
<th>NF Group (n=25)</th>
<th>AF vs. NF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>36.0 ± 5.7</td>
<td>36.3 ± 5.9</td>
<td>NS</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>164.3 ± 4.6</td>
<td>165.4 ± 7.0</td>
<td>NS</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>83.1 ± 13.6</td>
<td>84.9 ± 13.7</td>
<td>NS</td>
</tr>
<tr>
<td>Body Mass Index (kg/m²)</td>
<td>30.5 ± 4.7</td>
<td>31.0 ± 4.0</td>
<td>NS</td>
</tr>
<tr>
<td>Fat Mass (kg)</td>
<td>31.6 ± 8.4</td>
<td>32.6 ± 7.6</td>
<td>NS</td>
</tr>
<tr>
<td>Fat-Free Mass (kg)</td>
<td>47.6 ± 5.6</td>
<td>48.7 ± 6.9</td>
<td>NS</td>
</tr>
<tr>
<td>Abdominal FM (kg)</td>
<td>3.2 ± 1.3</td>
<td>3.4 ± 1.1</td>
<td>NS</td>
</tr>
<tr>
<td>TNF-α (pg/mL)</td>
<td>13.4 ± 3.7</td>
<td>12.0 ± 3.1</td>
<td>NS</td>
</tr>
<tr>
<td>IL-6 (pg/mL)</td>
<td>2.0 ± 0.7</td>
<td>1.8 ± 0.7</td>
<td>NS</td>
</tr>
<tr>
<td>CRP (ng/mL)</td>
<td>4043 ± 3552</td>
<td>4335 ± 3554</td>
<td>NS</td>
</tr>
<tr>
<td>IL-1β (pg/mL)</td>
<td>0.61 ± 0.14</td>
<td>0.63 ± 0.21</td>
<td>NS</td>
</tr>
<tr>
<td>8-epi-PGF$_{2α}$ (ng/dL)</td>
<td>1.9 ± 4.0</td>
<td>3.1 ± 6.2</td>
<td>NS</td>
</tr>
<tr>
<td>Serum OC (ng/mL)</td>
<td>10.2 ± 3.9</td>
<td>11.1 ± 6.2</td>
<td>NS</td>
</tr>
<tr>
<td>Serum OPG (mol/L)</td>
<td>2.6 ± 1.5</td>
<td>2.7 ± 1.0</td>
<td>NS</td>
</tr>
<tr>
<td>Serum NTx (nM BCE)</td>
<td>11.8 ± 3.2</td>
<td>12.5 ± 3.3</td>
<td>NS</td>
</tr>
<tr>
<td>Serum RANKL (pmol/L)</td>
<td>0.26 ± 0.16</td>
<td>0.32 ± 0.21</td>
<td>NS</td>
</tr>
<tr>
<td>Serum Calcium (mg/dL)</td>
<td>9.6 ± 0.6</td>
<td>9.8 ± 0.5</td>
<td>NS</td>
</tr>
<tr>
<td>Serum Phosphorus (mg/dL)</td>
<td>3.5 ± 0.5</td>
<td>3.4 ± 0.5</td>
<td>NS</td>
</tr>
<tr>
<td>Urine Calcium (mg/24hr)</td>
<td>121.3 ± 59.0</td>
<td>130.0 ± 54.6</td>
<td>NS</td>
</tr>
<tr>
<td>Urine Phosphorus (g/24hr)</td>
<td>1.10 ± 0.51</td>
<td>1.00 ± 0.40</td>
<td>NS</td>
</tr>
<tr>
<td>Total Body BMD (g/cm²)</td>
<td>1.25 ± 0.11</td>
<td>1.22 ± 0.08</td>
<td>NS</td>
</tr>
<tr>
<td>Lumbar Spine BMD (L1-L4) (g/cm²)</td>
<td>1.12 ± 0.14</td>
<td>1.10 ± 0.17</td>
<td>NS</td>
</tr>
<tr>
<td>Hip BMD (g/cm²)</td>
<td>1.03 ± 0.11</td>
<td>1.01 ± 0.10</td>
<td>NS</td>
</tr>
<tr>
<td>Forearm BMD (g/cm²)</td>
<td>0.58 ± 0.04</td>
<td>0.57 ± 0.04</td>
<td>NS</td>
</tr>
</tbody>
</table>

P-values are for between group differences at baseline using independent $t$-tests.
TNF-α, tumor necrosis factor-α; IL-6, Interleukin-6; CRP, C-reactive protein; IL-1β, Interleukin-1β; 8-epi-PGF₂α, 8-epi-Prostaglandin F₂α; OC, Osteocalcin; OPG, Osteoprotegrin; NTx, cross-linked N-teleopeptide of type I collagen; RANKL, Receptor Activator for Nuclear Factor κ B Ligand; BMD, body bone mineral density; AF, additional flavanols; NF, no additional flavanols.
Table 5.2 Estimated daily dietary intake by flavanol group at baseline and week 18

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Group</th>
<th>Baseline</th>
<th>Week 18</th>
<th>Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Daily Energy Intake (kcal)</td>
<td>AF</td>
<td>2023 ± 363</td>
<td>1607 ± 323</td>
<td>-416 ± 419 ***</td>
</tr>
<tr>
<td></td>
<td>NF</td>
<td>2106 ± 525</td>
<td>1577 ± 267</td>
<td>-529 ± 468 ***</td>
</tr>
<tr>
<td>Carbohydrate Distribution (%)</td>
<td>AF</td>
<td>50.1 ± 9.6</td>
<td>49.0 ± 4.0</td>
<td>-1.1 ± 10.4</td>
</tr>
<tr>
<td></td>
<td>NF</td>
<td>51.6 ± 7.6</td>
<td>51.9 ± 7.8</td>
<td>+0.3 ± 6.7</td>
</tr>
<tr>
<td>Fat Distribution (%)</td>
<td>AF</td>
<td>34.1 ± 5.9</td>
<td>33.3 ± 5.1</td>
<td>-0.8 ± 6.3</td>
</tr>
<tr>
<td></td>
<td>NF</td>
<td>32.5 ± 6.6</td>
<td>30.6 ± 7.7</td>
<td>-1.9 ± 7.0</td>
</tr>
<tr>
<td>Protein Distribution (%)</td>
<td>AF</td>
<td>15.7 ± 2.4</td>
<td>16.1 ± 2.7</td>
<td>+0.4 ± 2.7</td>
</tr>
<tr>
<td></td>
<td>NF</td>
<td>14.7 ± 2.3</td>
<td>16.5 ± 3.3</td>
<td>+1.8 ± 3.2 **</td>
</tr>
<tr>
<td>Alcohol Distribution (%)</td>
<td>AF</td>
<td>1.3 ± 2.3</td>
<td>1.6 ± 3.4</td>
<td>+0.3 ± 2.9</td>
</tr>
<tr>
<td></td>
<td>NF</td>
<td>1.2 ± 3.7</td>
<td>0.9 ± 2.6</td>
<td>-0.3 ± 4.0</td>
</tr>
<tr>
<td>Calcium Intake (mg/day)</td>
<td>AF</td>
<td>882 ± 393</td>
<td>770 ± 256</td>
<td>-112 ± 392</td>
</tr>
<tr>
<td></td>
<td>NF</td>
<td>830 ± 295</td>
<td>705 ± 215</td>
<td>-125 ± 315</td>
</tr>
<tr>
<td>Vitamin D (IU/day)</td>
<td>AF</td>
<td>78.2 ± 66.7</td>
<td>81.1 ± 66.6</td>
<td>+2.8 ± 70.4</td>
</tr>
<tr>
<td></td>
<td>NF</td>
<td>82.5 ± 65.5</td>
<td>93.9 ± 63.2</td>
<td>+11.4 ± 83.7</td>
</tr>
</tbody>
</table>

Values are means ± SD.
AF, additional flavanol, Group (n=25); NF, no additional flavanol, Group (n=25).
*P < 0.05, **P < 0.01, ***P < 0.001; P-value analyzed using paired t-tests for within group changes from baseline.
There were no significant differences between groups at baseline or week 18 or for the change over time between groups analyzed using independent t-tests.
Table 5.3 Anthropometric measurements by flavanol group at all intervals

