THE DOSE-RESPONSE EFFECTS OF LEAN BEEF IN A MEDITERRANEAN-
STYLE DIETARY PATTERN ON ESTABLISHED AND EMERGING
CARDIOVASCULAR DISEASE RISK FACTORS

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by
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ABSTRACT

A Mediterranean (Med) dietary pattern is widely recommended because of an extensive evidence base showing beneficial effects on cardiovascular disease (CVD) risk and mortality. As with many dietary patterns, much of the evidence has evolved from observational trials consistently showing major reductions in cardiovascular morbidity and mortality. Notably, due to a wide range of adherence scales, the benefits of a Med diet on cardiovascular risk factors (i.e. lipids, lipoproteins, vascular health) is largely based on findings from a variety of similar plant-based dietary patterns. Moreover, the benefits of a Med diet on emerging CVD risk factors is somewhat lacking and/or inconsistent. Specifically, limited evidence exists among a U.S. population. This is important as adherence to plant-based diets is often hampered by restrictions on red meat, a staple of the American diet.

Thus, the objective of this study was to evaluate the effects of a Med diet (CHO 42%, PRO 17%, FAT 41%, SFA 8%, MUFA 26%, PUFA 8%) with different quantities of lean beef (0.5, 2.5 and 5.5 oz/d) compared to an Average American diet (AAD; CHO 52%, PRO 15%, FAT 33%, SFA 12%, MUFA 13%, PUFA 8%) on multiple traditional and emerging CVD risk factors (lipids, lipoproteins, lipid subspecies, vascular health and HDL function) in a U.S. population. This was a multicenter, 4-period controlled feeding, randomized, crossover study conducted at Penn State University and USDA, Beltsville. Participants (n=66) included generally healthy normal to overweight/obese males and females (BMI= 20-38 kg/m²) aged 30 to 70 years. Participants were randomized to each of four diets for 4 weeks with an approximate 2-week break between treatments. Fasting blood samples were collected on two consecutive days at baseline (start of study) and at the end of each 4-week period.
All three Med diets elicited similar lowering of total cholesterol (TC; p<0.0001), LDL-C (p<0.001), non-HDL-C (p<0.0001) and apolipoprotein B (apoB) (p<0.0001) that was greater than the AAD. All diets (AAD, MED0.5, MED2.5 and MED5.5) decreased HDL-C (-3.46 ± 1.11, -4.93 ± 1.14, -4.44 ± 0.93, and -3.31 ± 1.20 mg/dl, respectively; p<0.01) and apolipoprotein A1 (apoA1) (-8.63 ± 1.77, -11.45 ± 1.72, -11.21 ± 1.71, and -7.97 ± 1.90 mg/dl, respectively; p<0.0001). However, the combined effects of the three Med diets versus AAD, on ABCA1 cholesterol efflux were significantly higher following a Med style diet (3.2 vs. 3.6; p=0.012, respectively). All three Med diets significantly reduced LDL particle number, however only the reductions by the MED0.5 and 2.5 were significantly different from AAD. Compared to AAD all three Med diets significantly decreased brachial systolic and diastolic pressures (p<0.01 for all). Both the 0.5 oz./day (-3.30 ± 0.76) and 2.5 oz./day (-2.94 ± 0.76) Med diets elicited greater reductions in central systolic blood versus the AAD. A similar pattern was observed for central diastolic pressure. There was a significant treatment effect for pulse wave velocity (p<0.01); pulse wave velocity was lower following consumption of a Med diet containing 0.5 oz. lean beef/day (6.86 m/sec ± 0.14; p<0.05) and 2.5 oz. of lean beef/day (6.84 m/sec ± 0.15; p<0.01) compared to the AAD (7.10 m/sec ± 0.14). A dose response analyses showed a significant difference between the MED0.5 and 5.5 in total cholesterol and LDL particle number with the MED0.5 eliciting a greater lipid lowering effect.

Our results demonstrate that lean beef at intakes equivalent to the average consumption in the U.S. (2.5 oz./day lean beef), may be incorporated into a Med style diet with no differences in cardiovascular risk benefits compared to a traditional Med diet with 0.5 oz./day lean beef. Despite there being no between treatment differences among the 3 Med diets, because lean beef intakes of 5.5 oz./day were similar to those for the AAD, our findings suggest that ≤2.5 oz./day
of lean beef can be included in a Med diet and not compromise the cardiovascular health benefits of a Med diet with 0.5 oz. lean beef. Furthermore, the observed improvements in cardiovascular risk markers were consistent with previous findings suggesting our U.S. population adhered well to the diet and as a result achieved similar cardiovascular benefits.
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ABBREVIATIONS

AAD Average American Diet
ABCA1, ATP-binding cassette subfamily A member 1
ABCG1, ATP-binding cassette subfamily G member 1
ALA α-linolenic acid
Apo Apolipoprotein
ApoAI, apolipoprotein A-I
ApoB, apolipoprotein B
BP Blood pressure
CDBP Central diastolic blood pressure
CHD Coronary heart disease
CSBP Central systolic blood pressure
CVD Cardiovascular disease
DASH Dietary Approaches to Stop Hypertension
DGAC Dietary Guidelines Advisory Committee
EVOO Extra virgin olive oil
HDL-C High-density lipoprotein cholesterol
IDL-C Intermediate-density lipoprotein cholesterol
LDL-C Low-density lipoprotein cholesterol
LDL-P Low-density lipoprotein particle number
Med Diet Mediterranean diet
MED0.5 Med diet with 0.5 oz./d lean beef
MED2.5 Med diet with 2.5 oz./d lean beef
MED5.5 Med diet with 5.5 oz./d lean beef
MI Myocardial Infarction
MUFA Monounsaturated fatty acids
NCEP National Cholesterol Education Program
PUFA Polyunsaturated fatty acids
PWA Pulse wave analysis
PWV Pulse wave velocity
RCT Randomized controlled trial
ROS Reactive oxygen species
SFA Saturated fatty acids
SR-B1 Scavenger receptor class B type 1
SREBP2 Sterol regulatory element-binding protein 2
TC Total cholesterol
TG Triglycerides
USDA U.S. Department of Agriculture
VLDL-C Very low-density lipoprotein cholesterol
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Chapter 1: Introduction

The most recent report from the Global Burden of Disease Study identifies CVD as being the leading cause of death worldwide (1). Several risk factors contribute to the development of CVD with dyslipidemia being one of the most influential modifiable risk factors (2). Lifestyle and in particular, unhealthy dietary patterns, represent a major contributor to the development of dyslipidemia. As such, dietary patterns have become the focus of human nutrition research and there is a growing evidence base to show the effectiveness of several dietary patterns in improving cardiovascular health, at least in the short term. It is the specific components of these patterns, such as the macronutrient distribution and types of foods included that impact adherence and ultimately long-term effectiveness.

Of the numerous dietary patterns evaluated, the Mediterranean (Med) dietary pattern is indeed one of the most well studied dietary and lifestyle patterns. Inspired from countries surrounding the Mediterranean Sea, the dietary pattern reflects the variety of foods traditionally consumed in the Mediterranean region during the mid-20th century. The staple foods in the Med dietary pattern include whole grains, fruits, vegetables, beans, herbs, spices, nuts and healthy oils, specifically olive oil. The Med dietary pattern also features moderate amounts of dairy (fermented cheese and yogurt) and lean protein, typically fish and poultry with very little red meat. In the 1960’s, the Harvard School of Public Health and the World Health Organization (WHO) became aware of the low chronic disease prevalence among these regions (3). This discovery, along with life expectancy exceeding world averages, was a surprise due to the limited available medical services. This led to the scientific investigation of the Med diet and its impact on chronic disease prevention and increasing life expectancy (4).
In recognition of the growing scientific evidence base surrounding the benefits of a Med diet, the U.S. now has identified the Med diet as one of three recommended dietary patterns, which is included in the 2015-2020 Dietary Guidelines for Americans. Some modifications from a traditional Med diet have been incorporated to accommodate the needs of the U.S. population, such as an increase in dairy consumption to ensure adequate calcium and vitamin D and a slightly lower fruit recommendation in consideration of the growing number of individuals diagnosed with diabetes and pre-diabetes (HHS & USDA, 2015). Unlike the Mediterranean regions, however, beef is a popular food in the U.S. and provides a source of key nutrients. The reduction of dietary saturated fat (SFA) has been a major focus of the guidelines for decades yet consumption in the U.S. continues to remain at levels greater than 10% of total calories.

Oftentimes a focus on one particular nutrient can lead to consumer confusion, such as the recommendation to restrict animal products to reduce SFA or the rise in refined carbohydrate intake in response to the low-fat recommendation. When a dietary pattern is considered overly restrictive long-term compliance becomes unattainable. However, adherence increases when certain staples of the U.S. diet (i.e. red meat) are incorporated into a healthy dietary pattern. For example, Nowson et al. (5) found that a DASH diet that included lean red meat was more accepted and therefore more effective in reducing blood pressure in older women, compared with a high-carbohydrate, low-fat diet. In addition, a growing body of clinical research has shown that lean red meat can be used interchangeably with other animal protein sources when total SFA is maintained at recommended levels, with no adverse effect on lipids and lipoproteins (6, 7).

Furthermore, dietary patterns should be designed to address a multitude of emerging CVD risk factors beyond our traditional targets (LDL-C and blood pressure). Therefore, the purpose of this
research is to examine the role of varying amounts of lean beef as part of a healthy Med-style diet on established and emerging CVD risk factors in a U.S. population.
Chapter 2: Literature Review

The Mediterranean Dietary Pattern

Cardiovascular disease (CVD) is considered the leading yet most preventable cause of mortality worldwide, accounting for 31% of all global deaths in 2016 (http://www.who.int/mediacentre/factsheets/fs317/en/). Nutritional status and dietary patterns are recognized as key factors in the development of disease, and among various dietary patterns, the Mediterranean diet (Med diet) has been shown to have a protective effect on cardiovascular health (8-10). The traditional Med diet represents the food patterns typical of Spain, Greece, and southern Italy in the early 1600’s. There are variations of this dietary pattern in Morocco, Portugal and Croatia. Although different regions have diets with unique characteristics, it is appropriate to consider these as variations of a single dietary pattern, the Med diet. One of the many dietary characteristics shared among the Mediterranean regions is the inclusion of olive oil as the predominant fat source (11).

As a key component of the Med diet, olive oil is associated with the preparation and consumption of an abundance of vegetables in the form of salads and sofrito, a traditional tomato based dish, as well as equally large quantities of legumes that serve as a major component of cooked meals (11). Thus, the Med diet as we know it may in part be identified as the dietary pattern that emerged in the olive-growing areas of the Mediterranean region in the late 1950s and early 1960s, before the Westernized fast food culture had spread to the region. In addition to olives and olive oil, the traditional Med diet, and in particular the Greek version of it, is characterized by the following components: 1) high MUFA/SFA ratio, 2) moderate wine consumption, 3) high legume intake, 4) high consumption of grains and cereals, including bread, 5) an abundance of fruits and vegetables, 6) low intake of meat and meat products, and 7)
moderate consumption of milk and dairy products. **Figure 2-1.** Total fat intake ranges by region with around 40% of total energy intake being typical in Greece, with intakes of approximately 30% of total energy intake being more common in Italy. This is likely a result of the higher olive consumption in Greece and pasta consumption in Italy, whereas fish consumption is more prevalent in some areas such as Spain (11). What remains consistent across regions is the very high ratio of monounsaturated fatty acids (MUFA) to saturated fatty acids (SFA) compared to other regions of the world, including northern Europe and North America.

Such differences in dietary pattern across the globe were first identified as part of an observational study conducted by Keys in the 1950’s (12). The study included 12,763 men aged 40 to 59 years that represented the populations of Greece and Southern Italy, the Netherlands, former Yugoslavia, Finland, United States of America (U.S.), and Japan. Over a period of 30 years, Keys and various collaborators (13-15) reported follow-up findings that focused on the role of diet in coronary heart disease (CHD). The dietary data were examined in relation to biochemical variables (mainly total cholesterol, because the importance of lipoprotein subfractions had not yet been identified as having different impacts on CHD risk). Interpretations of the Keys studies focused on the role of SFA as accounting for the variation of total cholesterol and, in turn, the incidence of CHD. Based on the low rates of other important causes of morbidity and mortality, the authors concluded that a diet consisting of less than 10% of calories from SFA was a predictive factor in reducing CVD risk. Some scientists from Mediterranean countries argued that the benefits of the dietary pattern was due to other factors beyond a low-SFA diet and had benefits for a wide range of diseases. It took many years for the scientific community to embrace the health benefits of the totality of the dietary pattern (11).
Several decades later, it is now recognized that several attributes of the Med diet contribute to the benefits observed against a variety of chronic diseases. Hence, a major focus of the research surrounding the Med dietary pattern has been understanding the benefits of the various components in order to differentiate this dietary pattern from other patterns found to be similarly protective (i.e. the “Prudent” and DASH diets) (17). Interestingly, the Med diet of the early 1960’s, as characterized by Trichopoulou and Lagiou, was “shaped by climatic conditions, poverty, and hardship rather than by intellectual insight or wisdom”. In a broader context it could also be described as “a set of skills, knowledge, practices and traditions ranging from
landscape to the table, including the crops, harvesting, fishing, conservation, processing, preparation and, particularly, consumption of food.” (UNESCO, 2013). Customary Greek meals include soups and salads served with whole-grain breads and plenty of olive oil, legumes, and vegetables, with fruit most commonly consumed as dessert. Dairy consumption is moderate with the predominant source being cheese, and feta in particular, and to a lesser extent yogurt. Low intake of meat was common because of the high cost, whereas high fish consumption was in relation to the proximity to the sea. The high consumption of vegetables, fresh fruits, and cereals and predominant use of olive oil provided a diet rich in many vitamins and minerals including beta-carotene, vitamin C, tocopherols, along with beneficial non-nutrient substances, such as polyphenols and anthocyanines. Wine is consumed with meals as part of the overall enjoyment of the foods (18, 19). Although the traditional Greek diet is rapidly adopting a more Westernized culture (20, 21) it still exists among much of the population, particularly in rural areas (22).

With the many healthful characteristics of a Med diet it is not surprising that the question of whether the Med diet is an essential entity or simply the sum of its components (e.g., high consumption of olive oil, high intake of vegetables and fruits) that may be equally beneficial, remains unknown. One of the first studies to address this question was a small study conducted among elderly Greeks. While the authors concluded that the Med dietary pattern as a whole was more important for longevity than were the individual components, the relatively small and targeted population limited its generalizability (18). Since that time, the study of the Med diet has advanced, and the definition originally introduced by Keys has evolved and varied. Numerous attempts to individualize whether the protective effects are reliant on individual food components or the dietary pattern as a whole have provided mixed results. Extensive research has shown that the benefits are attributed to the polyphenols in extra virgin olive oil (EVOO)
(23), the antioxidant compounds in fruit and vegetables (24) and the unsaturated fatty acids, vegetable proteins, antioxidants, and phytochemicals found in legumes and nuts (25, 26). The significant inverse associations of these specific components with CVD outcomes without the synergistic effects of the diet as a whole does suggest the individual role of these specific foods, yet the answer remains unclear. For example, the high alpha linolenic acid (ALA) intake in the Lyon Diet Heart Study (27); the abundance of fruits, vegetables and nuts in the Indo-Mediterranean Diet Study (28) and supplemental consumption of nuts and EVOO in the PREDIMED study (29) all elicited similar and favorable impacts on CVD outcomes, despite these slight variations in dietary pattern.

In large part, studying the Med dietary pattern has been challenging due to the lack of a universal definition of the Med diet. Nutrition epidemiologists define a dietary pattern in several ways, including general descriptions, dietary pyramids, a priori scoring systems, a posteriori dietary pattern formation, or by food and nutrient content (3, 18, 30-33). An a priori, system uses previously established dietary patterns and compares them to dietary data whereas a posteriori, uses principle component analyses to cluster dietary data into food groups. The disadvantage of a priori analysis is that it focuses on a few broadly defined food groups while a posteriori analysis, takes into account a much broader spectrum of foods and food groups (34).

In general, a priori scoring systems are most frequently used because they represent a simple way to assess adherence to the diet in relation to primary outcomes (8). Typically, a point system is used in which a greater number of points are awarded based on greater adherence to the selected dietary pattern or lower intakes of foods considered not to be in relation to the specified dietary pattern. From there a total adherence score is calculated. However, numerous Med diet adherence scales have been developed with different scoring criteria making
comparison across studies challenging. For example, as part of a study comparing the same nutritional data from 10 different a priori Med diet scales, mean adherence scores ranged from 22.7% to 87.7%, (8) with weak correlations between most indices (35). This illustrates the range of definitions used to calculate adherence to a Med diet and numerous variations of the diet that may exist, yet still achieve similar benefits.

Large differences also exist between studies using gram intakes of foods and food groups to assess adherence scores (36). The Greek population in the Seven Countries study reported a consumption of approximately of 191 g/day of vegetables, whereas the Greek individuals enrolled in the EPIC study had intakes greater than 500 g/day. Participants in the PREDIMED study reported an average consumption of 350 g/day (29, 37, 38). A focus on gram weight alone could be limiting our understanding of the mechanisms by which the Med diet confers its health benefits. For example, the biological activity of specific components of the Med diet, such as fatty acids, have been studied and show beneficial effects, yet the results are inconsistent (39). Such inconsistencies are likely the result of differences in amount of foods without regard to nutrient differences between studies. However, when the dietary pattern is assessed in terms of nutrient composition rather than amount of foods there is very little difference among studies. Davis et al. (36) gathered information from a wide range of previous Med diet studies and found that despite considerable variation in the quantity of each individual dietary component, the average nutrient content of the dietary pattern remained relatively consistent. This suggests that a variety of foods can provide similar nutrient profiles. In part, this may explain the variation among regions and the ability to preserve traditional foods and dishes consumed across the various Mediterranean countries while retaining the beneficial effects provided by the nutrients
and bioactive compounds. The authors concluded that there might be a “distinct advantage to defining the diet by nutrients rather than foods” (36).

Thus, the benefits of a Med diet are attributable to the dietary pattern and the inclusion of a wide variety of nutrients rather than any one individual food component. Notably, while the Med dietary pattern is considered plant-based, it also minimizes intake of nutritionally inadequate plant-based foods such as sugar, refined grains, sodium, and highly processed foods. Therefore, it is important to recognize that the dietary pattern appears to be effective because of the presence of nutritionally rich plant-based foods, rather than the exclusion or limitation of meat (40). Thus, a Med dietary pattern is not to be considered vegetarian, but rather a healthful dietary pattern abundant in plant foods with a combination of all components appearing to confer the greatest cardiovascular benefit. For example, results from the PREDIMED trial show that prediction of CVD events from the 14 individual score components is not always consistent with findings for prediction from the whole score, which suggests a potential synergistic effect among the individual components (41). This further demonstrates that the contribution of a variety of foods appears to enhance the robustness of the total score while also supporting the enjoyment of a varied diet as a major aspect of eating, a key component of the Mediterranean lifestyle.

**Epidemiological Evidence of the Benefits of a Mediterranean Diet on CVD**

Since its global recognition, a variety of dietary indices have been developed and regionally adapted to quantify level of adherence based on key features of the Med dietary pattern. Despite variation (and different scoring systems of Med dietary pattern adherence) numerous prospective cohort studies of total CVD risk in relation to adherence to a Med diet have been conducted. The extensive study of the Med diet is reflected in the publication of seven
meta-analyses of observational studies (8, 9, 42-46) since 2008. In total, the results consistently demonstrate an inverse association between adherence to the Med diet and the risk of total CVD. In the recent meta-analysis of 11 prospective cohort studies by Rosato et al. (46) a 19% lower risk of total CVD was reported when comparing the highest versus lowest categories of adherence to the Med diet (RR: 0.81; 95% CI: 0.74–0.88). These findings are consistent with those from a meta-analysis of 30 prospective studies conducted by Grosso et al. (9), which showed a 29% lower risk of CVD mortality (n=16; RR: 0.71; 95% CI: 0.65, 0.78) and 27% lower risk of CVD incidence (n=14; 0.73; 95% CI: 0.66, 0.80) when comparing the highest dietary adherence versus the lowest category. In a cumulative meta-analysis of both prospective cohort studies and randomized controlled trials (RCTs), Martinez-Gonzalez et al. (44) reported that each 2-point increment in a 9 point adherence scale was associated with an 11% relative risk reduction (RR: 0.89; 95% CI: 0.86, 0.91). These findings support those from an earlier meta-analysis by Soft et al. (8), which reported a 2-point increase in adherence score was associated with an 8% reduction of overall mortality (RR: 0.92; 95% CI: 0.91, 0.93), and a 10% reduced risk of CVD (RR: 0.90; 95% CI: 0.87, 0.92).

The extensive evidence base on the benefits of the Med dietary pattern has prompted its global recognition as one of the healthiest dietary patterns, demonstrating consistent beneficial outcomes with respect to CVD and longevity (47). Notably, an umbrella review conducted by Dinu et al., (48), concluded that the accumulated evidence is “robust” regarding the protective role of a Med style dietary pattern diet in reducing the risk for total mortality, CVD, cancer incidence, neurodegenerative diseases and diabetes. Moreover, an updated meta-analysis of prospective cohort studies (n=30) including studies published up to January 2018 verified an inverse association between adherence to the Med diet and all-cause mortality (49). As part of
their analysis, the authors estimated mortality ratios for each of the nine components included in
the Med diet score. Based on summary mortality ratios they reported inverse or null associations
for higher intakes of all Med diet components with the exception of meat and meat products,
which showed a slight positive association (RR: 1.07; 95% CI: 1.01, 1.13). The greatest
protection was observed from moderate alcohol intake as well as above the median consumption
of fruit (RR=0.88; 95% CI 0.83, 0.84). However, the authors suggest caution in interpreting the
results based on the influence of collection methods, the different number of studies included
within each individual component analysis and the varying descriptions of foods comprising the
individual components (49). For example, it is not known what meat and meat products were
represented.

Of the two other meta-analyses that have examined the role of the individual food
components, one in relation to incidence of and mortality from CVD (9) and the other on cancer
(50) neither one reported a positive association with meat. Similarities among all the analyses
included inverse associations with all-cause or disease specific incidence/mortality with higher
intakes of fruits and vegetables (49). In agreement with studies conducted within the
Mediterranean region, large prospective cohort studies conducted within the U.S., also have
demonstrated strong evidence for benefits of dietary patterns that are consistent with a Med style
dietary pattern on risk of total, CVD and cancer mortality (51-58).

**Clinical Evidence of the Benefits of the Mediterranean Diet on CVD**

Although much of the evidence base comes from epidemiological studies, two landmark
RCTs of dietary patterns have laid the groundwork for our current dietary guidelines (27, 29).
Findings from the Lyon Diet-Heart Study (27) and PREDIMED (29) trials, established that
certain dietary patterns can prevent or delay a significant amount of currently existing chronic
diseases and cardiovascular related diseases in particular.

The Lyon Heart Study, a secondary prevention trial of CHD (27, 59), showed a nearly
70% risk reduction (RR: 0.27 95% CI: 0.12, 0.59, p = 0.001) in cardiac events in those
individuals consuming a Med diet enriched with ALA, provided in the form of margarine,
compared with those in the control group (low-fat diet). The Indo-Mediterranean Diet Heart
Study (28), although later retracted, showed that a Med diet (rich in ALA from fruit, vegetables,
and nuts) was of benefit for secondary prevention of CHD in non-Mediterranean populations
demonstrating significant reductions in sudden cardiac death and non-fatal myocardial infarction
(MI). The Mediterranean Alpha-linolenic Enriched Groningen Dietary Intervention
(MARGARIN) Study (60, 61), a primary prevention study in hypercholesterolemic subjects, also
showed the benefit of a Med diet rich in ALA. The Mediterranean Diet, Cardiovascular Risks,
and Gene Polymorphisms (Medi-RIVAGE) trial was one of the first primary prevention studies
to show that it was the MUFA (specifically from olive oil) as part of a Med diet rich in soluble
fibers that was largely responsible for the reduction in cardiovascular risk. Specifically, the
Medi-RIVAGE study examined the effects of a Med-style or low-fat diet on risk factors in 212
individuals with moderate risk for CVD (62). Following three months of dietary intervention,
significant reductions were found in total and triacylglycerol-rich lipoprotein cholesterol,
triglycerides (TG), and apolipoprotein B (apoB) levels.

The largest, and perhaps most well known study to evaluate the cardiovascular effects of
the Med diet has been the multicenter PREDIMED trial (Prevención con Dieta Mediterránea).
Individuals (n=7,447) at high risk for CVD were randomized to receive a reduced-fat diet or a
Med diet supplemented with EVOO (1 liter per week per family or 50 g per day per participant)
or mixed nuts (30 g per day) for 4.8 years (63). With a primary composite endpoint of MI, stroke or death from CVD they found both Med diet groups (with nuts and with EVOO) experienced a 30% risk reduction in the primary endpoint compared to control. Several ancillary studies have since been conducted to identify the potential mechanisms that contributed to their findings. In a short-term ancillary study of 772 patients, Estruch et al. (64) found that compared to the reduced fat diet adherence to either Med diet resulted in greater reductions in the total cholesterol: HDL-C ratio. Of note, no treatment differences in LDL-C were observed among any of the interventions.

The results from several clinical trials of the Med diet have been summarized in different meta-analyses and have shown favorable effects on triglycerides (65), LDL-C (66), and blood pressure (65). Notably, the meta-analysis of 29 RCT’s conducted by Garcia et al. (65) suggested that interventions conducted in Europe demonstrated a greater beneficial effect on health outcomes than those conducted in the U.S. (65). This suggests that the U.S. population is less likely to realize the same benefits of a Med diet compared to populations within the Mediterranean region, or, more likely, that the U.S. population is unable to conform to a Med style dietary pattern. Currently, <1.0% of the U.S. population consumes an ideal dietary pattern that meets the recommendations for key components of a Med diet, while approximately 75% of the population is categorized as having poor adherence to a healthy eating pattern (67). Therefore, examining the benefits of a Med diet on cardiovascular risk in U.S. adults, as well as identifying ways to improve conformity, remain critical areas of research that warrant further investigation.
The Role of Red Meat on CVD Risk

Despite recommendations by the 2015-2020 Dietary Guidelines for Americans, the U.S. population continues to struggle with implementation and maintenance of a Med style dietary pattern. Currently, the typical American dietary pattern is characterized by excess added sugars, saturated fat, sodium, and calories. The poor diet quality of Americans is one of the primary causes of mortality and chronic disease in this country, with CVD being the most prevalent (43). Thus, in the U.S., where 1 out of 4 deaths are related to CVD, adopting healthful dietary patterns, such as the Med diet, is of utmost importance. The actual consumption of nutrients and foods in the average American diet versus what is recommended is presented in Table 2-1.

Table 2-1. U.S. consumption recommendations for key dietary factors (2009-2012)

<table>
<thead>
<tr>
<th>Dietary Factor</th>
<th>Actual Intake*</th>
<th>Recommended**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fruits (g/d)</td>
<td>115</td>
<td>300</td>
</tr>
<tr>
<td>Vegetables (g/d)</td>
<td>183</td>
<td>400</td>
</tr>
<tr>
<td>Nuts and seeds (g/d)</td>
<td>12 (&lt;3oz./wk)</td>
<td>20 (5 oz./wk)</td>
</tr>
<tr>
<td>Whole grains (g/d)</td>
<td>21</td>
<td>125</td>
</tr>
<tr>
<td>Unprocessed red meat (g/d)</td>
<td>47 (1.7 oz.)</td>
<td>&lt;100 g/wk (0.5 oz./d)</td>
</tr>
<tr>
<td>Processed meat (g/d)</td>
<td>31 (1.1 oz.)</td>
<td>Limit or avoid</td>
</tr>
</tbody>
</table>

*Source: Micha et al. 2017 (68)
**Source: USDA. 2015–2020 Dietary guidelines for Americans

In 2017, the average American consumed 56.9 pounds of beef (or approximately 2.5 oz/day) (https://www.nationalchickencouncil.org/about-the-industry/statistics/per-capita-consumption-of-poultry-and-livestock-1965-to-estimated-2012-in-pounds/). In contrast, the Med
dietary pattern recommends less than two servings per week of red meat, with an emphasis on lean cuts (http://dietamediterranea.com/en/piramide/). Therefore, in an effort to increase adherence to recommended dietary patterns, and specifically, the Med dietary pattern, the cardiovascular risk associated with inclusion of red meat in the diet will be reviewed.

**Updates on the Published Literature on Red Meat and CVD**

Based on a comparative risk assessment conducted by Micha et al. (68) nearly 50% of all cardiometabolic deaths are associated with poor dietary habits. Among 12 individual dietary factors, the greatest estimated mortality was associated with excessive sodium intake, low nut and seed intake and high processed meat consumption (Figure 2-2). In contrast, when separated from highly processed red meats, unprocessed red meats contributed the least amount (0.4%) to estimated mortality rates. This suggests that ingredients in the processing and preservation of red meat account for most of the risk (69) rather than nutrient composition. In general, the risk of red meat intake has been attributed to a high saturated fat and cholesterol content; however, recent evidence suggests red and processed meats have several important nutritional differences that may be contributing to increases in risk. The issue of confounding, which is highly common in observational data and often unavoidable, has failed to distinguish these differences. For example, the majority of observational studies demonstrating an adverse association between red meat and a number of major chronic diseases have failed to adequately account for fat content, meat processing, and cooking methods (70). These factors can influence the cardiovascular risk associated with lean red meat in the diet. In addition, red meats are often broadly classified into groups containing beef, pork as well as lamb (71), which is significantly higher in total and SFA (72). However, when the SFA content of the red meat is taken into consideration, the increased
disease risk is no longer statistically significant (73). Thus, it continues to remain unclear whether a causative relation exists.

Figure 2-2. Cardiometabolic mortality attributed to consumption of processed vs unprocessed meats Source: Micha et al. 2017 (68)

To adequately assess the impact of red meat intake on cardiovascular risk it is necessary to categorize red meat into two main groups: unprocessed (“fresh”) meat and processed meat (sausage, salami, hot dogs, bacon, and processed deli or luncheon meats). In meta-analysis of three prospective cohort studies and one case–control study with 56,311 individuals and 769 reported events, Micha et al. (69) reported no significant association between unprocessed red meat and cardiovascular risk (RR: 1.00 per 100 g serving/day, 95% CI: 0.81,1.23). In contrast, the analysis of processed meat consumption from the six observational studies, which included a total of 614,062 individuals and 21,308 events reported that each additional 50 g serving/day of
processed meats was associated with a 42% higher risk (RR: 1.42, 95% CI: 1.07, 1.89) of cardiovascular events. In the original analyses the standard serving of unprocessed meat was 100 g compared to a 50 g standard serving of processed meat, however when considering an equivalent portion (100 g serving/day of processed meats), there was an associated 2-fold increase in the risk of CVD (RR: 2.02, 95% CI: 1.14, 3.57).

**Observational Data on the Association between Red Meat and CVD**

The failure to differentiate higher fat red meat sources from lean red meat and/or not accounting for the level of processing (74) has resulted in mixed associations between red meat consumption and CVD mortality (75), acute MI, unstable angina, and metabolic syndrome (69, 71, 73, 74, 76). A prospective analysis using food frequency questionnaires (FFQs) from 47,976 men and 23,276 women aged 50 -71 years as part of the National Institute of Health-American Association of Retired Persons (NIH-AARP) Diet and Health Study (75), reported an increase in CVD mortality for men and women in the highest quintile of intake of both red (RR: 1.27 95% CI: 1.20, 1.35 and 1.50 95% CI: 1.37, 1.65, respectively), and processed meat (RR: 1.09, 95% CI: 1.03, 1.15 and 1.38, 95% CI: 1.26, 1.51, respectively). In their analyses, red meat included all types of beef and pork such as bacon, cold cuts, ham, hamburger, hot dogs, liver, sausage and steak while processed meat included bacon, red meat sausage, poultry sausage, luncheon meats (red and white meat), cold cuts (red and white meat), ham, regular hotdogs and low-fat hotdogs made from poultry. Likewise, a case-control study (n=1062) examining the effects of diet and heart disease found that a dietary pattern containing red meat (defined as beef, pork, bacon, and fried pork rind) was associated with an increased risk of nonfatal MI (OR = 3.7). However, this
dietary pattern also was characterized by increased palm oil, refined grains, added sugar, coffee, legumes, and organ meats (77).

In contrast, three meta-analyses of prospective studies that distinguished processed from unprocessed meat reported that processed meat consumption was significantly associated with a moderate increase in all-cause mortality, whereas the consumption of unprocessed red meat was not (78-80). In these studies, “unprocessed meats” were defined as fresh meat from beef, veal, lamb, or pork, excluding fish and poultry, whereas “processed meats” were defined as any meat preserved by smoking, salting, curing, or by the addition of chemical preservatives, such as bacon, sausages, hot dogs, salami, or ham.

Noting the inconsistencies in the literature, Mozaffarian (81) questioned the observed association between red meat and mortality reported by Sinha et al. (75). In his commentary he recognized that the AARP-NIH Diet and Health study used a negative control to correct for “all other deaths” (chronic pulmonary disease, pneumonia, influenza, diabetes, chronic liver disease and cirrhosis) and all but diabetes lacked a plausible biological mechanism linking these outcomes to increased meat consumption. The importance of this is reflected in the finding that consumption of red and processed meat had stronger associations to ‘all other deaths’, than CVD deaths. Mozaffarian identified the potential false assumption that is commonly made in epidemiologic studies of red meat and CVD. He asserted that increased red meat consumption was not associated with an increase in CVD mortality but that individuals with higher red or processed meat consumption also participate in unaccounted behaviors that increase their risk of CVD and mortality (81). Shang et al. (82) also recognized meat consumption as being a marker of an overall less healthy diet and lifestyle in their findings that high animal protein intake was associated with higher intakes of fat and lower nutritional adequacy, which likely contributed to
the positive association observed between animal protein intake and incident Type 2 Diabetes (T2DM).

**Clinical Evidence on the Role of Red Meat on CVD**

A review of RCTs examining the relationship between lean red meat consumption and CVD risk factors shows that within the context of a heart-healthy diet, there is no adverse effect on major CVD risk factors. Rather, a heart-healthy diet with lean beef results in improvements in CVD risk factors. A meta-analysis of 124 RCTs evaluated the lipid and lipoprotein changes when comparing beef against poultry or fish consumption and found no significant differences in CVD risk factors with similar reductions in LDL-C (83).