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Group</th>
<th>Baseline</th>
<th>Week 6</th>
<th>Week 12</th>
<th>Week 18</th>
<th>Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body Weight (kg)</td>
<td>AF</td>
<td>83.1 ± 13.6</td>
<td>79.6 ± 13.8***</td>
<td>77.2 ± 14.1***</td>
<td>78.0 ± 17.8***</td>
<td>-5.1 ± 2.9***</td>
</tr>
<tr>
<td></td>
<td>NF</td>
<td>84.9 ± 13.7</td>
<td>81.8 ± 13.9***</td>
<td>80.1 ± 14.3***</td>
<td>79.0 ± 14.2***</td>
<td>-5.9 ± 4.8***</td>
</tr>
</tbody>
</table>

|                     |         |           |           |           |           |            |
|体脂率 (kg/m²)       | AF      | 30.5 ± 4.7| 29.4 ± 4.9*** | 29.2 ± 4.2*** | 28.7 ± 5.3*** | -1.8 ± 1.1*** |
|                      | NF      | 31.0 ± 4.0| 29.8 ± 4.0*** | 28.4 ± 4.8*** | 28.8 ± 10.5*** | -2.2 ± 1.8*** |

| Waist Circumference  | AF      | 88.4 ± 10.0| 85.0 ± 10.6*** | 82.8 ± 9.7*** | 82.7 ± 10.9*** | -5.7 ± 5.1*** |
|                      | NF      | 92.1 ± 11.1| 88.1 ± 10.6*** | 86.4 ± 10.7*** | 86.0 ± 10.5*** | -6.1 ± 5.3*** |

| Hip Circumference    | AF      | 115.3 ± 10.1| 110.9 ± 10.0*** | 110.7 ± 10.2*** | 109.3 ± 11.3*** | -6.0 ± 3.9*** |
|                      | NF      | 116.3 ± 9.5 | 112.0 ± 9.8*** | 109.7 ± 10.9*** | 110.5 ± 10.0*** | -5.8 ± 3.8*** |

Values are means ± SD.
AF, additional flavanols, Group (n=25); NF, no additional flavanols, Group (n=25).
***P < 0.001; P-value analyzed using analysis of variance with repeated measures on the time factor for within group changes from baseline.
There were no differences between groups at any interval or in the change over time between groups using 2 x 4 analysis of variance with repeated measures on the time factor.
Table 5.4 Body composition and bone mineral density by flavanol group at baseline and week 18

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Group</th>
<th>Baseline</th>
<th>Week 18</th>
<th>Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fat Mass (kg)</td>
<td>AF</td>
<td>31.6 ± 8.4</td>
<td>27.8 ± 9.1</td>
<td>-3.8 ± 1.8***</td>
</tr>
<tr>
<td></td>
<td>NF</td>
<td>32.6 ± 7.6</td>
<td>28.4 ± 8.1</td>
<td>-4.2 ± 3.4***</td>
</tr>
<tr>
<td>Fat-Free Mass (kg)</td>
<td>AF</td>
<td>47.6 ± 5.6</td>
<td>47.2 ± 5.8</td>
<td>-0.4 ± 2.0</td>
</tr>
<tr>
<td></td>
<td>NF</td>
<td>48.7 ± 6.9</td>
<td>48.0 ± 8.1</td>
<td>-0.7 ± 1.6*</td>
</tr>
<tr>
<td>Abdominal Fat Mass (kg)</td>
<td>AF</td>
<td>3.2 ± 1.3</td>
<td>2.8 ± 1.4</td>
<td>-0.4 ± 0.3***</td>
</tr>
<tr>
<td></td>
<td>NF</td>
<td>3.4 ± 1.1</td>
<td>3.0 ± 1.2</td>
<td>-0.4 ± 0.5**</td>
</tr>
<tr>
<td>Total Body BMD (g/cm²)</td>
<td>AF</td>
<td>1.25 ± 0.11</td>
<td>1.25 ± 0.10</td>
<td>+0.01 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>NF</td>
<td>1.22 ± 0.08</td>
<td>1.22 ± 0.08</td>
<td>+0.01 ± 0.03</td>
</tr>
<tr>
<td>Spine BMD (g/cm²)</td>
<td>AF</td>
<td>1.12 ± 0.14</td>
<td>1.12 ± 0.14</td>
<td>0.0 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>NF</td>
<td>1.10 ± 0.17</td>
<td>1.08 ± 0.08</td>
<td>-0.02 ± 0.13</td>
</tr>
<tr>
<td>Hip BMD (g/cm²)</td>
<td>AF</td>
<td>1.03 ± 0.11</td>
<td>1.05 ± 0.10</td>
<td>+0.02 ± 0.03**</td>
</tr>
<tr>
<td></td>
<td>NF</td>
<td>1.01 ± 0.10</td>
<td>1.02 ± 0.11</td>
<td>+0.01 ± 0.03</td>
</tr>
<tr>
<td>Forearm BMD (g/cm²)</td>
<td>AF</td>
<td>0.58 ± 0.04</td>
<td>0.59 ± 0.04</td>
<td>+0.01 ± 0.01**</td>
</tr>
<tr>
<td></td>
<td>NF</td>
<td>0.57 ± 0.04</td>
<td>0.59 ± 0.04</td>
<td>+0.02 ± 0.02***</td>
</tr>
</tbody>
</table>

Values are means ± SD.
AF, additional flavanols, Group (n=25); NF, no additional flavanols, Group (n=25).
*P < 0.05, **P < 0.01, ***P < 0.001; P-value analyzed using paired t-tests for within group changes from baseline. There were no differences between groups at baseline or week 18 or in the change over time between groups using independent t-tests.
BMD, body bone mineral density.
Figure 5.1 Change in bone mineral density from baseline to week 18 by cohort

**P< 0.01; Forearm BMD was significantly greater in cohort 1 at week 18 as analyzed using independent t-tests.

Changes in hip (P < 0.01) and forearm BMD (P < 0.001) over time were significantly different between cohorts as analyzed using independent t-tests.

§The increases in hip and forearm BMD from baseline to week 18 were significant (P < 0.001) in cohort 1 but not in cohort 2 as analyzed using paired t-tests.

BMD, bone mineral density
Table 5.5 Markers of oxidative stress and inflammation by flavanol group at all intervals

<table>
<thead>
<tr>
<th>Marker</th>
<th>Group</th>
<th>Baseline</th>
<th>Week 6</th>
<th>Week 12</th>
<th>Week 18</th>
<th>Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>8-epi-PGF$_2$α (ng/dL)</td>
<td>AF</td>
<td>1.9 ± 4.0</td>
<td>2.2 ± 2.1</td>
<td>2.5 ± 2.7</td>
<td>4.0 ± 5.6</td>
<td>+2.1 ± 5.8</td>
</tr>
<tr>
<td></td>
<td>NF</td>
<td>3.1 ± 6.2</td>
<td>2.5 ± 3.6</td>
<td>5.6 ± 8.1</td>
<td>4.1 ± 5.6</td>
<td>+0.98 ± 4.3</td>
</tr>
<tr>
<td>TNF-α (pg/mL)</td>
<td>AF</td>
<td>13.4 ± 3.7</td>
<td>13.7 ± 7.6*</td>
<td>11.0 ± 2.9*</td>
<td>10.7 ± 1.0**</td>
<td>-2.7 ± 3.8**</td>
</tr>
<tr>
<td></td>
<td>NF</td>
<td>12.0 ± 3.1</td>
<td>15.3 ± 7.0</td>
<td>11.2 ± 2.9</td>
<td>10.8 ± 1.4</td>
<td>-1.2 ± 3.8</td>
</tr>
<tr>
<td>IL-6 (pg/mL)</td>
<td>AF</td>
<td>2.0 ± 0.7</td>
<td>1.1 ± 1.0***</td>
<td>0.9 ± 0.7***</td>
<td>1.0 ± 0.6***</td>
<td>-0.97 ± 0.69***</td>
</tr>
<tr>
<td></td>
<td>NF</td>
<td>1.8 ± 0.7</td>
<td>1.2 ± 0.8***</td>
<td>1.0 ± 0.7***</td>
<td>1.0 ± 0.6***</td>
<td>0.81 ± 0.74***</td>
</tr>
<tr>
<td>CRP (ng/mL)</td>
<td>AF</td>
<td>4043 ± 3552</td>
<td>3793 ± 4400</td>
<td>2723 ± 2429**</td>
<td>3242 ± 2306</td>
<td>-801 ± 2560</td>
</tr>
<tr>
<td></td>
<td>NF</td>
<td>4335 ± 3554</td>
<td>3350 ± 3616*</td>
<td>3427 ± 3355</td>
<td>3360 ± 2455</td>
<td>-976 ± 2693</td>
</tr>
<tr>
<td>IL-1β (pg/mL)</td>
<td>AF</td>
<td>0.61 ± 0.14</td>
<td>0.29 ± 0.28***</td>
<td>0.11 ± 0.0***</td>
<td>0.11 ± 0.06***</td>
<td>-0.50 ± 0.14***</td>
</tr>
<tr>
<td></td>
<td>NF</td>
<td>0.63 ± 0.21</td>
<td>0.25 ± 0.21***</td>
<td>0.13 ± 0.09***</td>
<td>0.13 ± 0.10***</td>
<td>-0.51 ± 0.24***</td>
</tr>
</tbody>
</table>

Values are means ± SD

AF, additional flavanols, Group (n=25); NF, no additional flavanols, Group (n=25).

* P < 0.05; **P < 0.01; ***P < 0.001; P-value analyzed using analysis of variance with repeated measures on the time factor for within group changes from baseline.

There were no differences between groups at any interval or in the change over time using analysis of variance with repeated measures on the time factor.