Two clinical trials conducted in a free-living setting found comparable cholesterol-lowering effects of a National Cholesterol Education Program Step I diet (total fat <30%, SFA <10% energy) containing lean red meat (LDL-C, -1.7 to -1.8%) or lean white meat (LDL-C, -2.0 to -2.9%) (84, 85). It should be noted that in both studies, subjects in the lean red meat group were more compliant to the cholesterol-lowering diet, as indicated by reported weekly meat intake, which was lower in the lean white meat group than in the lean red meat group (P<0.01). Similarly, Nowson et al. (5) examined the lipid and blood pressure (BP)-lowering effects of a DASH-like diet containing lean red meat in moderately hypertensive, post-menopausal women. After 14 weeks of dietary intervention (dietary instruction and lean red meat were provided), the investigators observed significant reductions in systolic BP (-5.6 mm Hg), with no changes in lipids or lipoproteins. The lack of an effect on lipids and lipoproteins was most likely due to the higher SFA intake (12-13% of total calories) in the DASH-like diet group.
Controlled feeding intervention trials with lean beef have shown improvements in lipids and lipoproteins including apolipoproteins A-I and C-III, as well as improved vascular health (7, 86). The Beef as Part of an Optimal Lean Diet (BOLD) study was a randomized 4 period crossover, controlled feeding trial. Thirty-six moderately hypercholesterolemic individuals (LDL-C >110mg/dL) completed each of 4 intervention diets for five weeks with a one week compliance break between diets. The intervention diets were as follows: 1) Healthy American Diet (HAD) [33% total fat, 12% SFA, 49% carbohydrate (CHO), 19% protein (PRO), 0.7 oz beef/d/2000 kcal]; 2) DASH Diet (27% total fat, 6% SFA, 50% CHO, 19% PRO, 1.0 oz beef/d/2000 kcal); 3) BOLD (28% total fat, 6% SFA, 54% CHO, 19% PRO, 4.0 oz beef/d/2000 kcal); and 4) BOLD+ (28% total fat, 6% SFA, 46% CHO, 28% PRO, 5.4 oz beef/d/2000 kcal). The BOLD study was designed to evaluate the effect of lean beef as part of a blood cholesterol-lowering diet on CVD risk factors (i.e., lipids, lipoproteins, and vascular health). Significant reductions (P<0.05) in total cholesterol and LDL-C were reported following the DASH (-19.0 ± 4.3 and -14.4 ± 3.7 mg/dL), BOLD (-18.6 ± 4.1 and -13.5 ± 3.6 mg/dL), and BOLD+ (-19.6 ± 4.1 and -13.5 ± 3.8 mg/dL) diets versus the HAD (-8.5 ± 4.1 and -5.5 ± 3.9 mg/dL). When compared with a DASH-like diet, the intervention diets containing lean beef demonstrated equivalent improvements on several CVD risk markers including lipids, lipoproteins and BP. Notably, compared with the HAD, the BOLD+ diet was the only treatment diet that significantly decreased apolipoprotein B, which is the predominant protein in non-HDL particles (i.e., the atherogenic lipoprotein particles).

Most recently, the randomized cross-over controlled feeding trial conducted by O’Connor et al. (87) evaluated the cardiometabolic response to a Med style diet (40% CHO, 22% protein, 40% total fat, 7% SFA and 20% MUFA), containing 500 g/week of lean beef and pork (Med-
Red), which represents typical U.S. consumption, versus a Med diet containing 200 g lean beef or pork per week (Med-Control). After 5 weeks of feeding they found greater reductions in both total and LDL-C following the Med Red vs Med Control (p<0.05 for both). This suggests that adopting a Med style diet with the inclusion of lean beef or pork may elicit similar cardioprotective benefits. Table 2-2 presents key trials demonstrating that lean red meat can be consumed interchangeably with other sources of lean protein, including white meat chicken and fish, when SFA is maintained at recommended levels with no attenuation of cardiovascular benefits.

**Table 2-2. Clinical studies that link beef consumption with heart health benefits**

<table>
<thead>
<tr>
<th>Reference</th>
<th>Study Design</th>
<th>Results for Beef</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scott et al. Arch Intern Med, 1994 (88)</td>
<td>85 g/d (3 oz.) beef (8% fat) vs. 85 g chicken (7% fat) in a heart healthy diet</td>
<td>↔ in lipid-lowering effects*#</td>
</tr>
<tr>
<td>Davidson et al. Arch Intern Med, 1999 (85)</td>
<td>6 oz./d lean red meat vs. 6 oz./d lean poultry or fish/d in heart healthy diet</td>
<td>↔ in lipid-lowering effects*#</td>
</tr>
<tr>
<td>Hunninghake et al. J Am Coll Nutr, 2000 (84)</td>
<td>≥6 oz/d as lean red meat vs. ≥6 oz./d lean white in a heart healthy diet</td>
<td>↔ in lipid-lowering effects*#</td>
</tr>
<tr>
<td>Beauchesne-Rondeau et al. AJCN, 2003 (89)</td>
<td>≥6 oz/d as beef, chicken, or white fish in Step I diet</td>
<td>↔ in lipid-lowering effects of all 3 diets*</td>
</tr>
<tr>
<td>Roussell et al. AJCN, 2012 (7)</td>
<td>0.7, 4.0 or 5.4 oz./d lean beef as part of a DASH diet</td>
<td>↔ in lipid-lowering effects of all 3 diets</td>
</tr>
<tr>
<td>O’Connor et al. AJCN, 2018 (87)</td>
<td>2.5 oz/d lean beef or pork vs poultry, eggs as part of a Mediterranean diet</td>
<td>↓ in lipid-lowering effects</td>
</tr>
</tbody>
</table>

*#Free Living Study; *Subjects only males

**Can Red Meat be Included as Part of a Mediterranean Dietary Pattern?**

Based on the evidence presented, the foundation for the recommendation to lower red meat intake in the context of a heart healthy diet remains unclear. In general, the U.S. population typically consumes less red meat (90, 91) than what has been reported in large population studies
of the Med diet (29, 92). According to Food and Agricultural Organization (FAO), as of 2013 red meat consumption in the Mediterranean regions is similar to current U.S. with amounts as high as 65 g (2.3 oz./day) in France and Italy (http://www.fao.org/faostat/en/#data/CL). In the European Prospective Investigation into Cancer and Nutrition (EPIC) project a mix of plant and animal products including eggs, fish, milk, unprocessed and processed meat (93) characterize the dietary patterns prevalent among Mediterranean countries (Italy, Greece, Spain, and France). Although red meat has always been a major part of the diet and there is a lack of consistent evidence supporting an association with increased cardiovascular risk, current dietary recommendations still suggest limiting red meat intake to as little as one to two servings per month highlight the importance of consuming mostly foods of plant origin (94-96).

Potential Health Risks Associated with Red Meat

Colorectal cancer

In general, unprocessed red meat refers to fresh meat, such as beef, veal, pork, lamb, among others, while processed meat describes any animal meat, which was treated by salting, curing, fermenting, smoking for preservation, or other processes to enhance flavor or improve shelf-life (International Agency for Research on Cancer, World Health Organization). For the past several decades, the health-related risks associated with red and processed meat intake has been a focus of debate in the scientific community. In a recent World Health Organization (WHO report), colorectal cancer was identified as a health risk linked to red and processed meat consumption (International Agency for Research on Cancer, World Health Organization). Colorectal cancer is the second leading cause of cancer death (49,920 deaths in 2009); in 2009 it
ranked fourth in diagnosed new cases (146,970 people) behind skin, breast, and prostate cancer (97).

In a review of nine cohort studies that examined the association between red meat intake and cancer risk, only two studies, the Nurses’ Health Study and the Health Professionals Follow-Up Study reported a positive association. The remaining seven cohort studies showed no relationship between beef intake and colorectal cancer (98). The Nurses’ Health Study reported a statistically significant dose-response relationship between red meat intake, defined as beef, pork, or lamb, and colorectal cancer (5th Quintile OR=1.8 at >134 g/d) (99). Giovannucci et al. (100) observed a similar dose response relationship between red meat intake and colorectal cancer in the Health Professionals Follow-Up Study. Men in the 5th quintile of red meat intake, defined as beef, pork, lamb, bacon, preserved meats, hot dogs, and hamburger, had a relative risk of colorectal cancer of 1.71. Over the past decade, several meta-analyses have been published and in general they have all produced similar positive but weak associations (101-107).

In response to the continued controversy, Alexander et al. (108) conducted an updated and expanded meta-analysis including dose response data from 27 independent prospective cohort studies. Inclusion criteria identified studies that classified meat intake as a composite “red meat” variable or individual red meat items, such as beef, pork, or lamb. Studies were excluded if they reported using a broad classification of meat that included poultry or fish, however, the categories of red meat across studies may have included some processed red meat items. In support of the previous meta-analyses they observed summary risk ratio estimates (SSRE) for red and colorectal cancer that were null or just above null (SRRE for men = 1.16; SRRE for women = 1.03). In conclusion, the authors report that red meat does not appear to an independent predictor of colorectal cancer risk (108).
The 2012 American Cancer Society Guidelines on nutrition and physical activity for cancer prevention recommends that individuals limit their intake of red and processed meats and to “prepare meat by baking, broiling, or poaching rather than by frying or charbroiling” (109). The recommendation is to “minimize consumption of processed meats such as bacon, sausage, luncheon meats, and hot dogs and choose fish, poultry, or beans as an alternative to red meat (beef, pork, and lamb).” However, it was noted that if red meat is consumed it is preferable to select lean cuts and eat smaller portions.

**Diabetes**

Over the last decade, a growing number of prospective studies have examined the association of red and processed meat with an increased risk of Type 2 Diabetes (T2DM). While the majority of these studies have consistently demonstrated an association between processed meat intake and a higher risk of incident diabetes (69, 110-114), the association of unprocessed red meat with T2DM risk is less certain, demonstrating much smaller (110, 114-116) or even no associations in the majority of studies (69, 111, 112, 117). In an analysis by Fretts et al. (118), they found that “the magnitude of the association of unprocessed red meat with fasting glucose was double that of processed meat”. In their analyses, however, unprocessed meat had a standard serving of 100 g, which was twice as much as the 50 g standard serving of processed meat; and after adjustment for BMI, the observed associations were attenuated and no longer statistically significant. In the meta-analysis by Feskens et al. (110), they reported an increased risk of T2DM associated with the consumption of red meat, which was mainly attributed to processed meat (110). **Figure 2-3** illustrates the results from meta-analyses conducted between 2010 and 2015 of the differences in outcomes between red and processed meat consumption on risk of T2DM and CVD.
Potential Mechanisms Thought to Contribute to Red Meats Adverse Effects

Heterocyclic amines

There currently are two hypotheses regarding the putative mechanisms to explain the associations between red meat intake and increased incidence of cancer – an increase in heterocyclic amine intake and an increase in heme intake. Heterocyclic amines are pro-cancerous in epidemiologic (120), animal (121), and cell culture studies (122). Processed meats and cooking red meat at high temperatures for extended periods of time causes the conversion of heterocyclic amines (HCA) to N-Nitrosamines; this by-product may initiate colorectal cancer (123-126). HCAs, once converted by cytochrome p450s (namely CYP1A2), readily react with
acetyltransferase and sulfotransferase enzymes to form DNA adducts with guanine. These DNA adducts result in base substitution or a mutation which leads to carcinogenesis; HCA can independently cause base substitutions as well (127). However, based on the dose of HCAs required to cause cancer in animals, it is unclear whether the amount of HCAs ingested in a typical diet is sufficient to cause colorectal cancer (128).

Le Marchand et al. (123) investigated the role of red meat preparation and genetic phenotype on colorectal cancer in a case-control study which included 727 Caucasian, Japanese, or native Hawaiians (matched for sex, age, and ethnicity). They examined phenotypes for enzymes that activate HCA, CYP1A2 and NAT2 (N-acetyltransferase-2). NAT2 is responsible for the O-esterification of heterocyclic amines following oxidation by CYP1A2. The rapid metabolizer CYP1A2 and NAT2 phenotypes (i.e., enzymes that acted quickly on their respective substrate) are polymorphisms that are hypothesized to increase risk for colorectal cancer (129). Individuals who preferred “well-done” red meat and who had the rapid metabolizer CYP1A2 and NAT2 phenotypes had a colorectal cancer odds ratio of 8.8. In comparison, individuals who preferred rare or medium-rare meat, and who had the slow metabolizer CYP1A2 and NAT2 phenotype had an odds ratio of 1.0. This study highlights the potential for genetic influences, in addition to red meat cooking methods, on colorectal cancer risk.

**Heme**

The second hypothesis by which red meat may increase colorectal cancer risk is via increased intake of heme. Previous research has shown that the heme molecule, and not heme iron, may cause increased cell proliferation (130). When heme is degraded by hydrogen oxide (HO), there is an increased production of reactive oxygen species (ROS), especially H$_2$O$_2$. Studies with colonic epithelial cells have shown that increases in H$_2$O$_2$ levels may further induce
inflammation, and cell proliferation. Whereas, Fe$^{2+}$ and bilirubin do not promote cell proliferation (130). As yet, the amount of dietary heme required to elicit clinically significant increases in abnormal colonic epithelial cell proliferation has not been identified.

**Red Meat, Heme and Diabetes**

Red meat is the main source of heme iron in the diet, which is more easily absorbed (25%) than non-heme iron (5–15%), and an elevated iron concentration has been associated with an increased T2DM risk by increasing glucose production and decreasing glucose utilization (131). As a pro-oxidant, iron is involved in the formation of ROS, which may increase cellular oxidative stress and contribute to the inhibition of insulin binding (132). ROS also damage pancreatic β-cells and previous observations have shown that iron accumulation in pancreatic β-cells contributes to impaired insulin secretion (133). It also has been speculated that elevations in hepatic iron stores may interfere with hepatic insulin extraction and increased hepatic glucose output (134). A summary result estimate from a meta-analysis of six prospective studies that included 41,091 control subjects and 4,366 T2DM cases demonstrated an increased risk of T2DM in those individuals with higher serum concentrations of the iron storage biomarker, ferritin (RRSE: 1.66, 95% CI: 1.1, 2.39 for the highest versus the lowest quintile; $P_{\text{trend}} = 0.01$) (135). Similarly, a meta-analysis of four prospective studies including 179,689 control subjects and 9,246 T2DM cases found that high intake of dietary heme iron was associated with an increased risk of T2DM (RRSE: 1.31, 95% CI: 1.21, 1.43 for the highest versus the lowest quintile) (135). Based on these findings there is evidence to suggest that increased dietary heme iron intake and increased serum ferritin concentration, a marker of iron
storage, are associated with a higher risk of T2DM. However, there is insufficient data from well-conducted RCTs to support a causative relationship between heme iron and T2DM.

**Red Meat, Heme and CVD**

The iron content of red meat varies by type and ranges from 1.7 mg in lean sirloin (per 3 oz. broiled) to 5.8 mg in beef liver (per 3 oz. braised) (USDA National Nutrient Database for Standard Reference Legacy Release, April 2018). The reported association between heme iron and the production of ROS was thought to promote the development of CVD (136). Yet, the evidence linking heme iron from red meat intake to CVD risk is limited. The relationship between iron and cardiovascular risk was first introduced in the findings by Sullivan et al. (137) in which he demonstrated that the incidence of CVD was higher in men and postmenopausal women compared with premenopausal women. From this, he concluded that the reduction in total iron via menstruation reduces CVD risk. Over a decade later, a four-year prospective trial was conducted to examine the risk of CHD in 44,933 US men aged 40 to 75 years with no previous history of CVD. In this study, neither total nor heme iron intake was associated with a higher incidence of fatal coronary disease or nonfatal MI. However, when comparing men in the highest quintile of intake to those in the lowest, incidence of fatal CHD or nonfatal MI was higher among men in the top quintile of heme iron intake compared with men in the lowest quintile (RR, 1.42; 95% CI, 1.02, 1.98). The authors concluded that the increased risk of MI among men with a higher intake of heme iron was due mainly to meat intake (138). In a study of both men and women, an inverse association was found between serum iron levels and CHD for both sexes as well as between iron level and women’s MI risk (139). In the Japanese cohort study of 58,615 healthy adults, Zhang et al. (140) showed no significant association between
dietary iron intake and mortality from CHD or MI in either sex. However, in a comparison of the highest versus lowest quintile of total iron intake, men in the highest quintile of iron intake had an increased risk of 1.43 (95% CI: 1.02, 2.00; P for trend = 0.01) for total stroke and 1.27 (1.01, 1.58; 0.02) for total CVD. There was no association for women. The limited and somewhat inconsistent data support the need for additional investigation into the association between dietary heme intake and CVD.

**Emerging CVD Risk Factors in Clinical Nutrition Research**

**Lipids and Lipoproteins**

Elevated LDL-C concentrations have been consistently associated with an increased risk of CVD, and therefore remains the primary target for cardiovascular preventive strategies (141). With the recent advancements in lipid testing, it is possible to identify the differences in the composition of the LDL particle, which provides information beyond that of LDL-C. Thus, it is now recognized that the risk information provided by LDL-C, LDL particle number (LDL-P), and LDL particle size may not be equivalent (142).

Advanced lipid testing allows for the measurement of the subpopulation of lipoproteins within a lipoprotein class. To date, four methods of advanced lipid testing have been developed commercially: nuclear magnetic resonance spectroscopy (NMR) (Liposcience, Inc., Raleigh, North Carolina), vertical rotor ultracentrifugation (VAP) (Atherotech, Birmingham, Alabama), and gradient gel electrophoresis and Ion Mobility (Berkeley Heart Lab, Inc., Berkeley, California). NMR and VAP methods are now becoming widely utilized in large clinical trials. The NMR lipid test directly measures lipoprotein subclasses particle numbers (**Figure 2-4**),
while the VAP test measures the cholesterol content in different lipoprotein subclasses (Figure 2-5).