8-epi-PGF$_2$α, 8-epi-Prostaglandin F$_2$α; TNF-α, tumor necrosis factor-α; IL-6, Interleukin-6, CRP, C-reactive protein; IL-1β, Interleukin-1β.
Values are means ± SEM

***P < 0.001; Tumor necrosis factor-α was significantly greater in cohort 1 at week 6 and week 12 as analyzed using 2 x 4 analysis of variance with repeated measures on the time factor. Changes over time were not significantly different between cohorts as analyzed using independent t-tests.

§The decrease in tumor necrosis factor-α from baseline to week 18 was significant (P < 0.01) in cohort 1 but not in cohort 2 as analyzed using paired t-tests.
Figure 5.3 Interleukin-1β concentration at each interval by cohort

Values are means ± SEM
*P < 0.05; Interleukin-1β was significantly greater in cohort 2 at week 6 as analyzed using 2 x 4 analysis of variance with repeated measures on the time factor.
Changes over time were not significantly different between cohorts as analyzed using independent $t$-tests.
.§The decrease in interleukin-1β from baseline to week 18 was significant (P < 0.001) in both cohorts as analyzed using paired $t$-tests.
Values are means ± SEM

*P < 0.05, **P < 0.01; Osteoprotegerin was significantly greater in cohort 2 at week 6 and 12 as analyzed using 2 x 4 analysis of variance with repeated measures on the time factor.

Changes over time were significantly different between cohorts (P < 0.001) as analyzed using independent t-tests.

§The increase in osteoprotegerin from baseline to week 18 was significant (P < 0.001) in cohort 2 but not in cohort 1 as analyzed using paired t-tests.
Figure 5.5 Cross-linked N-telopeptide of type I collagen concentration at each interval by cohort

Values are means ± SEM

*P < 0.05, **P < 0.01, ***P < 0.01; Cross-linked N-telopeptide of type I collagen was significantly greater in cohort 1 at baseline and at weeks 6 and 12 as analyzed using 2 x 4 analysis of variance with repeated measures on the time factor. Changes over time were significantly different between cohorts (P < 0.01) as analyzed using independent t-tests.

§The decrease in cross-linked N-telopeptide of type I collagen from baseline to week 18 was significant (P < 0.01) in cohort 1 but not for cohort 2 as analyzed using paired t-tests.
Figure 5.6 Receptor activator for nuclear factor κB ligand concentration at each interval by cohort

Values are means ± SEM

**P < 0.01, ***P < 0.001; Receptor activator for nuclear factor κB ligand was significantly greater in cohort 2 at week 6 and was significantly greater in cohort 1 at week 12 as analyzed using 2 x 4 analysis of variance with repeated measures on the time factor.

Changes over time were not significantly different between cohorts as analyzed using independent t-tests.

§The change in receptor activator for nuclear factor κB ligand from baseline to week 18 was not significant within either cohort as analyzed using paired t-tests.
Table 5.6 Markers of bone turnover and mineral metabolism by flavanol group at all intervals

<table>
<thead>
<tr>
<th>Marker</th>
<th>Group</th>
<th>Baseline</th>
<th>Week 6</th>
<th>Week 12</th>
<th>Week 18</th>
<th>Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum OC (ng/mL)</td>
<td>AF</td>
<td>10.2 ± 3.9</td>
<td>11.3 ± 5.0</td>
<td>10.7 ± 4.6</td>
<td>11.7 ± 4.1</td>
<td>+1.5 ± 3.9</td>
</tr>
<tr>
<td></td>
<td>NF</td>
<td>11.1 ± 6.2</td>
<td>11.4 ± 6.8</td>
<td>9.7 ± 3.3</td>
<td>11.8 ± 4.8</td>
<td>+0.7 ± 4.4</td>
</tr>
<tr>
<td>Serum OPG (mol/L)</td>
<td>AF</td>
<td>2.6 ± 1.5</td>
<td>2.1 ± 1.2**</td>
<td>2.4 ± 1.1</td>
<td>3.1 ± 1.6**</td>
<td>+0.5 ± 0.9**</td>
</tr>
<tr>
<td></td>
<td>NF</td>
<td>2.7 ± 1.0</td>
<td>2.5 ± 1.2*</td>
<td>2.9 ± 1.5</td>
<td>3.5 ± 1.6***</td>
<td>+0.8 ± 1.0***</td>
</tr>
<tr>
<td>Serum NTx (nM BCE)</td>
<td>AF</td>
<td>11.8 ± 3.2</td>
<td>11.9 ± 3.2</td>
<td>11.3 ± 2.1</td>
<td>11.9 ± 2.5</td>
<td>+0.1 ± 4.1</td>
</tr>
<tr>
<td></td>
<td>NF</td>
<td>12.5 ± 3.3</td>
<td>12.8 ± 2.9</td>
<td>11.4 ± 1.8</td>
<td>11.3 ± 2.8</td>
<td>-1.2 ± 4.2</td>
</tr>
<tr>
<td>Serum RANKL (pmol/L)</td>
<td>AF</td>
<td>0.26 ± 0.16</td>
<td>0.24 ± 0.21</td>
<td>0.27 ± .13</td>
<td>0.27 ± .13</td>
<td>+0.01 ± 0.20</td>
</tr>
<tr>
<td></td>
<td>NF</td>
<td>0.32 ± 0.21</td>
<td>0.26 ± 0.21</td>
<td>0.35 ± 0.28</td>
<td>0.28 ± .27</td>
<td>+0.04 ± 0.32</td>
</tr>
<tr>
<td>Serum Calcium (mg/dL)</td>
<td>AF</td>
<td>9.6 ± 0.6</td>
<td>9.4 ± 0.3**</td>
<td>9.6 ± 0.8</td>
<td>9.6 ± 0.5</td>
<td>0.0 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>NF</td>
<td>9.8 ± 0.5</td>
<td>9.4 ± 0.5</td>
<td>9.7 ± 1.0</td>
<td>9.6 ± 0.6</td>
<td>-0.2 ± 0.8</td>
</tr>
<tr>
<td>Serum Phosphorus (mg/dL) §</td>
<td>AF</td>
<td>3.5 ± 0.5</td>
<td>4.0 ± 0.4***</td>
<td>3.9 ± 0.5*</td>
<td>3.9 ± 0.7</td>
<td>+0.4 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>NF</td>
<td>3.4 ± 0.5</td>
<td>4.0 ± 0.5***</td>
<td>3.7 ± 0.4*</td>
<td>3.9 ± 0.6***</td>
<td>+0.5 ± 0.6***</td>
</tr>
<tr>
<td>Urine Calcium (mg/24hr)</td>
<td>AF</td>
<td>121.3 ± 59.0</td>
<td>131.5 ± 71.5</td>
<td>126.2 ± 71.3</td>
<td>125.8 ± 68.6</td>
<td>-4.5 ± 52.0</td>
</tr>
<tr>
<td></td>
<td>NF</td>
<td>130.0 ± 54.6</td>
<td>136.1 ± 54.5</td>
<td>128.2 ± 51.5</td>
<td>115.6 ± 56.8</td>
<td>-14.6 ± 58.1</td>
</tr>
<tr>
<td>Urine Phosphorus (g/24hr)</td>
<td>AF</td>
<td>1.10 ± 0.51</td>
<td>0.90 ± 0.40</td>
<td>1.16 ± 0.70</td>
<td>1.00 ± 0.58</td>
<td>-0.10 ± 0.58</td>
</tr>
<tr>
<td></td>
<td>NF</td>
<td>1.00 ± 0.40</td>
<td>0.97 ± 0.40</td>
<td>1.06 ± 0.52</td>
<td>0.90 ± 0.45</td>
<td>-0.10 ± 0.37</td>
</tr>
</tbody>
</table>

Values are means ± SD

AF, additional flavanols, Group (n=25); NF, no additional flavanols, Group (n=25).
*P < 0.05, **P < 0.01, ***P < 0.001; P-value analyzed using analysis of variance with repeated measures on the time factor for within group changes from baseline.

§Serum phosphorus was significantly greater in the AF group compared to the NF group at week 12 (P < 0.05) as analyzed using analysis of variance with repeated measures on the time factor.

There were no other significant differences between groups at any interval or for the change over time between groups as analyzed using analysis of variance with repeated measures on the time factor.

OC, Osteocalcin; OPG, Osteoprotegrin; NTx, cross-linked N-teleopeptide of type I collagen; RANKL, Receptor Activator for Nuclear Factor κ B Ligand.
CHAPTER 6
SUMMARY, STRENGTHS, LIMITATIONS AND FUTURE DIRECTIONS
Obesity is a problem here in the United States (US) as well as in other industrialized and developing nations. Obesity is associated with insulin resistance, hypertension and hyperlipidemia which are risk factors in the development of many chronic conditions. Losing BW can reduce fat mass (FM), ameliorate risk factors for disease and improve long-term health status. While there are a multitude of weight-loss programs available, many of them have overly restrictive dietary patterns which may reduce an individual’s ability and desire to adhere. However, removing restrictions and incorporating commonly enjoyed snack foods into an energy-restricted diet (ERD) may improve adherence and facilitate BW loss. Further, certain snack choices such as dark chocolate (DC) may contain bioactive nutrients that can provide additional health benefits.

This novel randomized controlled trial was conducted to determine the effects of incorporating either two daily DC snacks and a high flavanol (HF) cocoa beverage or two daily non-chocolate (NC) licorice snacks and a flavanol-free (FF) vanilla beverage into an ERD on BW, body composition, metabolic parameters, markers of oxidative stress and inflammation, markers of bone formation and resorption, mineral metabolism and bone mineral density (BMD). This 18-week diet intervention tested the 3 main hypotheses proposed by this dissertation. The first hypothesis was that overweight or obese premenopausal women who followed an ERD containing either two daily DC or two daily NC snacks could maintain an energy deficit and experience significant and positive changes in anthropometric and body composition measurements, with no differential effect of snack. The second hypothesis was that healthy yet overweight or obese women who consumed either two daily DC or two daily NC snacks within the context of an ERD would experience reductions in BW, improvements in body fat percentage and BP and reductions in glucose and insulin concentrations without negative effects on lipid levels. The third hypothesis was that all overweight and obese women who lost BW and FM would experience reductions in markers of oxidative stress and
inflammation. Additionally, this hypothesis further stated that overweight and obese women, who consumed additional flavanols in the form of two DC snacks and a HF cocoa beverage during BW loss, would have a greater reduction in markers of both oxidative stress and inflammation than women who consumed the FF snacks and beverage. The final part of this hypothesis was that overweight and obese women who consumed additional flavanols in the form of two DC snacks and a HF cocoa beverage would experience a greater decrease in markers of bone resorption, a greater increase in markers of bone formation and maintenance of mineral metabolism and BMD after BW loss.

In support of the first hypothesis, the pilot study showed that it was feasible for overweight or obese women to achieve an energy deficit, lose a significant amount of BW and improve body composition measurements while incorporating either two DC or two NC licorice snacks into a ERD daily. This pilot study indicated that premenopausal women who are overweight or obese can include energy-controlled sweet snacks daily within an ERD without hindering BW loss.

The 18-week intervention demonstrated that women who included either DC or NC snacks daily were able to achieve BW loss and improvements in body fat percentage, BP measurements and glucose and insulin concentrations. In addition, this intervention demonstrated that there were no adverse effects of either snack on lipid levels. There were no differential health benefits acquired from consuming one snack over the other, however. These findings demonstrate that consuming sweet snacks daily within the context of an ERD does not deter improvements in metabolic parameters typically achieved with BW loss.

In partial support of the third hypothesis, this intervention demonstrated that both groups had a reduction in two markers of inflammation, IL-6 and IL-1β while the group who consumed additional flavanols also had a reduction in TNF-α concentration. However, in contrast to our
hypothesis, neither group experienced a significant decrease in markers of oxidative stress with BW loss nor did the group who consumed the DC snacks or HF cocoa beverage have significantly greater reductions in markers of oxidative stress or inflammation. Therefore, it cannot be concluded that the reduction in inflammation demonstrated by the women in this intervention was attributed to the consumption of additional flavanols.

This intervention also demonstrated that women in both groups experienced increases in osteoprotegerin (OPG), a marker of bone formation and BMD of the forearm while women who consumed the additional flavanols also had an increase in hip BMD. Again, in contrast to the original hypothesis, the incorporation of additional flavanols into an ERD did not provide significantly greater benefit on markers of bone turnover, mineral metabolism or BMD following BW loss. On the other hand, this intervention demonstrated that short-term BW loss does not necessarily result in a deleterious effect on markers of bone formation and resorption, mineral metabolism or BMD in healthy, premenopausal women who are overweight or obese as long as the energy restriction is moderate and the nutrient adequacy of the ERD is maintained.

Finally, this 18-week intervention demonstrated that both groups of women who lost BW experienced similar changes in markers of oxidative stress, inflammation and bone turnover, mineral metabolism and BMD independent of flavanol consumption. These results are in contrast to the original hypothesis that stated the group who consumed additional flavanols during BW loss would experience greater reductions in markers of oxidative stress and inflammation. It was further stated in the hypothesis that this additional reduction in oxidative stress and inflammation would translate into a greater reduction in bone resorption, greater increase in bone formation and a maintenance of BMD compared to the group who did not consume the FF beverage and snacks. Cumulatively, this study indicates that there was no greater benefit of consuming additional flavanols during BW loss.
However, all women in this study who reduced inflammation had an increase in OPG and BMD indicating there may be a potential relationship between weight-loss induced inflammation reduction and improvements in bone health. This study also indicates that there were no adverse effects of consuming either of the sweet snacks during BW loss on markers of oxidative stress and inflammation, markers of bone formation or resorption, mineral metabolism or BMD.

Findings of this research project demonstrate that a reduction in energy intake and BW loss can be achieved while enjoying sweet snacks daily. The inclusion of energy-controlled sweet snacks daily did not inhibit the benefits acquired from BW loss including the reduction of BP measurements and glucose, insulin and pro-inflammatory cytokine concentrations. In addition, neither lipid levels nor skeletal health were adversely affected by BW loss or the consumption of daily snacks. Consuming DC snacks and a cocoa beverage with additional flavanols during BW loss did not provide greater benefits on metabolic parameters, markers of oxidative stress or inflammation, bone turnover or BMD. Excellent adherence to the ERD and compliance with the consumption of the daily snacks indicate that including energy-controlled sweet snacks is a feasible technique to help women lose BW. Finally, this intervention demonstrated that BW loss can improve many indicators of health even in generally healthy, overweight or obese premenopausal women. Therefore, young women who are presently overweight and obese should be encouraged to reduce BW in order to improve long-term health outcomes without concern for detrimentally affecting their bone health.

This research project has several strengths. The ERD designed for the women in this intervention were based on the current national guidelines which are founded in solid nutrition research. The ERD did not require the restriction of certain food groups or macronutrient components and it had only a moderate energy restriction specifically designed to induce a steady BW loss of 1-2 lbs per week. Use of a food exchange system allowed women to choose the types of
food they wanted to consume from each exchange group. The weekly nutrition classes which were created and taught by a registered dietitian helped the women incorporate healthier foods into their existing lifestyle and meal pattern without requiring them to change their eating habits appreciably. This intervention was designed to explore the feasibility and effectiveness of implementing a technique aimed at improving dietary adherence in a population who needs weight-loss assistance. While it addressed a large group within the dieting population, the application of these results are limited to Caucasian females in the US who are seeking weight-loss help due to the lack of racial and ethnic diversity within the study sample.

This research project also has limitations. The weight-loss intervention was conducted in a very specific population of overweight and obese premenopausal women within a limited age range. This may limit the ability to generalize study results to women in other stages of the lifespan, men of all ages and those with chronic health conditions. Another limitation was that only 50 women provided blood and urine samples at all four time points. This study was originally powered to detect significant differences in BW and BMD not in markers of oxidative stress, inflammation and bone turnover which can be much more variable. This may have contributed to the lack of significant differences between the two groups in these outcome markers. Further, the active intervention period of this study lasted only 18 weeks. A longer intervention may be necessary to observe more significant changes in markers of oxidative stress or inflammation, bone turnover or BMD.

This intervention also included nutrition education classes which may have resulted in greater health improvements than those demonstrated by women who would not be provided with weekly guidance. The positive outcomes may have been attributed to visits with a registered dietitian rather than from the ERD alone. This study did not control dietary intake and relied on self-
reported dietary intake which can be subjective and sometimes unreliable in overweight and obese individuals. However, positive changes in BW, FM and metabolic markers demonstrate evident adherence with the dietary intervention. Due to the design of the intervention, participants were not given a choice of the snack they were going to consume for 18 weeks. Allowing them to choose their favorite snack food could possibly lead to overconsumption and thus alter results or conversely, may further increase adherence. Finally, this study lacked a long-term follow-up period to determine whether women remained compliant with the consumption of daily energy-controlled snacks, maintained an energy deficit, continued to lose BW and maintained bone mass.

Future research in this area should explore the feasibility of incorporating more than one type of snack food into an ERD. This type of study would give participants the choice of several different types of snacks, both sweet and savory, to examine the ability to maintain an energy deficit in the presence of dietary variety. Will the variety make it easier to comply with an ERD or will the variety make it more difficult to remain adherent as there will be no sensory specific satiety to cease intake? Also, women in the current intervention were provided with only one week’s worth of snacks in a pre-portioned amount. A future study could provide the snacks for the entire intervention in a bulk form. The participants alone would be responsible for consuming only the assigned snack portion size. This would more closely resemble a real-life situation examining whether individuals would be able to adhere to the assigned snack food amount and maintain the energy deficit necessary for BW loss.

An additional future study could track long-term dietary adherence, continued BW loss and maintenance after the participants had completed the active part of the intervention. Participants could be contacted or reassessed at six months, one year or two years following the 18-week intervention to determine if they are still consuming a daily snack, if they continued to maintain an
energy deficit and lost additional BW, are in energy balance or had re-gained BW. This would examine the long-term feasibility of consuming a daily snack as useful technique for inducing an energy deficit and BW loss. Will individuals become tired of consuming the same snack each day and thus stop adhering? Will they disregard portion size when not monitored weekly? Or will they start consuming a snack of their own choosing in an energy-controlled portion?