**Figure 2-4.** NMR lipid test on classification of lipoprotein classes and subclasses

![NMR lipid test diagram](image)

**Figure 2-5.** VAP lipid test on classification of lipoprotein classes and subclasses

![VAP lipid test diagram](image)
Recent clinical evidence demonstrates that these novel lipid parameters can improve CVD risk assessment. In a subset of the Framingham Offspring Study (n=531), subjects with a low level of LDL-P (<25th percentile) had a lower CVD incidence (59 events per 1000 person years) than those with an equivalently low level of LDL-C or non-HDL-C (81 and 74 events per 1000 person-years, respectively) (143). In the Multi-Ethic Study of Atherosclerosis (MESA, n=5598), Mora et al. (144) reported that for those with discordant levels of LDL-C and LDL-P, only LDL-P was associated with incident CVD (LDL-P HR 1.45, 95% CI: 1.19-1.78; LDL-C HR 1.07, 95% CI: 0.88-1.30). Likewise, in the Women’s Health Study (n=27,533, median follow-up 17.2 years), CHD risk remained under or overestimated by ≈20-50% for women with discordant LDL-C and LDL-P levels after multivariable adjustment for potentially mediating factors including HDL-C and TG (145). The discordance between LDL-C and LDL-P is due to variation in cholesterol and TG content in LDL particles, which can be assessed by LDL particle size and density (Figure 2-6).

Figure 2-6. Differences in LDL-C and LDL-P measurements.
Large prospective studies have demonstrated that the measurement of small dense LDL (sdLDL) may be a marker for CHD risk that is typically undetected when using only standard lipid measurements in various populations (166-169). In the Atherosclerosis Risk in Communities (ARIC) study (n=11,419, follow-up 11 years), Hoogeveen et al. (146) found that sdLDL was significantly associated with CHD incidence after adjusting for standard CHD risk factors, even in a subgroup of individuals with an optimal LDL-C level (<100mg/dL). In a sub sample from the MESA study (n=4387, 8.5 years of follow up), Tsai et al. (147) reported that elevated sdLDL was a risk factor for developing CHD after adjusting for standard CHD risk factors in normoglycemic individuals. In contrast, some studies have failed to find an improved prediction of incident cardiovascular events using markers from advanced lipid testing (148, 149). Although measurements of sdLDL may not improve risk assessment beyond conventional lipid measures in all populations, their significant association with disease outcomes, as well as their pathological properties, suggests the potential use of advanced lipid testing in monitoring efficacy of lipid-altering therapy and dietary intervention for reducing CVD risk in individual patients.

The Med dietary pattern has been associated with reduced LDL-C concentrations (64, 150, 151), but the effects on LDL particle subclasses is an area that has not yet been explored. By assessing in changes in both LDL particle number and LDL size following consumption of a Med diet, our study adds novel evidence on the beneficial effects of this dietary pattern. Nuclear magnetic resonance testing is the most frequently used and recommended (by the ADA, ACC, AACC and NLA) test that can provide information about the number of LDL particles.
**Vascular health**

Vascular health is highly recognized as an important CVD risk marker, as cardiovascular events are largely driven by atherosclerotic cardiovascular disease. Although resting brachial blood pressure is the traditional measure used diagnostically, central or aortic blood pressure may improve risk stratification and better predict CVD outcomes. Conventional cuff sphygmomanometer measurements of brachial systolic and diastolic pressure are considered an accurate reflection of the pressure load in large conduit arteries, despite the fact that they are measured in a peripheral limb. Although diastolic pressure measurements remain almost steady throughout the arterial tree, as the pressure wave travels from the highly elastic central arteries to the stiffer brachial artery the arterial pulse narrows forming a more prominent peak; thus, systolic pressure and pulse pressure in the brachial vasculature is generally not the same as that in the central aorta (152). Due to anatomical proximity, the central aortic pulse pressure more closely reflects the pulsatile stress experienced by organs such as the heart, brain, and kidneys than the peripheral pulse pressure (153). There is growing evidence to suggest that the measurement of central blood pressure provides greater predictive value and may respond differently to an intervention than brachial blood pressure. For example, in the Strong Heart Study (n=3520), central pulse pressure was a better predictor of cardiovascular events, vascular hypertrophy, and the extent of atherosclerosis than brachial pulse pressure (154). Similarly, in the Conduit Artery Function Evaluation (CAFÉ) Study, they found that different blood pressure-lowering medications elicited substantially different effects on central aortic pressure - despite producing similar reductions in brachial pressures - and that this may better predict clinical outcomes, such as total cardiovascular events and the development of renal impairment (155). Central pressures can be measured non-invasively from peripheral pressure wave forms by using
a Fourier generalized transfer function (156). The SphygmoCor System (AtCor Medical) has become one of the most widely used methods for non-invasive measurement of central blood pressure and measures of arterial stiffness (156).

Pulse wave analysis is conducted using measurements obtained from a brachial blood pressure cuff and a generalized transfer function to derive central blood pressure characteristics. As the incident pulse generated by the contraction of the left ventricle travels towards the periphery, part of this wave is reflected back and merges with the incident wave. Thus, the central pulse pressure wave is formed by the combination of the incident wave generated by the left ventricle in systole and the wave that is reflected back from the periphery. In healthy, elastic arteries the reflected wave travels slower and returns later, merging with the incident wave at diastole. As arteries become stiffer, the rate and amplitude of the reflected wave are increased and the reflected wave returns to the heart sooner, merging with the incident wave during systole, prior to aortic closure (157). This results in an increase in central systolic pressure and greater left ventricular afterload that accelerates left ventricular hypertrophy (158). This increase in pressure is expressed as the augmentation index (AIX). The augmentation index depends primarily on: 1) the timing of the reflected wave, which is determined by pulse wave velocity and arterial stiffness, and 2) the intensity or amplitude of the reflected wave, which is determined largely by peripheral resistance. Thus, larger AIX values indicate greater wave reflection and/or earlier return of the reflected wave as a result of increased pulse wave velocity due to arterial stiffness. Higher AIX values are associated with coronary artery disease, cardiovascular risk, and other important cardiovascular intermediate endpoints (156).

Pulse wave velocity (PWV) is a measure of the time required for a pulse wave to travel from one point to another (158). The SphygmoCor PWV is performed by simultaneously
recording the wave forms at the carotid and femoral arteries. Pulse wave velocity is dependent on the stiffness of the vasculature, with increasing values signifying greater vessel stiffness. PWV was associated with coronary artery plaque burden in a cross-sectional analysis, and has been shown to be a strong predictor of mortality in multiple longitudinal studies (158).

**HDL Functionality**

HDL has several anti-atherosclerotic properties, including the regulation of macrophage cholesterol efflux, increase in antioxidant capacity and anti-inflammatory effects (159). HDL particles in particular are the critical acceptors of cholesterol from lipid loaded macrophages. However, the traditional measurement of absolute HDL-C only provides information about the size of the HDL pool, but does not convey anything about HDL function. Thus, growing evidence suggests that HDL functionality, specifically HDL-mediated cholesterol efflux from macrophages during reverse cholesterol transport seems to correlate better with atherosclerotic burden than HDL-C levels (160).

New insight into the functional role of HDL affirms it has several anti-atherogenic functions that may influence CVD risk, which, in contrast to previous views, are not dependent upon increases in HDL-C concentrations but rather improvements in HDL functionality. The reverse cholesterol transport function of HDL is important for cell cholesterol homeostasis, which is thought to be a protective mechanism against atherosclerosis. HDL function is measured by cholesterol efflux capacity, which is an assessment of HDL’s transport of cholesterol from peripheral cells to the liver for recycling or excretion (161). The major cholesterol efflux mechanisms are: 1) free cholesterol (FC) efflux to the HDL particles via passive diffusion through the plasma membrane (162); 2) cholesterol efﬂuxed to lipid-poor
apoA-1 or HDL particles by ABCA1 or ABCG1 transporters (162, 163); and 3) cholesterol efflux via scavenger receptor class B type I receptors (SR-BI) (164).

Cholesterol efflux, independent of HDL concentration, has been shown to be inversely related to coronary artery disease (165) and incident CVD events (166, 167) in several studies. To date, the largest and most rigorous study to measure HDL functionality as part of a dietary intervention was conducted on a sub-sample (n=296) of the PREDIMED study. This landmark randomized controlled trial comparing two Mediterranean diets (Med diet), one supplemented with mixed nuts and the other with extra-virgin olive oil (EVOO), to a reduced fat diet in a Spanish population (n=7447) reported a 30% risk lower risk of major cardiovascular events over a period of 5 years with a Med diet. In the sub-sample analyzed by Hernaez et al., (168) the authors found that both Med diet groups improved cholesterol efflux compared to baseline (p=0.018 and p=0.013 for the EVOO and mixed nuts group, respectively); no change was observed for the reduced fat control diet. Of note, both Med diet groups also experienced a trend toward a reduction in HDL-C concentration. In an earlier study by Hernaez et al. (169), the investigators reported similar increases in cholesterol efflux capacity following 3 weeks consumption of EVOO, which they attributed to the high polyphenol content. Similar to the previous study of the Med diet (168) there were no changes reported for absolute HDL-C concentration. Recognizing the strong relationship of diet to both HDL-C and CVD risk has prompted an increase in dietary interventions designed to assess the role of HDL functionality, yet results have been contradictory.
Rationale for Current Study

In response to the growing scientific evidence base surrounding the benefits of a Med diet; the Med diet has been included as one of three recommended healthy eating patterns in the 2015-2020 Dietary Guidelines for Americans. Some adjustments from a traditional Med diet have been incorporated to accommodate the needs of the U.S. population, such as an increase in dairy consumption to ensure adequate calcium and vitamin D and a slightly lower fruit recommendation in consideration of the growing number of individuals diagnosed with diabetes and pre-diabetes (HHS & USDA, 2015). Unlike the Mediterranean regions, however, beef is a popular food in the U.S. and provides a source of key nutrients, yet the recommendation to limit red meat remains resolute. Additionally, previous research has shown that lean beef can be included in a heart-healthy dietary pattern that is low in saturated fatty acids (SFA) and cholesterol (6, 7). Therefore, the purpose of this research is to examine the role of varying amounts of lean beef as part of a healthy Med-style diet on CVD risk factors in a U.S. population.

Objectives and hypotheses

1) To determine the effect of three different lean beef quantities (0.5 oz., 2.5 oz., and 5.5 oz. per day) in a Mediterranean-style dietary pattern on lipids and lipoproteins when compared to an Average American Diet.
   a. Hypothesis: Inclusion of three different quantities of lean beef in a Med-style dietary pattern will confer similar improvements in lipids and lipoproteins.
b. Hypothesis: Inclusion of three different quantities of lean beef in a Med-style dietary pattern will confer greater improvements in lipids and lipoproteins when compared to an Average American Diet.

2) To assess the effect of three different lean beef quantities (0.5 oz., 2.5 oz., and 5.5 oz. per day) in a Mediterranean-style dietary pattern on measures of vascular health when compared to an Average American Diet.
   a. Hypothesis: Inclusion of three different quantities of lean beef in a Med-style dietary pattern will confer similar improvements in vascular health.
   b. Hypothesis: Inclusion of three different quantities of lean beef in a Med-style dietary pattern will confer greater improvements in vascular health when compared to an Average American Diet.

3) To investigate the effects of three different lean beef quantities (0.5 oz., 2.5 oz., and 5.5 oz. per day) of lean beef in a Mediterranean-style dietary pattern on the biological and functional properties of HDL that extend beyond HDL-C concentrations
   a. Hypothesis: Inclusion of three different quantities of lean beef in a Med-style dietary pattern will confer similar improvements in cholesterol efflux.
   b. Hypothesis: Inclusion of three different quantities of lean beef in a Med-style dietary pattern will confer greater improvements in cholesterol efflux when compared to an Average American Diet.
Chapter 3: The Effect of Varying Quantities of Lean Beef as part of a Mediterranean-Style Dietary Pattern on Lipids and Lipoproteins: A Randomized Controlled Trial

ABSTRACT

Background: A Mediterranean (Med) dietary pattern is widely recommended because of an extensive evidence base showing beneficial effects on cardiovascular disease (CVD) risk and mortality. The reduction in cardiovascular mortality is due, in part, to the improvements in lipids and lipoproteins versus a Western dietary pattern. Plant-based diets such as a Med diet are recommended for CVD risk reduction. However, adherence to plant-based diets is often hampered by the limited or restricted intake of red meat, a staple of the American diet.

Objective: We evaluated the effects of a Med diet (CHO 42%, PRO 17%, FAT 41%, SFA 8%, MUFA 26%, PUFA 8%) with different quantities of lean beef (0.5, 2.5 and 5.5 oz/d) compared to an Average American diet (AAD; CHO 52%, PRO 15%, FAT 33%, SFA 12%, MUFA 13%, PUFA 8%) on CVD risk factors.

Design: This was a multicenter, 4-period controlled feeding, randomized crossover study conducted at Penn State University and USDA, Beltsville. Participants (n=66) included generally healthy normal to overweight/obese males and females (BMI= 20-38 kg/m²) 30 to 65 years. Participants were randomized to each of the 4 diets for 4 weeks with an approximate 2-week break between treatments. Fasting blood samples were collected on two consecutive days at baseline (start of study) and at the end of each 4-week period.

Results: All three Med diets elicited similar lowering of total cholesterol (TC; p<0.0001), LDL-C (p<0.001), non-HDL-C (p<0.0001) and apolipoprotein B (apoB) (p<0.0001) that was greater than the AAD. All diets (AAD, MED0.5, MED2.5 and MED5.5) decreased HDL-C (-3.46 ± 1.11, -4.93 ± 1.14, -4.44 ± 0.93, and -3.31 ± 1.20 mg/dl, respectively; p<0.01) and apolipoprotein
A1 (apoA1) (-8.63 ± 1.77, -11.45 ± 1.72, -11.21 ± 1.71, and -7.97 ± 1.90 mg/dl, respectively; p<0.0001). All three MED diets significantly reduced LDL particle number, however only the reductions by the MED0.5 and 2.5 were significantly different from AAD.

**Conclusions:** A healthy Med style diet containing 2.5 oz/day of lean beef elicits similar improvements in multiple CVD risk factors compared to a traditional Med style diet containing 0.5 oz/day. It is possible that the replacement of antioxidant and polyphenol rich foods when additional quantities of lean beef (5.5 oz/day) are included in the diet will attenuate the benefits in some risk factors.

**Keywords:** Mediterranean diet, lean beef, lipids, lipoproteins, cardiovascular disease
INTRODUCTION

Current dietary guidelines now focus on healthy dietary patterns for the prevention of chronic disease risk versus traditional nutrient based recommendations recognizing that people do not eat foods in isolation but rather in combination. The components of the eating pattern can have interactive and potentially cumulative effects on health. (170). Two dietary patterns consistently linked with improved cardiovascular health include the Dietary Approaches to Stop Hypertension (DASH) and Mediterranean (Med) dietary patterns. Both dietary patterns are plant-based (i.e. abundant in fruits, vegetables, grains, cereals, nut, and seeds) and low in red meat and saturated fat with varying amounts of unsaturated fats. As two recommended heart healthy dietary patterns included in the 2015-2020 Dietary Guidelines, Americans are now encouraged to adopt a dietary pattern that is lower in red meat and low in processed meats. However, red meat remains a staple of the typical American diet and provides several key nutrients, some of which otherwise may be lacking (e.g. vitamin B12, niacin, vitamin B6, iron, zinc and phosphorus) (171). The recommendation to limit red meat may make long-term compliance to a healthy eating pattern challenging and potentially exacerbate certain nutrient shortfalls. Furthermore, there is a growing clinical evidence to show that lean, unprocessed red meat may be included as part of a heart healthy eating pattern with no adverse effects on cardiovascular disease (CVD) risk factors (5, 7, 86). Nevertheless, reducing animal proteins, and particularly red meat, is progressively encouraged to attain cardiovascular benefits.

The dietary patterns followed in the Mediterranean regions (Italy, Greece, Spain, and France), which are represented in the European Prospective Investigation into Cancer and Nutrition (EPIC) project, are characterized by a variety of plant foods including legumes, vegetables oils, fruits and vegetables as well as animal products including eggs, fish, milk, along
with unprocessed and processed meat (93). According to Trichopoulou et al. (18), the average daily intakes of red meat in Greece in 1990, which represents the traditional Med diet, were ~100 g per day. As a whole, the U.S. population typically consumes less red meat (90, 91) than what has been reported in large population studies of the Med diet (29, 92), yet CVD accounts for about 1 of every 3 deaths in the U.S. (67). Currently, <1% of the U.S. population consumes an ideal dietary pattern that meets the recommendations for key components of a Med diet, while approximately 75% of the population is categorized as having poor adherence to a healthy eating pattern (67). However, increases in dietary adherence have been demonstrated when certain staples of the U.S. diet (i.e. red meat) are incorporated into a healthy dietary pattern (5). The elimination and/or restriction of favored foods is often cited by individuals as major challenges or reasons for lack of adherence to recommended dietary guidelines (84).