A snack intervention similar to the current study could be conducted with the inclusion of a group that does not receive a snack of any kind. The additional group would be given extra “real food” exchanges in place of the snack to account for the energy difference from the beverage and twice daily snack. This type of intervention would determine whether an ERD that incorporates a portion-controlled sweet snack can increase dietary adherence to a greater extent than an ERD that does not include daily snacks. This type of study design could examine whether individuals who plan sweet snacks into their day significantly reduce consumption of other snack foods, have a greater reduction in energy intake and have greater BW loss compared to those who do not plan portion-controlled snacks into their day. Metabolic and inflammatory outcomes could also be examined following the consumption of these two different ERDs to determine whether consuming the “real food” exchanges produce greater benefits in these outcomes than an “empty-calorie” snack food.

The type of additional exchanges given to the participants in the non-snack group could also vary. For example, the non-snack group could be given one and a half more dairy exchanges in place of the snack to determine the effect on bone health and FM loss during BW reduction. The non-snack group could be also given two to three additional protein exchanges to examine the whether there is greater FM loss or FFM sparing during BW loss compared to the snack group. Or
the additional group could be given 2 extra fruit exchanges and 2 extra vegetable exchanges to examine the addition of other types of flavanoids on oxidative stress and inflammation.

The current intervention did not find a significant difference between snack groups in relation to reductions in BP measurements, or insulin, glucose or inflammatory protein concentrations. Previous research interventions with individuals who added HF cocoa products isocalorically to the diet demonstrated improvements in these metabolic parameters. Because of this, future studies may need to incorporate a greater dose of flavanols into the ERD to produce a biological effect greater than the effect of weight loss. Future interventions may also need to be conducted in a population that has elevated baseline BP, lipid, insulin, glucose or pro-inflammatory cytokine concentrations.

Finally, to further differentiate between the benefits acquired from cocoa flavanol consumption and the benefits acquired from BW loss, two additional experimental groups could be added. One group would maintain BW while consuming a similar amount of flavanols as the HF plus weight loss group and the other group would maintain BW and consume no additional flavanols. This would simultaneously address four objectives: the effect of isocalorically supplementing flavanols in the diet compared to normal flavanol consumption without weight loss; the effect of regular flavanol consumption compared to regular flavanol consumption during BW loss; the effect of incorporating additional flavanols into a weight-loss diet compared to BW loss alone; and the effect of supplementary flavanol consumption alone compared to supplementary flavanol consumption during BW loss.

In summary, this randomized intervention explored the feasibility of incorporating two different types of sweet snacks into an ERD and their subsequent effect on markers of health. Women were able to reduce BW and experience a multitude of metabolic benefits while enjoying
either two dark chocolate or two licorice snacks daily. In addition, there were no detrimental effects of consuming either sweet snack on lipid levels or bone health. Further, all overweight or obese women who reduced inflammation had small but significant increases in bone formation markers and BMD indicating a possible link between inflammation reduction and improvements in bone health. Finally, the consumption of additional flavanols during BW loss did not induce greater health improvements for any of the parameters measured in this intervention.
APPENDIX A

SCREENING FORMS AND INFORMED CONSENT
Date: December 23, 2008
From: Tracie L. Kahler, IRB Administrator
To: Kathryn E. Piehowski
Subject: Results of Review of Proposal - Full (IRB #29543) 
Approval Expiration Date: October 15, 2009
“Effects of Incorporating Dark Chocolate into a Weight Loss Diet on Biomarkers of Inflammation, Oxidative Stress, and Bone Metabolism”

The Biomedical Institutional Review Board (IRB) has reviewed and approved your proposal for use of human participants in your research. By accepting this decision, you agree to obtain prior approval from the IRB for any changes to your study. Unanticipated participant events that are encountered during the conduct of this research must be reported in a timely fashion.

Enclosed is the dated, IRB-approved informed consents and recruitment materials to be used when recruiting/enrolling participants for this research. Participants must receive a copy of the approved informed consent form to keep for their records.

If signed consent is obtained, the principal investigator is expected to maintain the original signed consent forms along with the IRB research records for this research at least three (3) years after termination of IRB approval. For projects that involve protected health information (PHI) and are regulated by HIPAA, records are to be maintained for six (6) years. The principal investigator must determine and adhere to additional requirements established by the FDA and any outside sponsors.

If this study will extend beyond the above noted approval expiration date, the principal investigator must submit a completed Continuing Progress Report to the Office for Research Protections (ORP) to request renewed approval for this research.

On behalf of the IRB and the University, thank you for your efforts to conduct your research in compliance with the federal regulations that have been established for the protection of human participants.

Please Note: The ORP encourages you to subscribe to the ORP listserv for protocol and research-related information. Send a blank email to: L-ORP-Research-L-subscribe-request@lists.psu.edu

TLK/tlk
Enclosure
cc: Sharon M. Nickols-Richardson
Title of Project: Effects of Incorporating Dark Chocolate into a Weight Loss Diet on Biomarkers of Inflammation, Oxidative Stress, and Bone Metabolism

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1. Purpose of the study: The purpose of this research is to compare two different weight loss interventions (“diets”) on various measures of health including markers of inflammation, cholesterol levels, blood pressure and bone mineral density in women between the ages of 25 and 45 years. A total of 90 premenopausal women, with a body mass index (BMI) between 25 and 43, will participate in this study. This research study is being funded by Hershey Corporation but they will have no influence over study results.

2. Procedures to be followed: As a participant, you will be asked to complete screening forms and seek physician clearance to participate; if enrolled in the study, you will then attend one informational meeting, six data collection sessions, weekly diet education sessions, and one follow-up meeting. During this 18-week study, you will complete several procedures:

   Informational Meeting – 1 hour of your time.  
   You will attend an initial informational meeting where the details of the study and the different diet groups will be explained. You will have plenty of time to ask questions of the investigators and will have up to 2 weeks after the meeting to decide not to be in the study. No further measurements will be taken until we receive your final agreement or consent to be in the study.

   At the informational meeting, you will:

   1) Review your signed Informed Consent Form and reaffirm or agree again your desire and willingness to be in the study (within two weeks of the meeting);  
   2) Ask questions about the study;  
   3) Be instructed on how to properly complete a 4-day food record and 7-day physical activity record; and  
   4) Receive a container for your first 24-urine sample.
After the informational meeting and your agreement to be in the study, you will undergo baseline testing. After this testing, you will be randomized into a diet group. This means that you will be put into one of the two diet groups by chance. Once you are assigned in a diet group, you will attend another meeting specifically for your diet. At this meeting that will take 1 hour of your time, we will discuss the diet guidelines, answer any of your questions, and give you a schedule for your testing sessions, weekly diet meetings, and the date on which to start your diet. You will stop using or taking any vitamin and mineral supplements and eating or drinking any cocoa/chocolate products until the end of the study, unless these products are part of your assigned diet. We will tell you if you are supposed to eat and drink cocoa/chocolate products or not.

Diet Groups

The two diet groups include: 1) a weight loss plus daily non-chocolate sweet snack (NCS) diet group; and 2) a weight loss plus daily dark chocolate product (DCP) diet group. Randomized means you will be placed in one of these three groups by chance.

If you are randomized to the NCS diet, you will follow a low-fat, high-carbohydrate weight-loss diet designed to produce a 2-pound weight loss per week. We will set calorie goals for you at 1,500; 1,600; 1,700; or 1,800 kilocalories per day based on your own energy needs. We will instruct you on your diet, with the help of exchange lists and portion sizes for servings per day from various food groups. Handouts with food choices, menus, and other guidelines will be given to you. You will eat one non-chocolate sweet snack at two different times each day. You will also drink a sugar-free beverage in your diet each day. These snacks and sugar-free beverage mix will be provided to you by the investigators. There will be no cocoa or chocolate in this diet, and you will not consume chocolate or cocoa products while you are on this diet.

If you are randomized to the DCP diet, you will follow the same low-fat, high-carbohydrate weight-loss diet as described for the NCS diet group. Your kilocalorie goal will be based on your own energy needs. If you are in this group, you will consume one dark chocolate tasting square at two different times each day. You will also drink one, 8-ounce sugar-free cocoa beverage every day.

Test Session 1 (Week 0) – 2 hours of your time.
During this test session, you will need to:

1) Arrive in Chandlee Lab on the campus of the Pennsylvania State University (University Park) at your scheduled appointment day and time;
2) Turn in your 24-hour urine sample, 4-day food record, and 7-day physical activity record;
3) Have your height, weight, waist circumference, and hip circumference measured;
4) Complete the current illness survey, a general symptoms questionnaire, and the SF-36 health status questionnaire;
5) Complete hunger and mood ratings, D-SAT questionnaire, the Eating Inventory, and a food frequency questionnaire;
6) Have your blood pressure measured;
7) Have 30 mL (about 2 Tablespoons) of whole blood drawn from your arm by a registered nurse who is trained in drawing blood from people;
8) Eat breakfast foods and beverages if desired;
9) Provide a urine sample for pregnancy testing; and
10) Undergo a peripheral quantitative computed tomography (pQCT) scan of your tibia (lower leg bone).