The primary target for CVD risk reduction is low density lipoprotein cholesterol (LDL-C). The recommended approach for lowering LDL-C is the reduction of SFA (<7%), which can be achieved by following a plant-based dietary pattern that limits red meat. However, there is little evidence directly linking lean red meat intake with increases in LDL-C (84, 85, 89). Previous research by Roussell et al. (7) comparing a DASH diet containing 1.0 oz/beef/day/2100 kcal to a similar diet containing approximately 5.4 oz/beef/day/2100 kcal (BOLD+ diet) with equal amounts of saturated fat (<7%) elicited equivalent improvements of several CVD risk factors including LDL-C. Additionally, when compared to a healthy average American diet only the BOLD+ significantly reduced apolipoprotein B (ApoB), which is the predominant protein of the non-HDL, or atherogenic, particles. Similarly, in an evaluation of a Med style diet (40% CHO, 22% protein, 40% total fat, 7% SFA and 20% MUFA), containing 500 g/week of lean beef and pork, which represents typical U.S. consumption, the authors reported reductions of 8% in
LDL-C and 6% in apoB. There was no change in response to the Med diet with the same macronutrient composition containing ~ 200 g of lean beef and pork per week (87). This study was not designed to identify the mechanisms by which a Med style diet with lean beef or pork might differentially affect lipids and lipoproteins. In many trials apoB is often used for estimation of LDL particle number and has been shown to be more strongly associated with CVD than LDL-C (172). However, apoB does not distinguish between total numbers of LDL particles and very low density lipoprotein (VLDL) particles. Therefore, quantifying LDL particle number by nuclear magnetic resonance spectroscopy can help to advance our knowledge of the mechanism(s) that account for the benefits associated with a traditional Med diet as well as variations of it which incorporate lean beef.

The main objective of this controlled feeding trial was to assess the effects of varying amounts (0.5 to 5.5 oz/day) of lean part as part of a Med style diet on CVD risk factors when compared to an average American control diet (AAD), containing approximately 2.5 oz. beef/day/2100 kcal. The three doses of lean beef were 0.5 oz./beef/day/2100 kcal (MED0.5), which represents an amount recommended in the Mediterranean Diet Pyramid, 2.5 oz./beef/day/2100 kcal (MED2.5), which represents current consumption patterns in the U.S. and 5.5 oz./beef/day/2100 kcal, which represents an amount previously shown to elicit heart health benefits when consumed as part of a DASH like diet. We hypothesized that all three Med diets would elicit greater improvements in lipids and lipoproteins compared to AAD in generally healthy U.S. adults. We further examined the dose response relationship between the Med diets with varying quantities of lean beef on the lipid and lipoprotein response.
METHODS

Experimental Design

This was a 4-period, randomized, crossover, controlled-feeding study conducted at two centers: Penn State University and USDA-Beltsville Human Nutrition Research Center. Subjects received each 4-week test diet in random order with a 1-2 week compliance break between diet periods where subjects could resume their self-selected diet. (Figure 3-1). Due to the study design, the participants were not blinded; however, the study coordinator and investigators were blinded for purposes of statistical analysis. At baseline (start of study) and at the end of each diet period, subjects completed a series of clinical and physical assessments [blood draw, weight, vascular function/arterial stiffness (by Sphygmocor), and BP] at the Clinical Research Center (CRC). These endpoints were collected over two consecutive days to control for variations in blood biomarkers including lipids, lipoproteins, inflammatory markers and other CVD risk factors. An independent staff member of the USDA performed the block randomization procedure. Participants were allocated to one of twelve sequences to ensure that treatments were assigned in a balanced and random order. Sequences were coded for the purpose of blinding the investigators during analyses. This trial is registered at clinicaltrials.gov #NCT02723617.
**Figure 3-1. Study design**

AAD: Average American Diet with 2.5 oz. per day; MED 0.5: U.S. Mediterranean Diet with 0.5 oz. per day of lean beef; MED 2.5: MED Diet with 2.5 oz. per day of lean beef; MED 5.5: MED Diet with 5.5 oz. per day of lean beef based on a 2100 kcal diet

= Clinical assessments conducted across two consecutive days

Study Population

Healthy nonsmoking normal to overweight [body mass index (BMI): >20 and <40 kg/m²] men and women (n=66; ~30 per site) aged 30-70 years were recruited. Exclusion criteria included: triglycerides > 350 mg/dL; HDL-C < 15th percentile of U.S. population (men < 34 mg/dL, women < 41 mg/dL); fasting glucose > 126 mg/dL; BP >160/100, participants on BP medications were considered eligible provided they met the specified blood pressure range of <160/100 mmHg and had been stable for at least 6 months; presence of kidney disease, liver disease, gout, untreated or unstable hyper- or hypothyroidism certain cancers, gastrointestinal disease, pancreatic disease, other metabolic diseases, or malabsorption syndromes, active CVD; cholesterol-lowering medication use; refusal to discontinue intake of putative cholesterol-lowering supplements (psyllium, fish oil capsules, soy lecithin, niacin, fiber, flax, and phytoestrogens); vegetarianism or other dietary practices that are inconsistent with the test diets;
weight change of ≥ 10% of body weight within 6 months prior to enrolling in the study; women
who were pregnant, lactating, planning to become pregnant or who had given birth in the past
year. Participants were required to maintain weight and physical activity levels.
The Institutional Review Board at the Pennsylvania State University approved the experimental
protocol, and all participants provided written informed consent.

**Dietary Interventions**

Participants consumed a controlled weight maintenance, full-feeding diet with a fixed
macronutrient composition that varied only between the Med diets (41% fat, 42% carbohydrate,
17% protein) and the Average American diet (33% fat, 52% carbohydrate, and 15% protein).
They were provided with three meals and two snacks daily using a seven day rotating menu for
the complete duration of each intervention period. The four intervention diets included: 1) a Med
diet (MED0.5) providing 0.5 oz. per day of lean beef; 2) a matched MED diet (MED2.5) with the
same fatty acid profile as the MED diet, but contained 2.5 oz. per day of lean beef; 3) a matched
MED diet (MED5.5), providing 5.5 oz. per day of lean beef; and 4) an Average American Diet
(AAD). The amount of lean beef consumed was based on the calculated energy requirements of
the participants, with a 2100 kcal diet providing 0.5, 2.5 and 5.5 oz /per day for the MED0.5,
MED2.5 and MED5.5 respectively. The nutrient composition of the treatment diets is presented
in Table 3-1.

Study diets were prepared in the metabolic kitchen facility located at each site.
Participants were encouraged to eat one meal per day (Monday–Friday) on site, and their other
meals were prepared and packed for off-site consumption for the remaining weekday and
weekend meals. Participants were instructed to consume only the foods provided and to limit
their consumption of alcohol (≤ 2 drinks/week) and caffeinated beverages (< 40 oz. per day).

Diets were planned for every subject according to their energy requirements and were
nutritionally adequate. Energy requirement was calculated using the Harris-Benedict equation.

Block menus were created in 200 kcal increments for a 7-day diet cycle across a range of calorie
levels (1600-3800 kcal/day). Body weights were monitored daily, and if participants gained or
lost weight energy adjustments were made. Menus were developed using FOOD PROCESSOR
(ESHA Research, Salem, Oregon, United States) and the nutrient content of the diet was
analyzed to verify macronutrient composition and assure protocol accuracy. In brief,

homogenized samples of each treatment menu across two calorie levels were analyzed by
Covance Laboratories, Inc. (Madison, WI). See Table 3-2 for complete analysis. Compliance
during the trial was monitored based on the remaining amounts of food intended for off-site
consumption, and by daily and weekly questionnaires asking about the consumption of study and
non-study foods and beverages, as well as daily weigh-ins.

Table 3-1. Proposed nutrient composition of the treatment diets†

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>AAD</th>
<th>MED0.5</th>
<th>MED2.5</th>
<th>MED5.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total fat (%)</td>
<td>33</td>
<td>41</td>
<td>41</td>
<td>41</td>
</tr>
<tr>
<td>SFA (%)</td>
<td>12</td>
<td>8</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>MUFA (%)</td>
<td>13</td>
<td>25.5</td>
<td>25.5</td>
<td>25.5</td>
</tr>
<tr>
<td>PUFA (%)</td>
<td>8</td>
<td>7.5</td>
<td>7.5</td>
<td>7.5</td>
</tr>
<tr>
<td>Carbohydrate (%)</td>
<td>52</td>
<td>42</td>
<td>42</td>
<td>42</td>
</tr>
<tr>
<td>Protein (%)</td>
<td>15</td>
<td>17</td>
<td>17</td>
<td>17</td>
</tr>
<tr>
<td>Cholesterol (mg)</td>
<td>&lt;300</td>
<td>&lt;300</td>
<td>&lt;300</td>
<td>&lt;300</td>
</tr>
<tr>
<td>Sodium (mg)</td>
<td>~3500</td>
<td>&lt;2300</td>
<td>&lt;2300</td>
<td>&lt;2300</td>
</tr>
<tr>
<td>Beef (oz/day)</td>
<td>≈ 2.5 oz.</td>
<td>0.5 oz.</td>
<td>2.5 oz.</td>
<td>≈ 5.5 oz.</td>
</tr>
<tr>
<td>ALA (g)</td>
<td>1.5</td>
<td>1.5</td>
<td>1.5</td>
<td>1.5</td>
</tr>
<tr>
<td>Marine n-3 (g)</td>
<td>&lt;0.1</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
</tbody>
</table>

†AAD: Average American Diet; MED: Healthy Mediterranean Diet (0.5, 2.5 or 5.5 oz. per day of
lean beef). Food Processor Nutrient Analysis Software (Version 10.10) was used to estimate the
total fat and fatty acid composition of the diets, rather than chemical analysis.
The Med diet used in this study was representative of the Med diet described by Fundación Dieta Mediterránea (https://dietamediterranea.com/en/nutrition/) (Table 3-3) and consistent with dietary guidelines for dietary saturated fatty acids and sodium. The three MED diets each contained similar foods with the exception of the amount of beef included and other protein equivalents. Each of the MED diets included 7oz. equivalents of protein, of which 0.5, 2.5 or 5.5 oz. came from beef and the remainder from fish, poultry, pork, nuts, eggs, and legumes. All MED diets provided 250 mg/day of EPA and DHA by varying the type of fish provided on each test diet. In addition, all MED diets contained < 300 mg/day of cholesterol, and <2300 mg/day of sodium. All MED diets provided ≈ 17% of calories from protein, 42% carbohydrate and 41% fat.

Table 3-2. Nutrient analysis data of treatment diets (based on 2000 kcals)

<table>
<thead>
<tr>
<th>Nutrient Targets</th>
<th>MED 0.5</th>
<th>MED 2.5</th>
<th>MED 5.5</th>
<th>Nutrient Targets</th>
<th>AAD</th>
<th>PSU</th>
<th>USDA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein (% energy)</td>
<td>17%</td>
<td>19.7%</td>
<td>17.7%</td>
<td>19.6%</td>
<td>18.2%</td>
<td>18.8%</td>
<td>19.6%</td>
</tr>
<tr>
<td>Carbohydrate (% energy)</td>
<td>42%</td>
<td>46.7%</td>
<td>40.7%</td>
<td>44.6%</td>
<td>40.4%</td>
<td>42.2%</td>
<td>38.4%</td>
</tr>
<tr>
<td>Fat (% energy)</td>
<td>41%</td>
<td>40.8%</td>
<td>41.6%</td>
<td>44.7%</td>
<td>41.3%</td>
<td>43.1%</td>
<td>42.0%</td>
</tr>
<tr>
<td>SFA (% energy)</td>
<td>8%</td>
<td>6.5%</td>
<td>7.4%</td>
<td>7.1%</td>
<td>7.5%</td>
<td>7.8%</td>
<td>8.8%</td>
</tr>
<tr>
<td>MUFA (% energy)</td>
<td>26%</td>
<td>24.0%</td>
<td>23.0%</td>
<td>25.0%</td>
<td>23.1%</td>
<td>22.7%</td>
<td>21.8%</td>
</tr>
<tr>
<td>PUFA (% energy)</td>
<td>8%</td>
<td>7.4%</td>
<td>7.8%</td>
<td>7.2%</td>
<td>7.1%</td>
<td>6.8%</td>
<td>6.6%</td>
</tr>
<tr>
<td>ALA (g)</td>
<td>1.5</td>
<td>1.69</td>
<td>1.48</td>
<td>1.58</td>
<td>1.31</td>
<td>1.54</td>
<td>1.17</td>
</tr>
<tr>
<td>Marine n-3 (g)</td>
<td>0.5</td>
<td>0.32</td>
<td>0.21</td>
<td>0.28</td>
<td>0.09</td>
<td>0.27</td>
<td>0.08</td>
</tr>
</tbody>
</table>
Additional characteristics of the MED diet that were similar across diets included the use of olive oil as the predominant fat and the consumption of: 3-6 servings of fruit daily, ≥ 6 servings of vegetables daily, ≥ 2 servings of legumes per week and 1-2 servings of nuts/seeds/olives per day (on a 2100 kcal diet). Total number of servings varied slightly in order to maintain a consistent protein level (17% of total kcal) across the experimental diets. A dietary pattern comparison is shown in Table 3-3.

Table 3-3. Dietary pattern comparison based on ~2100 kcal diet

<table>
<thead>
<tr>
<th></th>
<th>Fundación Dieta Mediterránea</th>
<th>MED0.5 goal/day</th>
<th>MED5.5 goal/day</th>
<th>AAD*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Goal listed in Pyramid</td>
<td>Goal/day</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grains</td>
<td>1-2/meal</td>
<td>3-6</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Whole grains</td>
<td>Preferably whole grain</td>
<td>Preferably whole grain</td>
<td></td>
<td>0.6 oz/day</td>
</tr>
<tr>
<td>Fruit</td>
<td>1-2/meal</td>
<td>3-6</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>Vegetables</td>
<td>≥ 2/meal</td>
<td>≥ 6</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Olive Oil (1 tsp = 1 svg)</td>
<td>1-2/meal</td>
<td>3-6</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Olives/Nuts/Seeds</td>
<td>1-2/day</td>
<td>1-2</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Dairy</td>
<td>2/day</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Sweets</td>
<td>≤ 2/week</td>
<td>≤ 0.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Potatoes</td>
<td>≤ 3/week</td>
<td>≤ 0.4</td>
<td>≤ 0.4</td>
<td>≤ 0.4</td>
</tr>
<tr>
<td>Protein</td>
<td></td>
<td>7 oz</td>
<td>7 oz</td>
<td></td>
</tr>
<tr>
<td>Eggs</td>
<td>2-4/week</td>
<td>0.3-0.6</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Red meat</td>
<td>&lt; 2/week</td>
<td>&lt; 0.3</td>
<td>.5 oz</td>
<td>5.5 oz</td>
</tr>
<tr>
<td>Processed meat</td>
<td>≤ 1/week</td>
<td>≤ 0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Legumes</td>
<td>≥ 2/week</td>
<td>≥ 0.3</td>
<td>0.3</td>
<td>0.3</td>
</tr>
<tr>
<td>White meat</td>
<td>2/week</td>
<td>0.3</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Fish/Seafood</td>
<td>≥ 2/week</td>
<td>≥ 0.3</td>
<td>1</td>
<td>0.4</td>
</tr>
</tbody>
</table>

*Source of AAD (Average American Diet): Usual U.S. Intake Adults, Dietary Guidelines for Americans 2010 (Table 5-1).
‡Amount listed is for total oil (breakdown of types of oil not determined).
‡‡Amount listed is for meat.
The lean and extra-lean beef used were cuts that are available in local grocery stores. According to the USDA, “extra-lean” beef is defined as 100 g of beef with less than 5 g of fat, less than 2 g of saturated fat, and less than 95 mg of cholesterol and “lean” beef is defined as 100 g of beef that contains less than 10 g of total fat and 4.5 g of saturated fat and 95 mg of cholesterol. On a selected basis, some higher fat cuts of beef were used, however, this was done such that total beef consumption on average, met the lean beef definition (since some extra-lean cuts were used). Beef was incorporated into meals in a manner that reflects typical consumption patterns of consumers. For example, 1 oz. of lean beef in a chili dish, 2 oz. in a lean beef sandwich or salad, 3 oz. in a fajita dish, and 4 oz. in a steak meal.

Clinical Visits and Blood Sample Collection

All clinical assessments were conducted on two consecutive days at baseline (start of study) and at the end of each diet period. For the 48 hours prior to each clinical assessment, participants were asked to refrain from alcohol consumption and the use of anti-inflammatory medications. For the 24 hours prior they were asked to refrain from vigorous exercise and asked not to consume any food or drink (except water) for the 12 hours before their visit. All blood samples were collected after an overnight fast according to a standardized protocol. Serum and plasma aliquots were collected and stored at -80 °C until time of analysis.

Lipids & Lipoproteins

Serum total, HDL and LDL cholesterol, and TG concentrations were determined by enzymatic procedures using a Vitros Clinical Chemistry Analyzer (VITROS® 5,1; Ortho-Clinical Diagnostics, Inc.). Serum Apo A-I and ApoB were measured by immunoturbidimetric assay (VITROS® 5,1: Ortho-Clinical Diagnostics, Inc.).
Lipoprotein particle number and size were measured by a proton magnetic resonance spectroscopy assay (NMR; LabCorp, Morrisville, NC). The analysis by NMR involves measurement of the 400 MHz proton NMR spectrum of a plasma or serum sample, deconvolution of the composite signal at ~0.8 ppm to produce the signal amplitudes of the lipoprotein subclasses that contribute to the composite plasma signal, and conversion of these subclass signal amplitudes to lipoprotein subclass concentrations (173). The ~0.8 ppm plasma NMR signal arises from the methyl group protons of the lipids carried in the LDL, HDL, and VLDL subclasses of varying diameter. The NMR signals from the various lipoprotein subclasses have unique and distinctive frequencies and line shapes, each of which is accounted for in the deconvolution analysis model. Each subclass signal amplitude is proportional to the number of subclass particles emitting the signal, which enables subclass particle concentrations to be calculated from the subclass signal amplitudes derived from the spectral deconvolution analysis.

**Statistical Analysis**

A sample size of 60 participants (n=30 per site) was determined based on LDL-C as the primary endpoint. The sample size was estimated to detect a 5% change in LDL-C (assuming a mean LDL-C of 120 mg/dL in the recruited cohort) with the following assumptions: power of 0.9, alpha of 0.05, the expected standard error of the difference is 13 mg/dL, and a two tailed test. Based on these assumptions, a sample size of 52 was considered sufficient to test the primary LDL-C hypothesis. However, this sample size was increased to account for an expected dropout rate of approximately 15%.

All statistical analyses were performed using SAS 9.4 (Statistical Analyses System, Cary, NC). Using PROC UNIVARIATE the residuals for each variable were used to assess normality
as well as visual inspection of distributions (histograms and stem and leaf plots), skewness value, and Shapiro-Wilk p-value. Logarithmic transformations were used for non-normally distributed variables. The analytic plan was designed a priori and described a mixed-effects model for analysis of the data for repeated measurements. For each variable the mean of two sample measurements taken at the end of each feeding period was used for analysis. Between-subject differences were accounted for by including baseline values as a covariate, and by including subject as a random effect in the models. Model covariance structures were based on optimizing fit statistics (evaluated as lowest Bayesian Information Criterion). The mixed model procedure (PROC MIXED) in SAS was used to assess between-treatment mean differences for each endpoint while adjusting for covariates such as treatment order, age and gender. Treatment and period and their interaction were included as fixed effects. To assess whether a dose response relationship was present, dose (0.5, 2.5, 5.5) was nested within diet (AAD or MED) and included as a fixed effect. Change scores were calculated by subtracting baseline values from endpoint values. Means values are reported as means ± SEM. The primary outcome is the change in LDL-C on the experimental diets compared to the AAD; Tukey-Kramer adjusted P-values were used for all dependent variables to adjust for multiple comparisons and determine whether the differences between the diets were significant (p<0.05).

RESULTS

Sixty-six participants were enrolled in the study. A total of nine individuals withdrew from the study. Of those, six dropped out before completing the first diet period. The remaining three dropped after diet period one. Individuals completing only baseline testing were dropped from analyses (n=6) along with one of the three subjects who completed only one diet period
because she did not complete both days of endpoint testing. The major reason for withdrawing from the study was inability to comply with controlled feeding protocol due to social obligations. The overall population was healthy with multiple CVD risk markers within recommended ranges at the start of the study. Baseline participant characteristics are presented in Table 3-4.

**Table 3-4.** Baseline characteristics of study participants

<table>
<thead>
<tr>
<th></th>
<th>Mean ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>49 ± 1.6</td>
</tr>
<tr>
<td>Males:Females</td>
<td>28:31</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>27 ± 0.5</td>
</tr>
<tr>
<td>TC (mg/dL)</td>
<td>193 ± 4.8</td>
</tr>
<tr>
<td>LDL-C (mg/dL)</td>
<td>109 ± 3.5</td>
</tr>
<tr>
<td>HDL-C (mg/dL)</td>
<td>55 ± 1.9</td>
</tr>
<tr>
<td>TG (mg/dL)</td>
<td>105 ± 7.9</td>
</tr>
<tr>
<td>Glucose (mg/dL)</td>
<td>99 ± 1.1</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>117 ± 1.7</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>77 ± 1.2</td>
</tr>
</tbody>
</table>

**Lipids, lipoproteins, and apolipoproteins**

TC, non-HDL-C, and LDL-C, were significantly lower following the MED0.5, MED2.5, and MED5.5 compared to the AAD (p<0.0001) (Table 3-5). Compared to the AAD, LDL-C was significantly decreased by -10.3 ± 1.9 mg/dL, -9.1 ± 1.9 mg/dL and -6.9 ± 1.9 mg/dL by the MED0.5, MED2.5, and MED5.5 diets, respectively (Figure 3-2, p<0.005). TC was reduced on the MED0.5, MED2.5, and MED5.5 diets by -12.8 ± 2.3 mg/dL, -10.9 ± 2.3 mg/dL and -6.9 ± 2.3 mg/dL respectively, compared to the AAD (p<0.05) and non-HDL-C was decreased on the
MED0.5, MED2.5, and MED5.5 diets by -11.2 ± 2.1 mg/dL, -9.8 ± 2.1 mg/dL and -7.0 ± 2.1 mg/dL respectively, compared to the AAD (p<0.01). There were no differences in HDL-C among treatments. All diets significantly decreased TG from baseline (p<0.01). In addition, TG was significantly lower following the MED5.5 compared to the MED0.5 (p<0.05). With the exception of TG, there were no differences in any of the lipid and lipoprotein changes among the three MED diets (MED0.5, MED2.5, MED5.5) (p>0.01). A dose-response effect was not detected for increasing lean beef dose on total cholesterol, LDL-C, HDL-C, non-HDL-C or TG. However, greater reductions in total cholesterol (p<0.05) were observed following the MED0.5 versus the MED5.5; the reduction following MED2.5 was not significantly different from MED0.5 or MED5.5.

Figure 3-2. Change from baseline in lipids and lipoproteins

Bars represent means and error bars represent standard errors of the means; *indicates significant change from baseline (P<0.01); different letters are significantly different; P ≤ 0.0001
Table 3-5. Between treatment differences in lipids, lipoproteins and apolipoproteins

<table>
<thead>
<tr>
<th>Endpoint</th>
<th>Baseline</th>
<th>AAD</th>
<th>MED 0.5</th>
<th>MED 2.5</th>
<th>MED 5.5</th>
<th>P for treat</th>
</tr>
</thead>
<tbody>
<tr>
<td>TC</td>
<td>192.6 ± 4.8</td>
<td>185.0 ± 4.4a</td>
<td>172.8 ± 4.1b</td>
<td>174.4 ± 4.4b</td>
<td>178.3 ± 3.9b</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>non-HDL-C</td>
<td>137.5 ± 4.6</td>
<td>133.5 ± 4.4a</td>
<td>123.0 ± 4.1b</td>
<td>124.1 ± 4.3b</td>
<td>126.9 ± 3.9b</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>LDL-C</td>
<td>109.4 ± 3.5</td>
<td>108.5 ± 3.8a</td>
<td>98.7 ± 3.5b</td>
<td>99.8 ± 3.8b</td>
<td>102.0 ± 3.2b</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>HDL-C</td>
<td>55.0 ± 1.9</td>
<td>51.6 ± 1.5</td>
<td>49.8 ± 1.5</td>
<td>50.3 ± 1.5</td>
<td>51.4 ± 1.5</td>
<td>0.0341</td>
</tr>
<tr>
<td>TG</td>
<td>105.4 ± 7.9</td>
<td>92.9 ± 5.6ab</td>
<td>94.2 ± 5.5a</td>
<td>93.9 ± 6.4ab</td>
<td>88.5 ± 5.3b</td>
<td>0.0437</td>
</tr>
<tr>
<td>ApoA1</td>
<td>148.5 ± 3.1</td>
<td>139.7 ± 2.7ab</td>
<td>136.7 ± 2.5a</td>
<td>136.8 ± 2.5a</td>
<td>140.0 ± 2.6b</td>
<td>0.0034</td>
</tr>
<tr>
<td>ApoB</td>
<td>94.4 ± 2.8</td>
<td>91.4 ± 2.7a</td>
<td>85.5 ± 2.5b</td>
<td>85.8 ± 2.7b</td>
<td>87.8 ± 2.4b</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

Bars represent means and error bars represent standard errors of the means; *indicates significant change from baseline (P<0.01); different letters are significantly different; P ≤ 0.01

For the non-HDL-C subparticles, total LDL particle number and large LDL particles were significantly reduced following the MED0.5 and MED2.5 compared to the AAD (p<0.005).

**Figure 3-3.** The reduction following the MED5.5 was not different from the other MED diets or AAD. There were no treatment effects for IDL or small LDL; however, the MED5.5 reduced a composite marker of VLDL, TG and chylomicron remnants compared to the MED0.5 (p<0.05). A dose response effect was not observed however, there were greater reductions in LDL particle number (p<0.05) and large LDL particles (p<0.0001) following the MED0.5 versus the MED5.5; reduction in these outcomes following MED2.5 were not significantly different from MED0.5 or MED5.5.

There were no treatment effects for large HDL particles, medium HDL particles or small HDL particles. **Figure 3-4.** Following the MED5.5 total HDL particle number was greater than following the MED0.5 (33.4 ± 0.6 vs. 32.6 ± 0.6, p<0.05, respectively). Compared to baseline,
all treatments significantly reduced total HDL particle number (p<0.01); however, the reduction from baseline for MED0.5 was greater than the reduction for MED5.5 (p<0.01). A dose response analysis confirmed this treatment effect. In addition the analysis revealed a greater reduction in medium HDL particles following the MED0.5 compared to the MED5.5 (p<0.05). However, the reductions following MED2.5 were not significantly different from MED0.5 or MED5.5.

**Figure 3-3.** Change from baseline in non-HDL subparticles

Bars represent means and error bars represent standard errors of the means; *indicates significant change from baseline (P<0.01); different letters are significantly different; P ≤ 0.0001

Treatment effects on apoB and apoA1 reflected lipoprotein changes; the MED0.5, MED2.5 and MED5.5 decreased apoB by -6.3 ± 1.2 mg/dL, -5.9 ± 1.2 mg/dL, -3.9 ± 1.2 mg/dL
compared to AAD, respectively (p<0.01). **Figure 3-5.** There was no treatment effect among the three MED diets. Reductions in apoA1 were greater for the MED0.5 and MED2.5 compared to MED5.5; AAD was not significantly different from any of the MED diets. A dose response analysis revealed similar results. When compared to the Med diets with low (MED0.5) and moderate (MED2.5) amounts of lean beef, the MED5.5 attenuated the reduction in apoA1 observed in the other two MED diet groups (p<0.05 for both). No effect of dose was found for apoB.

**Figure 3-4.** Change from baseline in HDL subclasses

Bars represent means and error bars represent standard errors of the means; *indicates significant change from baseline (P<0.01); different letters are significantly different; P ≤ 0.01
Figure 3-5. Change from baseline in apolipoproteins

Bars represent means and error bars represent standard errors of the means; *
indicates significant change from baseline ($P<0.01$); different letters are significantly different; $P \leq 0.01$
DISCUSSION

Our results show that the consumption of a healthy Med style dietary pattern with lean red meat in amounts that represent current intake in the U.S. (~2.5 oz./day), improve CVD risk factors when compared to a typical American dietary pattern containing equivalent amounts of red meat. These findings are consistent with previous research that shows consuming lean, unprocessed red meat (≤ 5.4 oz./beef/day/2100 kcal) as part of a DASH diet does not attenuate CVD risk reduction (7). Similar findings also were observed with the inclusion of lean beef and pork (500 g/week) as part of a Med style diet compared to a Med diet containing 200 g/week of beef or pork (87).

Over the past few decades, dietary guidance has recommended a shift to increasing energy from plant sources at the expense of animal sources, and red meat in particular. In 2017, the average American consumed 56.9 pounds of beef (or approximately 2.5 oz./day) (https://www.nationalchickencouncil.org/about-the-industry/statistics/per-capita-consumption-of-poultry-and-livestock-1965-to-estimated-2012-in-pounds/). In contrast, the Med Diet, a Healthy Eating Pattern featured in the current 2015-2020 Dietary Guidelines, recommends less than two servings per week of red meat, with an emphasis on lean cuts (https://dietamediterranea.com/en/nutrition/). However, according to the Food and Agricultural Organization (FAO), as of 2013 red meat consumption in the Mediterranean regions is similar to current U.S. consumption with amounts as high as 65 g (2.3 oz./day) in France and Italy. Consistent with the findings of our study this suggests that red meat in quantities equivalent to current consumption patterns in the U.S. do not attenuate the cardiovascular benefits of a traditional Med style diet. Hence, the adoption of a dietary pattern abundant in antioxidant and polyphenolic rich foods will allow for continued consumption of low to moderate (≤2.5 oz./day)
quantities of lean beef, a finding which should be integrated into U.S. dietary guidelines. To maintain energy balance with increasing quantities of lean beef caution is advised to avoid the replacement of other nutrient dense foods.

For example, the three Med diets in the present study were designed to maintain a consistent macronutrient breakdown (Table 3-1). However, as illustrated in Table 3-6, an analysis of the Med diets using the 14-point Mediterranean Diet Assessment Scale (174) showed a substantial point differentiation between the MED0.5 (12 points) and MED5.5 (7 points) as a result of the increase in lean beef. Specifically, as the quantity of lean beef increased we observed a considerable reduction in the servings of nuts, legumes and fish. This suggests that it is not the inclusion of lean red meat that contributed to the observed differences in lipids and lipoproteins between the MED0.5 and MED5.5; rather it was the reduction in several key components of the Med dietary pattern. Based on findings from the PREDIMED study a score of 10 or more points corresponds to high dietary adherence. In the present study, both the MED0.5 (12 points) and MED2.5 (10 points) met the definition for high adherence to a Med dietary pattern. Therefore, based on the evidence presented we believe intakes of 0.5 to 2.5 oz./day of can achieve the benefits observed with a traditional Med diet.

Current recommendations rely largely on observational data demonstrating an adverse association between red meat and major chronic disease risk, however the researchers did not adequately account for calorie content, type of fat, sodium content, meat processing additives (i.e., nitrites, sodium), and cooking methods (70). These factors can modify the health effects of lean red meat in the diet (175). Results from the PREDIEMD randomized controlled trial showed that differences existed in the association between red meat consumption and metabolic syndrome based on level of processing. When processed meats were consumed in amounts ≥ 44
g/day as part of a Med diet there was a twofold higher risk of metabolic syndrome, whereas the consumption of unprocessed red meats showed no association (176). Similarly, in the meta-analysis by Micha et al., (177) processed meat consumption (50 g/day) was associated with a 42% increase in CHD (RR=1.42; 95% CI, 1.07 to 1.89; p=0.04), whereas 100 g/day of unprocessed red meat intake showed no association with CHD. These results suggest that the effects associated with red meat consumption will vary based on type of meat. While the authors speculated that the significantly greater amount of sodium in processed meats might be contributing to the difference in outcomes observed between processed and unprocessed red meats, there are several other important nutritional differences that may be associated with increases in risk (e.g., specific fats and additives).

Despite the robust evidence for the benefits of a Med diet on CHD, the effects of a Med dietary pattern on lipids and lipoproteins remains inconclusive. Based on the 2013 AHA/ACC Guideline on Lifestyle Management to Reduce Cardiovascular Risk the strength of evidence is categorized as “low” for the benefits of a Med dietary pattern on lipids and is not recognized as one of the dietary patterns recommended for LDL-C lowering. Yet, elevated LDL-C have been consistently associated with an increased risk of CHD and is a primary target for cardiovascular risk reduction. The inconsistencies in the Med diet research have been attributed, at least in part, to the substantial differences and limitations of the available studies, including how the Med dietary pattern and adherence levels are defined (178).

To date, a reduction in LDL-C as the primary target for CVD risk reduction is accepted by most scientific societies. However, there are large differences in the lipoprotein particle core, which can lead to discrepant findings for total LDL-C, LDL particle number and LDL particle size. In the systematic review by Ip et al., (149) the measures of LDL particle number were
more strongly associated with CHD than LDL particle size. Similar findings have been reported in the Multi-Ethnic Study of Atherosclerosis (MESA, n=5598), which found that for those with discordant levels of LDL-C and LDL particle number, only LDL particle number was associated with incident CVD (LDL-P HR 1.45, 95% CI: 1.19-1.78; LDL-C HR 1.07, 95% CI: 0.88-1.30). These findings suggest that a reduction in LDL particle number may signal an antiatherogenic event not otherwise indicated by LDL-C concentration alone.

The present highly controlled crossover feeding trial allowed for the examination of a Med diet pattern with three levels of lean beef on lipids and lipoproteins; shifts in the lipoprotein particle profile also were assessed using NMR spectroscopy. Notably, all three Med diets elicited significant reductions in both total LDL-C concentration and LDL particle number. Given the emerging evidence about the importance of LDL particle number, this finding provides valuable insight into the potential mechanisms by which the Med diet elicits beneficial cardiometabolic effects. Moreover, a dose response analysis revealed no attenuation of the LDL lowering response with increasing quantities of lean beef when incorporated into a Med diet. However, when compared to the AAD control diet, only the MED0.5 and MED2.5 elicited greater reductions in LDL particle number, whereas all three Med diets significantly reduced LDL-C compared to AAD. Based on the findings of Damasceno et al., (179) in which greater reductions in both LDL-C concentration and LDL particle number were demonstrated by the inclusion of nuts as part of a Med diet, it is possible that the reduction in nuts to compensate for the increase in lean red meat in the MED5.5 may have contributed to a decrease in LDL particle number that was not different from the AAD. This illustrates the importance of establishing a healthy Med dietary pattern that encompasses all nutrient rich components, which can include lean beef.
However, it is evident that consuming any one food or nutrient in excess leads to increased energy intake or the reduction of other valuable nutrients when energy intake is constant.