Test Session 2 (Week 0) – 30 minutes of your time.
During this test session, you will need to:
1) Arrive at the General Clinical Research Center on the campus of the Pennsylvania State University (University Park) at your scheduled appointment day and time;
2) Provide a urine sample for pregnancy testing; and
3) Undergo a dual-energy X-ray absorptiometry (DXA) scan of your whole body, spine, hip and forearm.

After test session 2, you will begin your assigned diet. This will require that you spend time each day thinking about the food that you eat, purchasing foods that fit with your assigned diet, and preparing foods that fit with your assigned diet.

Test Session 3 and 4 (Week 6 and Week 12) – 2 hours of your time at each of these weeks (4 hours total). During this test session, you will need to:

1) Arrive in Chandlee Lab on the campus of the Pennsylvania State University (University Park) at your scheduled appointment day and time;
2) Turn in your 24-hour urine sample, 4-day food record, and 7-day physical activity record;
3) Have your height, weight, waist circumference, and hip circumference measured;
4) Complete the current illness survey, a general symptoms questionnaire, the SF-36 health status questionnaire, and the Zung Scale;
5) Complete hunger and mood ratings, D-SAT questionnaire, and the Eating Inventory;
6) Have your blood pressure measured;
7) Have 30 mL (about 2 Tablespoons) of whole blood drawn from your arm by a registered nurse who is trained in drawing blood from people; and
8) Eat breakfast foods and beverages if desired;

Test Session 5 (Week 18) – 2 hours of your time.
During this test session, you will need to:

1) Arrive in Chandlee Lab on the campus of the Pennsylvania State University (University Park) at your scheduled appointment day and time;
2) Turn in your 24-hour urine sample, 4-day food record, and 7-day physical activity record;
3) Have your height, weight, waist circumference, and hip circumference measured;
4) Complete the current illness survey, a general symptoms questionnaire, the SF-36 health status questionnaire, and the Zung Scale;
5) Complete hunger and mood ratings, D-SAT questionnaire, the Eating Inventory, and a food frequency questionnaire;
6) Have your blood pressure measured;
7) Have 30 mL (about 2 Tablespoons) of whole blood drawn from your arm by a registered nurse who is trained in drawing blood from people;
8) Eat breakfast foods and beverages if desired;
9) Provide a urine sample for pregnancy testing; and
10) Undergo a peripheral quantitative computed tomography (pQCT) scan of your tibia (lower leg bone).

Test Session 6 (Week 18) – 30 minutes of your time.
During this test session, you will need to:

1) Arrive at the General Clinical Research Center on the campus of the Pennsylvania State University (University Park) at your scheduled appointment day and time;
2) Provide a urine sample for pregnancy testing; and
3) Undergo a dual-energy X-ray absorptiometry (DXA) scan of your whole body, spine, hip and forearm.

Weekly Diet and Nutrition Education Sessions – 1 hour of your time each week.
At these sessions, we will discuss food purchasing and preparation, dining in restaurants, recipe modification, and basic nutrition knowledge. We will provide ways to problem-solve in difficult diet situations, and we will help you stay motivated. Sessions will be specific to diet groups, although topics will be the same for all groups. At these weekly sessions, we will also give the non-chocolate sweet snacks and sugar-free beverage mix to the NCS group and the dark chocolate products and sugar-free cocoa mix to the DCP group.

Follow-up Meeting – 1 hour of your time.

Once everyone has finished the whole study, we will have one last meeting. At this meeting, you will be given your study results, and we will provide nutrition education to help you continue your diet, if you wish. Any snack and drink mix products provided during the study will no longer be given to you, once the study is finished.

Bone Density Scans and Weight Loss during Pregnancy

While pQCT and DXA scans are not harmful to an unborn baby, any woman who is pregnant or planning to become pregnant should not participate in this study. Pregnancy is not a time to go on a diet to lose weight. Therefore, if you are pregnant or planning to become pregnant during the study, you should inform the Primary Investigator or Faculty Advisor immediately.

Clothing during Test Sessions

You should wear a short-sleeved or loose-fitting shirt for the blood draw and elastic-waisted pants for the waist and hip circumference measurements. Do not wear expensive jewelry or many clothing accessories to the test sessions. During bone density scans, you will have to take any jewelry off of your body and remove any metal zippers or snaps, metal buttons, or other metal materials.

Length of Time for Study Procedures

Your appointments may take more or less time than estimated to complete each procedure. You will be given plenty of time to complete measurements and blood draws and to understand your diet.

3. Discomforts and risks: There are two potential risks: 1) blood draws, and 2) radiation exposure from DXA and pQCT (bone density scans). There is minimal risk involved in blood draws. A bruise or a little bleeding may result from blood collection procedures with no known harmful effects to your health or wellbeing. In order to minimize bruising and bleeding, a registered nurse trained to do blood draws will draw all blood samples. You may become slightly lightheaded or nauseous during blood draws, but you may sit or recline for as long as you need to minimize discomfort. After each blood draw, you will be provided with breakfast foods and beverages. Two attempts to draw blood (or two needle sticks) will be allowed. If a second attempt is unsuccessful, no further tries for blood collection will be performed. Universal blood precautions will be taken by research personnel during handling of all blood samples.

This protocol calls for a series of pQCT leg scans and whole body, spine, hip and forearm Dual Energy X-ray Absorptiometry (DXA) scans. The pQCT and DXA bone density procedures will expose you to a small amount of radiation where the X-ray beam crosses the body. This radiation exposure is not necessary for your medical care and is for research purposes only.

The dose for the two pQCT leg scans is equivalent to a whole body radiation exposure of 0.4 millirem. The dose for the eight DXA scans is equivalent to a whole body radiation exposure of 9.0 millirem. The total dose for all the scans would be 9.4 millirem. A millirem is a unit of whole-body radiation dose. For comparison purposes, the average person in the United States receives a radiation exposure of 300 mrem per year from natural background sources, such as from the sun, outer space, and from radioactive materials that are found naturally in the earth’s air and soil. 9.4 mrem is less than you would receive from 12 days of natural background radiation.
The investigator is currently unaware of any specific risks associated with following any of the diets in this study. Please inform the investigator if you do not like chocolate or have cocoa intolerances or allergies. If you experience any changes in your health during the study, you must tell the investigators.

4. Abnormal Test Results: Your blood pressure result will be shared with you by the registered nurse at each test session. If your systolic (top number) blood pressure measurement is 140 mmHg or more and/or if your diastolic (bottom number) blood pressure measurement is 90 mmHg or more, you will be told to seek immediate medical attention from your physician, and the investigator will remove you from the study.

The blood samples will not be analyzed until the study has been completed. However, after all participants have completed their blood draws and bone density scans, we will have a follow-up meeting. At this meeting, you will be given your results. If there are any study results that suggest that medical attention is needed (for example, high blood cholesterol), you will be made aware of this within 30 days of analysis and recommended to contact your primary care physician for a follow-up examination.

5. Benefits to individual: You may benefit from participation in this research in several ways including: 1) determination of body composition by DXA; 2) assistance with a diet with supervision by a Registered Dietitian; 3) measurement of bone density; 4) analysis of cholesterol level, blood sugar, insulin, and inflammatory markers; 5) blood pressure measurements; and 6) dietary intake analysis. You may or may not lose weight, but you will receive nutrition education by a Registered Dietitian. You will be provided with your results from every procedure at the completion of the study. Referral to appropriate health care professionals will be provided if necessary based on your results after completion of the study.

6. Benefits to society: Society will benefit from this research as new understandings of links among different approaches to weight loss and disease risks will be identified from this study. We expect to show that weight loss is helpful for the heart and the skeleton.

7. Duration/time of the procedures and study: The study will consist of one informational meeting, six data collection sessions, weekly diet meetings and a follow-up meeting. The initial informational meeting will last no more than 60 minutes, four data collection sessions will last 2 hours each, the two DXA scans sessions will last 30 minutes each, the diet meetings will last 60 minutes each and the follow-up meeting will last no more than 60 minutes. The total time for this study is estimated at 29 hours over a 6-month period. You will also spend some time each day thinking about, preparing, and eating food.

8. Statement of confidentiality: Your participation in this study is confidential. The data will be stored and secured at an investigator’s office in a locked file cabinet. A three-digit code number will be assigned to you and used in place of your name. A master list of participants' code numbers will be kept in a separate locked file cabinet. In the event of any publication or presentation resulting from the research, no personally identifiable information will be shared. Only the investigators of this study or students of the faculty advisor will be allowed access to any data. It is up to you to share your individual results with your Primary Health Care Provider, if you so choose.

The following may review records related to this research: Penn State’s Office for Research Protections, Penn State’s Institutional Review Board, and the Office of Human Research Protections in the U.S. Dept. of Health and Human Services.

9. Right to ask questions: Please contact the Principal Investigator, Kathryn Piehowski or Dr. Shelly Nickols-Richardson (Faculty Advisor) at 814-863-2920 with questions, complaints, or concerns about this research. You can also call this number if you feel this study has harmed you. If you have any questions, concerns, or problems about your rights as a research participant or would like to offer input, please contact
Penn State University’s Office for Research Protections (ORP) at (814) 865-1775. The ORP cannot answer questions about research procedures. Questions about research procedures can be answered by the research team.

10. *Payment for participation:* You will receive $10 per hour that you participate in the four, 2-hour test sessions. These four sessions are 2 hours in length, so at $10 per hour, this is equal to $80 for the whole study.

11. *Voluntary participation:* Your decision to be in this research is voluntary. You can stop at any time. You do not have to answer any questions you do not want to answer. Refusal to take part in or withdrawing from this study will involve no penalty or loss of benefits you would receive otherwise. There may be reasons for which the investigators find that you should discontinue the study.

12. *Event of injury:* In the unlikely event you become injured as a result of your participation in this study, you will be referred to your own primary care physician or health specialist. It is the policy of this institution to provide neither financial compensation nor free medical treatment for research-related injury. By signing this document, you are not waiving any rights that you have against The Pennsylvania State University for injury resulting from negligence of the University or its investigators.

You must be between the ages of 25 to 45 years to take part in this research study. If you agree to take part in this research study and with the information outlined above, please sign your name and indicate the date below.

You will be given a copy of this signed and dated consent form for your records.

______________________________________________  _____________________
Participant Signature       Date

______________________________________________  _____________________
Person Obtaining Consent      Date
MEDICAL HISTORY FORM
BONE LAB – PSU
Title of Project: Effects of Incorporating Dark Chocolate into a Weight Loss Diet on Biomarkers of Inflammation, Oxidative Stress, and Bone Metabolism

<table>
<thead>
<tr>
<th>Date: __________________________</th>
</tr>
</thead>
<tbody>
<tr>
<td>Name (please print): ____________________________________________________________________</td>
</tr>
<tr>
<td>Name of physician (please print): __________________________________________________________</td>
</tr>
<tr>
<td>Date of Birth: _____________  Age (years): ____________</td>
</tr>
<tr>
<td>Gender:     FEMALE     MALE</td>
</tr>
<tr>
<td>Ethnicity: _____________________________</td>
</tr>
</tbody>
</table>

Medical History
Please indicate any current or previous conditions or problems you have experienced or have been told by a physician that you have had:

<table>
<thead>
<tr>
<th>Condition</th>
<th>Yes</th>
<th>No</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart disease or any heart problems:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Respiratory disease or breathing problems:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Circulation problems:</td>
<td></td>
<td></td>
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<tr>
<td>Kidney disease or problems:</td>
<td></td>
<td></td>
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<tr>
<td>Urinary problems:</td>
<td></td>
<td></td>
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<tr>
<td>Reproductive problems:</td>
<td></td>
<td></td>
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<tr>
<td>Muscle problems:</td>
<td></td>
<td></td>
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<tr>
<td>Skeletal problems including osteoporosis or osteopenia:</td>
<td></td>
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<tr>
<td>Fainting or dizziness, especially with exertion:</td>
<td></td>
<td></td>
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<tr>
<td>Neurological problems/disorders:</td>
<td></td>
<td></td>
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<tr>
<td>High blood pressure:</td>
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<tr>
<td>Low blood pressure:</td>
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<td></td>
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<tr>
<td>High blood cholesterol:</td>
<td></td>
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<tr>
<td>Diabetes mellitus:</td>
<td></td>
<td></td>
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<tr>
<td>Thyroid problems:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eating disorders (bulimia, anorexia):</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crohn's disease:</td>
<td></td>
<td></td>
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<tr>
<td>Allergies:</td>
<td></td>
<td></td>
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<tr>
<td>Insomnia:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unusual sleep patterns:</td>
<td></td>
<td></td>
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<tr>
<td>Other (Please list): ______________________________</td>
<td></td>
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</tr>
</tbody>
</table>
If “yes” to any of the above please indicate the date, explain, and describe:

________________________________________________________________________

________________________________________________________________________

________________________________________________________________________

Please list any hospitalizations/operations/recent illnesses (Type/Date):

________________________________________________________________________

________________________________________________________________________

________________________________________________________________________

**Medications**

Please indicate any current medications that you are taking on a daily or weekly basis:

<table>
<thead>
<tr>
<th>Medication</th>
<th>Yes</th>
<th>No</th>
</tr>
</thead>
<tbody>
<tr>
<td>Steroids (such as Prednisone):</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thyroid medications (such as Synthroid):</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bisphosphonates (such as Fosamax):</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anticonvulsants (such as Dilantin):</td>
<td></td>
<td></td>
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<tr>
<td>Glucocorticoids (such as Dexamethasone):</td>
<td></td>
<td></td>
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<tr>
<td>Other bone medications (such as Miacalcin):</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Please list any nutritional supplements, herbal products, or other medications, (prescription and over-the-counter) you are currently taking on a daily or weekly basis and the doses per day:  

________________________________________________________________________

**Family Health History**

Has anyone in your family (blood relatives only) been diagnosed or treated for any of the following?

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Yes</th>
<th>No</th>
<th>Relationship</th>
<th>Age</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart attack</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heart disease</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>High blood pressure</td>
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<td></td>
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</tr>
<tr>
<td>Stroke</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>Kidney disease</td>
<td></td>
<td></td>
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<tr>
<td>Diabetes</td>
<td></td>
<td></td>
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<tr>
<td>Crohn's disease</td>
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<tr>
<td>Thyroid disorders</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Osteoporosis</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Osteopenia</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>
Have you broken any bone(s)?  Yes _____  No ____
If “yes,” please list bone(s) and age(s) at time of break: ________________________________
_________________________________________________________________________________
_________________________________________________________________________________

Health Habits
Do you add salt to your food?  Yes _____  No _____
Are you on any special type of diet?  Yes _____  No _____
If “yes,” please describe: _________________________________________________________________
_____________________________________________________________________________________
_____________________________________________________________________________________

Do you drink caffeinated beverages?  Yes _____  No _____  If “yes,” how many cups per day? _______
Do you drink alcoholic beverages?  Yes _____  No _____  If “yes,” how many cups per day? _______
What is the average number of alcoholic drinks that you consume on the weekend? __________
Did you use tobacco products in the past (more than 12 months ago)?  Yes _____  No _____
Do you currently use tobacco products?  Yes _____  No _____  If “yes,” what type of tobacco products do you
use, how frequently do you use them, and what number do you use per day? _________________
_____________________________________________________________________________________

Work Schedule and Patterns
Do you engage in night-time work?  YES  NO
If yes, please explain: ________________________________________________________________
_____________________________________________________________________________________

Exercise Habits
Do you engage in regular exercise?  Yes _____  No _____
If “yes” please list:

<table>
<thead>
<tr>
<th>Activity</th>
<th>Frequency (times per week)</th>
<th>Duration (minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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</tr>
</tbody>
</table>

Do you ever feel faint, short of breath, or chest discomfort with exertion?  Yes _____  No _____
If “yes,” please explain: ________________________________________________________________
_____________________________________________________________________________________

196
Are there any orthopedic limitations you have that may restrict your ability to exercise? Yes ___ No ___
If "yes" please explain: ________________________________________________________________

Questions Related to Reproductive Function
Do you use an oral contraceptive? Yes _____ No _____
If "yes" what brand and dose of oral contraceptive? __________________________________________
If "yes," for how long have you used this oral contraceptive? ______________________________
If "yes," do you use oral contraceptives due to menstrual cycle irregularities? Yes ____ No ____
Have you undergone a hysterectomy and/or ovariectomy? Yes _____ No _____
If "yes," when? ________________________________________________________________
If "yes," do you use hormone replacement therapy? Yes _____ No _____
If "yes," what brand and dose of estrogen or hormone replacement therapy do you use? ________
______________________________________________________________________________
If "yes," for how long have you used this estrogen or hormone replacement therapy?
______________________________________________________________________________
When was the first day of your last menses? _____________________________
Have you had any abnormal menses or absence of menses in the last 12 months? Yes _____ No _____
If "yes", describe: __________________________________________________________________
_________________________________________________________________________________

Are you pregnant or do you think that you may be pregnant? YES  NO
Are you attempting to become pregnant? YES  NO
How many menstrual cycles do you have per year?
a) 12 to 14 per year
b) 9 to 11 per year
c) 6 to 8 per year
d) 3 to 5 per year
e) < 3 per year

Do you have children? YES  NO  If "yes" how many children do you have? ________________
Are you currently breastfeeding? YES  NO

Weight History
What is your current weight? __________
How much did you weigh six months ago? ______________
How much did you weigh one year ago? ___________
During the last 2 years, how many times have you lost 5 pounds?

NEVER   ONCE   TWICE   THREE OR MORE

During the last 2 years, how many times have you gained 5 pounds?