We also observed a significant reduction from baseline in the large buoyant LDL particles for each of the 3 Med diets, with treatment differences between the MED0.5 and MED2.5 compared to AAD. When similar results have been found in studies replacing dietary SFA with MUFA one potential mechanism that has been proposed is an increase in LDL clearance—for example animal and tissue culture studies have shown that SFAs decrease the activity of the LDL receptor while MUFAs increase the activity of the LDL receptor (180). Given that large LDL particles have a greater affinity for the LDL receptor this represents a plausible mechanism for explaining our results. The greater reductions observed for apoB across all three Med diet groups compared to AAD are consistent with the LDL lowering effect as plasma apoB provides a measure of the number of circulating LDL particles. These findings also are in agreement with those reported by Richard et al., (181) in which the investigators observed decreases in plasma LDL-C and LDL-apoB following a Med diet for 5 weeks under controlled feeding conditions. Although our study was not designed to test fractional catabolic, the authors suggest that consumption of Med diet may be contributing to the increase in catabolism of LDL.

All treatment diets were associated with reductions in HDL-C concentration and HDL particle number. The decrease in HDL particle number without an effect on HDL size suggests a decrease in the number of circulating HDL particles during all the diets and a possible decrease in production rates (182). Recent research into HDL function shows that the size and composition of HDL particles differ in how they affect HDL function, however the evidence is mixed regarding which particle size offers the greatest cardioprotection. Some research supports the notion that the smaller the HDL particle, the shorter time in the bloodstream and its
contribution to reverse cholesterol transport. A reduction in HDL particle size has been found to be positively associated with CVD (183) and a greater number of small HDL particles are shown to be associated with increased CVD risk in healthy adults (184). These contradict some cholesterol efflux studies that suggest the smaller HDL particles are highly efficient to promote cholesterol efflux via the ABCA1 transporter (185).

In contrast, the large, spherical HDL are found to be inversely correlated with CVD risk (183) and are considered to be the preferred acceptors of the cholesterol that effluxes from macrophages and is modulated by the ABCG1-mediated pathway (186). In the present study the only significant changes from baseline occurred for the small particles with reductions of -1.13 ± 0.58 (p<0.05), -1.01 ± 0.47 (p=0.05), and -1.45 ± 0.57 (p<0.01), for the AAD, MED5.5 and MED2.5 diets, respectively. Therefore, despite the reduction in HDL concentration and particle number these findings suggest the decrease was driven by the loss of small HDL particles, implying that HDL functionality may have been maintained. The reductions in apoA1, are consistent with the reductions observed in HDL particle number. Kinetic studies have suggested potential mechanisms for the reduction in apoA1 include dietary fat-induced changes in HDL and apoA1 production rate while others demonstrate a change in apoA1 fractional catabolic rate (187-189).

In an animal study conducted by Brousseau et al (187) the authors demonstrated that the isocaloric substitution of dietary SFA with either MUFA or PUFA resulted in significant reductions of plasma TC, HDL and apoA1 concentrations. The reductions in apoA1 were directly attributable to enhanced apoA1 catabolism. Thus, this demonstrates that apoA1 is cleared at a faster rate during consumption of a high MUFA diet relative to a high SFA diet.
Although the majority of evidence is consistent with this finding, variation in content or source of MUFA have shown opposing results.

In the study by Richard et al., (190) the investigators found reductions in apoA1 concentration and pool size following consumption of a Med diet. They also reported a lowering of the apoA1 production rate, which they believe may be partly attributable to the reduction in SFA intake. Additionally, the authors hypothesized that the reduction in apoA1 production rate may be related to the significant decrease in LDL-C and ApoB, as previous evidence has shown a positive correlation between these markers (190). Thus, it is likely there may be less need for reverse cholesterol transport when plasma LDL concentrations have been reduced.

The results from this study provide further support for the benefits of a Med dietary pattern on CVD risk factors and advances our knowledge of the specific effects on lipid and lipoprotein metabolism. As a tightly controlled clinical trial, we were able to assess the effect of varying amounts of lean red meat as part of a Med dietary pattern. Based on our results, the benefits of a Med style diet are not attenuated with the inclusion of small to moderate amounts of lean beef, a finding which may help to improve dietary adherence to a Med style diet in a U.S. population. This study adds to the growing evidence base of clinical trials designed to specifically test the effects of including unprocessed lean red meat in a healthy diet on CVD risk factors. Currently, the evidence used to advocate reductions in red meat intake is driven by observational data, which is subject to confounding. Therefore, future dietary guidance should take into consideration differences in the processing and fat content of red meat when establishing guidelines. Dietary recommendations have made a positive transition to a focus on dietary patterns with the goal of promoting consumer compliance. Thus, given that red meat is a popular food in the U.S. diet, our findings have shown that lean beef in the amounts similar to
average US consumption can be included in a healthy Med style dietary pattern. On the contrary, it need not be eliminated in a heart healthy diet.

**Strengths and Limitations**

A major strength of the study is the randomized controlled crossover design and low dropout rate (<15%). High levels of dietary compliance were reported as verified by the completion of daily and weekly monitoring forms. This study is the first to our knowledge to examine the effects of Med diet pattern containing three levels of lean red meat on CVD risk factors in a U.S. population. Additionally, our population was generally healthy, with near optimal LDL-C levels, which made our findings more notable and generalizable. Finally, the lean red meat included in the study was purchased through local grocers and easily accessible to any consumer. Limitations of the study include the lack of biological measures of compliance as we relied solely on self-reported measures. Additionally, with the controlled feeding design participants received all of their foods. In a free-living setting, individuals would be expected to purchase and prepare all Med diet recipes and properly select lean ground meats and meat cuts and incorporate red meat in recommended amounts in a healthy dietary pattern. For many participants the elaborate menu items may be challenging to prepare perhaps lessening the lipid lowering benefits achieved by the dietary pattern as a whole. Finally, our study population was a convenience sample of predominately Caucasian individuals, thus limiting the generalizability to other races and ethnicities.

**Conclusion**

In conclusion, the consumption of a Med style dietary pattern containing lean beef in amounts equivalent to or less than current consumption (≤ 2.5 oz) significantly reduces TC, LDL-C, non-HDL-C, LDL particle number and apoB compared to a typical American style diet.
Notably, all Med diets achieved similar lipid lowering responses, however small changes in the dietary pattern to accommodate the increased lean beef may have contributed to the lack of treatment differences between the MED5.5 and AAD for measures of LDL particle number. These findings support the transition to dietary pattern-based recommendations and demonstrate that lean beef can be part of a Med style dietary pattern without attenuating the cardiovascular benefits.

**Table 3-6.** 14-point Mediterranean diet adherence score of treatment diets

<table>
<thead>
<tr>
<th>Component</th>
<th>MED 0.5</th>
<th>MED 2.5</th>
<th>MED 5.5</th>
<th>AAD</th>
<th>PREDI MED</th>
</tr>
</thead>
<tbody>
<tr>
<td>Olive oil main fat</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Olive oil (≥ 4 tbsp.)</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Vegetables ≥ 2 svg/d (svg=200 g)</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Fruits ≥ 3 servings/d</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Red or processed meats &lt;100-150 g)/d</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Butter, cream. margarine &lt; 12 g/d</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Soda drinks &lt; 1/d</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Wine glasses ≥ 7/wk</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Legumes ≥3 svg /wk (svg=150 g)</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Fish / seafood ≥ 3 svg/wk (svg=100-150 g)</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Commercial bakery ≤ 2/wk</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Nuts ≥ 3 svg/wk (svg=30 g)</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1/0</td>
</tr>
<tr>
<td>Poultry more than red meats</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Use of sofrito sauce ≥ 2 /wk</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

**TOTAL SCORE:** 12 10 7 6 11/12
Chapter 4: A Mediterranean-Style Diet with Lean Beef Lowers Blood Pressure and Improves Vascular Function: A Randomized Controlled Trial

ABSTRACT

Background: The Mediterranean (Med) dietary pattern is now widely accepted because of extensive evidence that demonstrates beneficial effects on cardiovascular risk factors. Increased central systolic pressure and arterial stiffness are newly identified independent predictors of CVD. The effect of a Med diet on these measures of vascular health is lacking.

Objective: To evaluate the effects of a Med diet with different quantities of lean beef (0.5, 2.5 and 5.5 oz/day) compared to an Average American diet (AAD) on CVD risk factors (brachial and central blood pressure, pulse wave velocity).

Design: This was a multicenter, 4-period controlled feeding, randomized crossover study conducted at Penn State University and USDA-Beltsville. Participants were generally healthy normal to overweight/obese males and females (n=66). PWV, central systolic and diastolic blood pressure were measured using the SphygmoCor ECEL-System. Endpoints were assessed at baseline and the end of each 4-week diet period.

Results: There was a significant treatment effect for PWV (p<0.01); PWV was lower following consumption of a Med diet containing 0.5 oz. lean beef/day (6.86 m/sec ± 0.14; p<0.05) and 2.5 oz. of lean beef/day (6.84 m/sec ± 0.15; p<0.01) compared to the AAD (7.10 m/sec ± 0.14). Compared to the AAD both the 0.5 oz./day (-3.30 ± 0.76) and 2.5 oz./day (-2.94 ± 0.76) Med diets elicited greater reductions in central systolic blood. A similar pattern was observed for central diastolic pressure. Compared to AAD all three Med diets significantly decreased brachial systolic and diastolic pressures (p<0.01 for all).
**Conclusions:** Our results demonstrate that lean beef at intakes equivalent to the average consumption in the U.S. (2.5 oz/day lean beef), may be incorporated into a Med style diet with no differences in vascular health benefits compared to a traditional Med diet with 0.5 oz/day lean beef. Despite there being no treatment differences among the 3 Med diets and that lean beef intakes of 5.5 oz/day were similar to those for the AAD, our findings suggest that ≤ 2.5 oz/day of lean beef can be included in a Med diet and not compromise the vascular health benefits of a Med diet.

**Key Words:** Mediterranean diet, lean beef, blood pressure, central blood pressure, arterial stiffness, cardiovascular disease
INTRODUCTION

Among individuals aged 60 years and older the leading contributor to the global disease burden is cardiovascular disease (CVD), representing 30% of the total burden. Yet, Mediterranean populations in southern Europe, such as Greece and Italy, have significantly lower rates of mortality from CVD than populations in northern Europe and the United States (U.S.) (191). Growing evidence suggests that this disparity is due to diverse dietary patterns across populations, and that a Mediterranean diet (Med diet) beneficially affects chronic disease thereby increasing life expectancy (192, 193).

During aging, structural and functional changes occur in the vessel wall that decrease arterial function and increase the susceptibility of developing CVD (194, 195). Structural changes that occur in the large elastic arteries during aging promote increases in collagen formation (fibrosis), degradation of elastin, and the build-up of oxidatively damaged proteins and advanced glycation end-products, which leads to a stiffening of the arterial wall and an increase in pulse wave velocity (PWV)/pressure (196). Likewise, two important functional changes that are associated with aging include a stiffening of the large elastic arteries (the aorta and carotid arteries), and a decline in systemic vascular endothelial function (197). There is growing evidence that a healthy dietary pattern, and specifically a Med diet, may delay vascular aging (198) and improve endothelial function (199).

Over the past decade, research examining the role of diet on CVD risk has advanced our understanding of how to assess vascular health. Specifically, the measure of PWV is now recognized as a surrogate measure of arterial stiffness and is evaluated by measuring pulse transit time from the pressure waveforms between two sites (carotid and femoral artery, for example), with a higher PWV signifying stiffer arteries. As an accepted global measure of arterial stiffness...
PWV has been shown to be an independent predictor of mortality in the general population as well as an independent predictor of coronary heart disease (CHD) and stroke in healthy populations (200, 201). In a systematic review and meta-analysis of 17 longitudinal studies (n = 15,877) PWV was shown to be an independent predictor of adverse cardiovascular events and all-cause mortality (202). In this analysis an increase of aortic PWV of 1 meter per second (m/s) increased CVD risk by more than 10% (202). Moreover, there is evidence for an association between heart healthy lifestyles and reduced PWV (203, 204). Previous research on adherence to a Med-style diet suggests it is beneficial for preventing the development of hypertension; however, research on the role of the Med diet in improving PWV, as a measure of arterial stiffness, is lacking.

A consequence of arterial stiffening is an increase in pulsatile and central blood pressure. Although traditional measures of brachial systolic and diastolic blood pressure are assumed to accurately reflect the pressure load in the large conduit arteries, the arterial pulse is modified as it travels away from the heart to the periphery. Based on anatomical proximity, the central aortic pulse pressure more closely reflects the pulsatile stress experienced by the organs such as the heart, and as such, is considered to be a better predictor of CVD events (205). Findings from two major cardiovascular outcomes trials, the Conduit Artery Functional Endpoint (CAFE) (155) study and Strong Heart (154) study were first to establish the importance of central blood pressures in assessing cardiovascular risk and the effects of pharmacologic and nutritional therapy on clinical outcomes. These studies both showed central pressure to be superior to brachial cuff pressure. Specifically, the Strong Heart sub-study used SphygmoCor® to investigate central as well as brachial blood pressure, and followed 2,409 subjects for a mean of 4.0 years. The study concluded that non-invasively determined central aortic systolic pressure...
was an independent predictor of incident CVD, yet these events were not related to brachial systolic pressure. Thus, central aortic pressure is a better predictor of incident CVD, likely because it directly reflects the vascular load on the left ventricle.

To date, little evidence exists regarding the effect of the Med dietary pattern on central pressure, however, large prospective cohort studies, such as the Seguimiento Universidad de Navarra (SUN) Study, have reported an association between adherence to a Med diet and risk of hypertension. With more than 41,000 person-years of follow-up, an inverse association between dietary adherence and changes in both systolic and diastolic blood pressure levels were observed over time. Notably, the Med dietary pattern followed by this population included an average consumption of ~3.5 oz/1000 kcal of red meat, as reported by the Scientific Report of the 2015 Dietary Guidelines Committee (206). In a randomized controlled trial conducted in a U.S. population, inclusion of 500 g/week (representative of current U.S. intake) of lean beef or pork as part of a Med diet elicited similar improvements in blood pressure, when compared to a traditional Med diet with ~ 200 g/week of lean beef. Likewise, reductions in blood pressure also were reported in the Beef as part of an Optimal Lean Diet (BOLD) controlled feeding trial (86) in which 5.4 oz/lean beef/day/2100 kcal was provided as part of a DASH diet. Thus, there is some evidence to suggest that the consumption of plant-based diets, including the Med diet, may improve blood pressure and these improvements are not attenuated with the inclusion of lean beef.

Given the emerging evidence regarding the role of central blood pressure and PWV in the assessment of cardiovascular risk, we believe the inclusion of these measures will provide valuable insight beyond that of standard brachial pressures. Thus, the objective of the present study was to evaluate the effects of a traditional Med diet (0.5 oz/day/lean beef/2100 kcal) as
well as two Med diets containing 2.5 oz. and 5.5 oz/day/lean beef/2100 kcal compared with an average American control diet on central systolic and diastolic pressure, arterial stiffness and brachial pressures. In addition, our study provides important findings to the evidence base about the role of lean beef as part of a healthy dietary pattern.

METHODS

Experimental Design

This was a 4-period, randomized, crossover, controlled-feeding study conducted at two centers: Penn State University and USDA-Beltsville Human Nutrition Research Center. Participants (n=60; 30 per site) included generally healthy males and females aged 30-65 years. Detailed methods and cohort characteristics were described previously (Chapter 3). Briefly, all meals and snacks were provided to participants. Treatment diets included: 1) a Med diet (MED0.5) providing 0.5 oz. per day of lean beef; 2) a matched MED diet (MED2.5) with the same fatty acid profile as the MED diet, but contained 2.5 oz. per day of lean beef; 3) a matched MED diet (MED5.5), providing 5.5 oz. per day of lean beef; and 4) an Average American Diet (AAD). The amount of lean beef consumed was based on the calculated energy requirements of the participants, with a 2100 kcal diet providing 0.5, 2.5 and 5.5 oz /per day for the MED0.5, MED2.5 and MED5.5 respectively. The nutrient composition of the treatment diets is presented in Table 4-1.

Clinical Visits and Blood Sample Collection

All clinical assessments were conducted on two consecutive days at baseline (start of study) and at the end of each diet period. For the 48 hours prior to each clinical assessment,
participants were asked to refrain from alcohol consumption and the use of anti-inflammatory medications. For the 24 hours prior to each clinical assessment, they were asked to refrain from vigorous exercise and instructed not to consume any food or drink (except water) for the 12 hours before their visit. All blood samples were collected after an overnight fast according to a standardized protocol. Serum and plasma aliquots were collected and stored at -80 °C until time of analysis. Vascular assessments were conducted on the first of the two consecutive days of endpoint testing.