NEVER   ONCE   TWICE   THREE OR MORE

Do you desire to lose weight?  YES  NO

What is your height? _____________

Body mass index: ________________ (kg/m²) (Please leave this line blank; an investigator will calculate.)

Chocolate/Cocoa Product Consumption

Do you consume chocolate or cocoa products? Yes _____  No _____

If “yes,” how often do you consume chocolate or cocoa products and how much?

a) one chocolate bar or cocoa drink per day
b) one or two chocolate bars or cocoa drinks per week
c) two chocolate bars or cocoa drinks per month
d) on special occasions
e) do not consume chocolate
f) If “other” frequency, please describe: ________________________________

Do you have a chocolate allergy or intolerance?  Yes _____  No _____

Do you avoid chocolate or cocoa products? Yes _____  No _____

If “yes” to either of these questions, please explain: ________________________________

I confirm that the above information is correct.

________________________________________  ________________________  ________________
Print Name                     Signature                     Date
<table>
<thead>
<tr>
<th>Exclusion/Inclusion Criteria</th>
<th>YES</th>
<th>NO</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Title of Project:</strong> Effects of Incorporating Dark Chocolate into a Weight Loss Diet on Biomarkers of Inflammation, Oxidative Stress, and Bone Metabolism</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age: 25 to 45 years</td>
<td>YES</td>
<td>NO</td>
</tr>
<tr>
<td>Female</td>
<td>YES</td>
<td>NO</td>
</tr>
<tr>
<td>Moderate physical activity (≤ 5 hours per week)</td>
<td>YES</td>
<td>NO</td>
</tr>
<tr>
<td>Eumenorrhea (9 to 14 cycles per year)</td>
<td>YES</td>
<td>NO</td>
</tr>
<tr>
<td>Stable body weight (in last 6 months)</td>
<td>YES</td>
<td>NO</td>
</tr>
<tr>
<td>Zung Scale Score (&lt;50)</td>
<td>YES</td>
<td>NO</td>
</tr>
<tr>
<td>Absence of chocolate allergy/intolerance/aversion/high consumption</td>
<td>YES</td>
<td>NO</td>
</tr>
<tr>
<td>Desire to lose weight</td>
<td>YES</td>
<td>NO</td>
</tr>
<tr>
<td>Alcohol intake (≤ 2 drinks/day, twice/wk)</td>
<td>YES</td>
<td>NO</td>
</tr>
<tr>
<td>Body mass index (between 25.0 to 43.0)</td>
<td>YES</td>
<td>NO</td>
</tr>
<tr>
<td>Absence of cigarette smoking</td>
<td>YES</td>
<td>NO</td>
</tr>
<tr>
<td>Absence of pregnancy/attempting to become pregnant</td>
<td>YES</td>
<td>NO</td>
</tr>
<tr>
<td>Absence of osteopenia or osteoporosis</td>
<td>YES</td>
<td>NO</td>
</tr>
<tr>
<td>Absence of steroid use</td>
<td>YES</td>
<td>NO</td>
</tr>
<tr>
<td>Thyroid hormones</td>
<td>YES</td>
<td>NO</td>
</tr>
<tr>
<td>Bisphosphonates</td>
<td>YES</td>
<td>NO</td>
</tr>
<tr>
<td>Anticonvulsants</td>
<td>YES</td>
<td>NO</td>
</tr>
<tr>
<td>Glucocorticoids</td>
<td>YES</td>
<td>NO</td>
</tr>
<tr>
<td>Absence of Crohn’s disease</td>
<td>YES</td>
<td>NO</td>
</tr>
<tr>
<td>Diabetes</td>
<td>YES</td>
<td>NO</td>
</tr>
<tr>
<td>Thyroid disorders</td>
<td>YES</td>
<td>NO</td>
</tr>
<tr>
<td>Impaired renal function</td>
<td>YES</td>
<td>NO</td>
</tr>
<tr>
<td>Hysterectomy w/o HRT</td>
<td>YES</td>
<td>NO</td>
</tr>
<tr>
<td>Ovariectomy w/o HRT</td>
<td>YES</td>
<td>NO</td>
</tr>
<tr>
<td>Oral contraceptive use (&lt;2 years)</td>
<td>YES</td>
<td>NO</td>
</tr>
</tbody>
</table>

**All answers must be YES**
Title of Project: Effects of Incorporating Dark Chocolate into a Weight Loss Diet on Biomarkers of Inflammation, Oxidative Stress, and Bone Metabolism

Physician Clearance Form

Dear Physician:
This patient would like to participate in a study titled, “Effects of Incorporating Dark Chocolate into a Weight Loss Diet on Biomarkers of Inflammation, Oxidative Stress, and Bone Metabolism,” conducted by Kathryn E. Piehowski RD, CBDT of the Nutritional Sciences Department at The Pennsylvania State University. The purpose of this study is to determine effects of following a weight-loss diet on blood markers of inflammation, bone turnover, mineral metabolism, cholesterol and blood pressure, and overall bone mineral density in overweight and obese women ages 25 to 45 years during an 18-week intervention period. A low-fat, high-carbohydrate diet designed to induce 2 pounds of weight loss per week will be assigned to each woman. Please indicate below that this patient has completed the enclosed Medical History Form in a manner that is consistent with the medical records of this patient. If this patient participates in this study, she will discontinue use of vitamin and mineral supplements for the 18-week study period. The patient will undergo a series of fasting blood draws at 6-week intervals over 18 weeks and bone densitometry scans at an 18-week interval.

Results of Physician Screening: Please make certain that all questions on this form are completed in a manner consistent with the medical records of this patient. If unusual problems are present or not disclosed that may affect the candidate’s safety or eligibility for the study, note this/these finding(s) below and submit to the investigator.

**By signing this form you are agreeing that this woman is free of osteoporosis, osteopenia, impaired renal function, abnormal lipid metabolism, diagnosed cardiovascular or metabolic (e.g., diabetes) disease, including hypertension, and is premenopausal and in good physical health to participate in an assigned dietary intervention.**

THIS CANDIDATE QUALIFIES FOR PARTICIPATION IN THE STUDY, SUBJECT TO FINAL VERIFICATION BY THE INVESTIGATOR.

Yes: ___ No: ___

If “No,” please explain reasons for which individual is not eligible to participate in this study.
_________________________________________________________________________________
_________________________________________________________________________________

Please sign to indicate that the above information is correct:

________________________________  __________________________________
Print Name       Signature    Date

You may contact the investigators at:
Kathryn Piehowski, RD (Primary Investigator) or Sharon Nickols-Richardson PhD, RD
kep158@psu.edu or 814-865-5926          smn13@psu.edu
The Pennsylvania State University                    The Pennsylvania State University
### Exchanges per Day by Energy Level

<table>
<thead>
<tr>
<th>Energy Level</th>
<th>1300</th>
<th>1400</th>
<th>1500</th>
<th>1600</th>
<th>1700</th>
<th>1800</th>
</tr>
</thead>
<tbody>
<tr>
<td>Starch/Bread/Rice</td>
<td>6.5</td>
<td>7</td>
<td>8</td>
<td>9</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Meat/Poultry/Fish/Beans</td>
<td>5</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>Non-Starchy Vegetables</td>
<td>3 or more</td>
<td>3 or more</td>
<td>3 or more</td>
<td>3 or more</td>
<td>3 or more</td>
<td>3 or more</td>
</tr>
<tr>
<td>Dairy</td>
<td>2</td>
<td>2</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Fruit</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Fat</td>
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</table>

### Exchanges per Meal

<table>
<thead>
<tr>
<th></th>
<th>Breads/Cereals</th>
<th>Meat/Fish Beans</th>
<th>Vegetables</th>
<th>Dairy</th>
<th>Fruit</th>
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<td>Lunch</td>
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<tr>
<td>Dinner</td>
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<tr>
<td>Snack 1</td>
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<tr>
<td>Snack 2</td>
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</table>
VITA
Kathryn E. Piehowski, PhD, RD, CBDT

EDUCATION
- PhD – Graduate Program in Nutritional Sciences, The Pennsylvania State University, August 2006 – August 2011

AWARDS AND HONORS
- 2008 Billye June Eichelberger Scholarship, The American Dietetic Association Foundation
- 2008 Jeannette H. Crum National Graduate Fellowship, The American Association of Family and Consumer Sciences
- 2009 Crum-Koehler National Graduate Fellowship, The American Association of Family and Consumer Sciences
- 2009 Grace Henderson Scholarship, College of Health and Human Development, The Pennsylvania State University
- 2009 Invited Participant, National Institutes of Health Graduate Research Festival, National Institutes of Health, Bethesda, MD, November, 2009
- 2010 Dye-Leverton National Graduate Fellowship, The American Association of Family and Consumer Sciences
- 2010-11 Kligman Fellowship Endowment, College of Health and Human Development, The Pennsylvania State University
- 2010-11 Graduate Scholarship, The Central Pennsylvania Dietetic Association
- 2010-11 The American Dietetic Association Foundation Scholarship, The American Dietetic Association
- 2010 Graduate Student Award, the American Dietetic Association Research Dietetic Practice Group

PUBLICATIONS AND PRESENTATIONS