**Table 4-1.** Nutrient composition of the treatment diets‡

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>AAD</th>
<th>MED0.5</th>
<th>MED2.5</th>
<th>MED5.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total fat (%)</td>
<td>33</td>
<td>41</td>
<td>41</td>
<td>41</td>
</tr>
<tr>
<td>SFA (%)</td>
<td>12</td>
<td>8</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>MUFA (%)</td>
<td>13</td>
<td>25.5</td>
<td>25.5</td>
<td>25.5</td>
</tr>
<tr>
<td>PUFA (%)</td>
<td>8</td>
<td>7.5</td>
<td>7.5</td>
<td>7.5</td>
</tr>
<tr>
<td>Carbohydrate (%)</td>
<td>52</td>
<td>42</td>
<td>42</td>
<td>42</td>
</tr>
<tr>
<td>Protein (%)</td>
<td>15</td>
<td>17</td>
<td>17</td>
<td>17</td>
</tr>
<tr>
<td>Cholesterol (mg)</td>
<td>&lt;300</td>
<td>&lt;300</td>
<td>&lt;300</td>
<td>&lt;300</td>
</tr>
<tr>
<td>Sodium (mg)</td>
<td>~3500</td>
<td>&lt;2300</td>
<td>&lt;2300</td>
<td>&lt;2300</td>
</tr>
<tr>
<td>Beef (oz/day)</td>
<td>≈ 2.5 oz.</td>
<td>0.5 oz.</td>
<td>2.5 oz.</td>
<td>≈ 5.5 oz.</td>
</tr>
<tr>
<td>ALA (g)</td>
<td>1.5</td>
<td>1.5</td>
<td>1.5</td>
<td>1.5</td>
</tr>
<tr>
<td>Marine n-3 (g)</td>
<td>&lt;0.1</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
</tbody>
</table>

‡AAD: Average American Diet; MED: Healthy Mediterranean Diet (0.5, 2.5 or 5.5 oz. per day of lean beef). Food Processor Nutrient Analysis Software (Version 10.10) was used to estimate the total fat and fatty acid composition of the diets, rather than chemical analysis.
Vascular Health

Resting Blood Pressure

Following a 5-minute seated rest period, brachial artery systolic and diastolic blood pressure were measured in the left arm using an automated blood pressure cuff (Omron HEM-705CP; Omron Healthcare, Vernon Hills, IL).

Vascular Function Measures

Vascular function, in terms of central blood pressure and arterial stiffness indices, was assessed using the SphygmoCor System pulse waveform analysis (AtCor Medical, Sydney, Australia). Measures were taken at baseline (start of study) and at the end of each 4-week treatment period in a quiet, dimly lit room.

Pulse Wave Analysis (PWA): Central (aortic) Blood Pressure and Augmentation Index

Following a 5-minute seated rest period, aortic (central) blood pressure and wave reflection characteristics were derived from brachial pressure waveforms using a validated generalized transfer function. At each time point, three PWA measures were taken, following JNC 7 blood pressure guidelines, with 1 minute between each reading. The last two PWA results were averaged and used for analysis. The augmentation index (AI) was standardized to a heart rate of 75 beats per minute to correct for the independent inverse effect of heart rate on the pulse wave form (207).

Pulse Wave Velocity (PWV): Arterial Stiffness

Aortic stiffness was assessed by carotid-femoral PWV. Carotid and femoral arterial pressure waveforms were measured simultaneously via an applanation tonometry sensor manually held in place above the right common carotid artery and a blood pressure cuff placed on the right femoral artery. Distance measurements were taken from the sternal notch to the
carotid artery, sternal notch to the top of the femoral cuff, and femoral artery to the top of the femoral cuff. Based on these measurements, the SphygmoCor System automatically calculates PWV as distance over transit time. Transit time between the carotid and femoral pressure waves is determined by the SphygmoCor System using the foot-to-foot method (208).

**Statistical Analysis**

All statistical analyses were performed using SAS 9.4 (Statistical Analyses System, Cary, NC). Using PROC UNIVARIATE the residuals for each variable were used to assess normality as well as visual inspection of distributions (histograms and stem and leaf plots), skewness value, and Shapiro-Wilk p-value. Logarithmic transformations were used for non-normally distributed variables. Individual differences were accounted for using the baseline value as a covariate, and by including a random subject effect in the model. Model covariance structures were based on optimizing fit statistics (evaluated as lowest Bayesian Information Criterion). A mixed model procedure (PROC MIXED) in SAS was used to compare the means of endpoints between groups while adjusting for covariates such as treatment order, age and gender. The mixed models procedure was used to test the effects of treatment, period, and their interaction on outcome measures. Dose response effects were measured using a nested design in the mixed model procedures. Change scores were calculated by subtracting baseline values from endpoint values. Means values are reported as means ± SEM. The primary outcome is the change in vascular health indices on the experimental diets compared to the AAD; Tukey-Kramer adjusted P-values were used for all dependent variables to adjust for multiple comparisons and determine whether the differences between the diets were significant (p<0.05).
**RESULTS**

Sixty-six individuals were enrolled in the study. A total of nine individuals withdrew from the study. Of those, 6 dropped out before completing the first diet period. The remaining three dropped after diet period one. Individuals completing only baseline testing were dropped from analyses (n=6) along with one of the three subjects who completed only diet period one because she did not complete the SphygmoCor testing. The overall population was healthy with multiple CVD risk markers within recommended ranges at the start of the study. Baseline subject characteristics are presented in Table 4-2.

**Table 4-2.** Baseline characteristics of the study participants

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Mean ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>49 ± 1.6</td>
</tr>
<tr>
<td>Males:Females</td>
<td>28:31</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>27 ± 0.5</td>
</tr>
<tr>
<td>TC (mg/dL)</td>
<td>193 ± 4.8</td>
</tr>
<tr>
<td>LDL-C (mg/dL)</td>
<td>109 ± 3.5</td>
</tr>
<tr>
<td>HDL-C (mg/dL)</td>
<td>55 ± 1.9</td>
</tr>
<tr>
<td>TG (mg/dL)</td>
<td>105 ± 7.9</td>
</tr>
<tr>
<td>Glucose (mg/dL)</td>
<td>99 ± 1.1</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>117 ± 1.7</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>77 ± 1.2</td>
</tr>
</tbody>
</table>

**Brachial Blood Pressure**

Brachial systolic pressure decreased (p<0.05) in participants on the MED0.5, MED2.5 and MED5.5 (111.9 ± 1.5, 112.0 ± 1.7, and 112.4 ± 1.6, respectively) versus AAD (115.3 ± 1.6 mm Hg). Brachial diastolic decreased (p<0.05) in participants on the MED0.5, MED2.5 and
MED5.5 (73.2 ± 1.1, 73.0 ± 1.1, and 73.7 ± 1.1 mm Hg, respectively) versus AAD (75.5 ± 1.2 mm Hg).

Compared to AAD brachial systolic pressure was decreased by 3.45 ± 1.0, 3.14 ± 1.0, and 2.66 ± 1.0 mm Hg for the MED0.5, MED2.5 and MED5.5 respectively (p<0.05) and diastolic by 2.79 ± 0.7, 2.73 ± 0.7, and 1.96 ± 0.74 mm Hg, respectively (p<0.05); no treatment differences were observed between the three Med diets.

**Central Aortic Pressure**

Central aortic systolic pressure was significantly reduced by 3.30 ± 0.76 and 2.94 ± 0.76 following the MED0.5 and MED2.5, respectively compared with the AAD (p<0.001). Central diastolic pressure was significantly reduced by 2.83 ± 0.59 and 2.20 ± 0.60 following the MED0.5 and MED2.5, respectively compared with the AAD (p<0.01). There were no treatment differences in central systolic or diastolic between the three MED diets (Table 4-3). All treatment diets elicited significant reductions compared to baseline (Figure 4-1), AAD (p<0.05), MED0.5, MED2.5 and MED5.5 (for all MED diet, p<0.0001).

**Table 4-3.** Effects of a Med diet with varying quantities of lean beef on central and brachial pressures.

<table>
<thead>
<tr>
<th>Endpoint</th>
<th>Baseline</th>
<th>AAD</th>
<th>MED 0.5</th>
<th>MED 2.5</th>
<th>MED 5.5</th>
<th>P for treat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Central SBP (mm Hg)</td>
<td>113 ± 1.68</td>
<td>111 ± 1.55&lt;sup&gt;a&lt;/sup&gt;</td>
<td>108 ± 1.49&lt;sup&gt;b&lt;/sup&gt;</td>
<td>108 ± 1.41&lt;sup&gt;b&lt;/sup&gt;</td>
<td>109 ± 1.62&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Central DBP (mm Hg)</td>
<td>75 ± 1.0</td>
<td>74 ± 1.09&lt;sup&gt;a&lt;/sup&gt;</td>
<td>71 ± 1.03&lt;sup&gt;b&lt;/sup&gt;</td>
<td>71 ± 0.89&lt;sup&gt;b&lt;/sup&gt;</td>
<td>72 ± 0.98&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Brachial SBP (mm Hg)</td>
<td>117 ± 1.74</td>
<td>115 ± 1.63&lt;sup&gt;a&lt;/sup&gt;</td>
<td>112 ± 1.49&lt;sup&gt;b&lt;/sup&gt;</td>
<td>112 ± 1.67&lt;sup&gt;b&lt;/sup&gt;</td>
<td>112 ± 1.58&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.0022</td>
</tr>
<tr>
<td>Brachial DBP (mm Hg)</td>
<td>77 ± 1.17</td>
<td>75 ± 1.16&lt;sup&gt;a&lt;/sup&gt;</td>
<td>73 ± 1.11&lt;sup&gt;b&lt;/sup&gt;</td>
<td>73 ± 1.11&lt;sup&gt;b&lt;/sup&gt;</td>
<td>74 ± 1.09&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.0006</td>
</tr>
</tbody>
</table>

Values are means ± SEMs. Values with different letters are different, P < 0.01
Arterial Stiffness

There were no treatment differences among any of the groups for augmentation index (Table 4-4). Compared to baseline all MED diets improved PWV (p<0.01), whereas there was no change for AAD (Figure 4-2). There were no treatment differences among the three MED diet groups, however only the MED0.5 and MED2.5 significantly decreased PWV (0.239 ± 0.08 and 0.269 ± 0.08 m/s, respectively) compared to AAD (p<0.01).

Figure 4-1. Changes in central and brachial pressure following 4 weeks of a Med diet with varying quantities of lean beef compared to an average American control diet

Means with standard error bars; ‡ values are different from baseline, P <0.05; * values are different from baseline, P < 0.0001; values with different letters are different, P < 0.01
Table 4-4. Effects of a Med diet with varying quantities of lean beef on PWV and augmentation index (AI@75 bpm)

<table>
<thead>
<tr>
<th>Endpoint</th>
<th>Baseline</th>
<th>AAD</th>
<th>MED 0.5</th>
<th>MED 2.5</th>
<th>MED 5.5</th>
<th>P for treat</th>
</tr>
</thead>
<tbody>
<tr>
<td>PWV (m/s)</td>
<td>7.1 ± 0.15</td>
<td>7.1 ± 0.14&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.9 ± 0.14&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.8 ± 0.15&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.9 ± 0.14&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.003</td>
</tr>
<tr>
<td>AI@75 bpm</td>
<td>22.2 ± 1.7</td>
<td>20.2 ± 1.8</td>
<td>19.8 ± 1.7</td>
<td>21.0 ± 1.6</td>
<td>21.3 ± 1.6</td>
<td>0.1383</td>
</tr>
</tbody>
</table>

Values are means ± SEMs. Values with different letters are different, P < 0.01

Figure 4-2. Changes in PWV following 4 weeks of a Med diet with varying quantities of lean beef compared to an average American control diet

Means with standard error bars; *values are different from baseline, P < 0.01; values with different letters are different, P < 0.01
DISCUSSION

A healthy dietary pattern is essential for the prevention and treatment of hypertension (178). This is the first study to our knowledge to assess measures of central pressure and arterial stiffness following a traditional Med diet as well as two Med diets containing varying quantities of lean beef. In the present study, both a traditional Med diet, containing 0.5 oz/lean beef and a Med diet containing amounts equivalent to current intakes in the U.S. (2.5 oz/day) were associated with significant reductions in central aortic systolic and diastolic pressures as well as arterial stiffness (as assessed by PWV) when compared to a typical American diet containing 2.5 oz./day red meat. Previous research suggests that the reduction in risk of hypertension associated with a Med diet may be attributed to the beneficial changes associated with a plant-based dietary pattern versus any one food or nutrient, such as those reported with the DASH diet. For example, the Med diets provided in this study resulted in reductions in blood pressure (−3.4, −2.8 and -2.7 mm Hg for the MED0.5, MED2.5 and MED5.5, respectively, compared to AAD p<0.05) that were similar to those reported in the original DASH trial, which lowered systolic blood pressure by −3.5 mm Hg (209). Our findings also are consistent with those from the aforementioned BOLD study which showed that inclusion of lean beef (5.4 oz/day) as part of a DASH diet yielded similar reductions in systolic blood pressure (-4.2 mm hg) compared to a traditional DASH diet (-2.8 mm Hg).

However, aside from a few clinical trials specifically conducted to assess lean beef as part of a healthy dietary pattern, the majority of observational evidence associate red meat consumption to increases in CVD risk. More recently, it has been recognized that observational data are highly subject to confounding and there is now a growing evidence base regarding the impact of processed meats versus fresh meats on cardiometabolic risk. In large part the CVD
risk associated with red meat has often been attributed to the SFA content, however, in addition to SFA there are other constituents of processed meats that are not found in fresh meats such as sodium and nitrates, which have been shown to impact CVD risk. As reported by Micha et al., (177) there is substantially higher amounts of sodium per 50 g of processed meat (621.7 ± 7.6 mg) compared to 50 g of red meat (154.8 ± 3.4 mg). Chronic consumption of excess dietary sodium has been linked to significant increases in blood pressure, a reduction in arterial compliance and promotion of arterial stiffness (210-212). Likewise, nitrates and their byproducts, also found in much higher amounts in processed meats, have been linked to the development of atherosclerosis and vascular dysfunction (213). Based on the blood pressure lowering effects observed in this study, inclusion of lean red meats as part of a healthy dietary pattern does not attenuate the benefits observed with plant-based dietary patterns and should therefore be distinguished from the CVD effects of processed meats.

Measures of arterial stiffness, such as PWV, provide greater insight into the potential functional mechanisms that may be contributing to the vascular health benefits associated with a Med dietary pattern. Plant-based dietary patterns are high in polyphenols and flavonoids that have been shown to enhance the production and synthesis of the vasoactive mediator, nitric oxide (NO), which regulates endothelial function. Therefore, dietary interventions designed to increase NO bioavailability may enable the relaxation of large-artery smooth muscle, potentially reducing arterial stiffness and cardiovascular risk. A post-prandial study performed in the elderly demonstrated that the consumption of a Med diet increases NO bioavailability promoting improvements in endothelium-dependent endothelial function (214). Likewise in an older (aged 55 to 80 years) subpopulation (n=200) of the PREDIMED study the investigators found that increases in plasma NO were associated with significantly lowers brachial blood pressure
following a one year intervention with a Med diet supplemented with either nuts or extra virgin olive oil (EVOO) compared to a reduced fat control diet. Together, these findings suggest that improved NO bioavailability following a Med diet may be an underlying mechanism contributing to the reductions observed in central pressures and arterial stiffness.

More recently, in a subpopulation of non-smoking women with moderate hypertension from the PREDIMED study, the investigators aimed to assess whether the improvement of blood pressure following a Med diet supplemented with EVOO or nuts would be mediated by the modulation of NO bioavailability (215). After 1 year of the intervention, participants had an increase in serum NO, providing further evidence for the beneficial role of NO on blood pressure following a Med diet supplemented with nuts or EVOO. Of note, the consumption of nutrients such as fiber, cereals, fruits, vegetables, legumes, meat and meat products, fish and alcohol were similar across the three groups at baseline, and throughout the course of the intervention. This suggests that the reduction in blood pressure may be a result of the main dietary changes from the intervention, which includes an increase in olive oil and nut consumption and the substitution of olive oil by EVOO in the corresponding Med diet groups.

Both olive oil, EVOO in particular, and nuts are typical of a traditional Med diet and both are rich sources of phytochemicals and phenolic compounds, which likely account for their cardiovascular benefits. Specifically, the high polyphenol content of EVOO is thought to inactivate the effects of free radicals and lipid peroxidation, therefore beneficially affecting arterial stiffness (216, 217). In a study of 23 hypertensive patients, Ferrara et al. (218) reported that when compared to sunflower oil, a diet rich in EVOO was shown to reduce the need for daily antihypertensive medication by 48% versus 4% during the sunflower oil diet. The authors attributed the reduction in blood pressure to the polyphenol content in the EVOO, which
increases NO concentrations and contributes to a reduction in blood pressure. In contrast, there are no polyphenols in sunflower oil.

Although these findings suggest EVOO and/or nuts may be the driving force behind the vascular health benefits, much epidemiological evidence supports the benefits of a polyphenol rich diet in the prevention of hypertension (219). For example, polyphenols from several other sources, such as berries, wine or tea have been shown to promote endothelium-dependent NO-mediated relaxations of the arteries. Such changes in plasma NO are directly associated with significant reductions in both systolic and diastolic pressures. These results are consistent with the findings of the present study, which suggests that a polyphenol rich dietary pattern improves multiple measures of vascular health including central systolic and diastolic pressure as well as arterial stiffness. Moreover, the inclusion of lean beef within a Med dietary pattern does not attenuate these beneficial effects. Specifically, the beneficial effect of the MED0.5 and MED2.5 diets compared to an AAD control diet on central systolic and diastolic pressures shows that lean beef in amounts that represent typical consumption in the U.S. can be included as part of a MED diet. Potential reasons for the lack of treatment effect between the MED5.5 and the AAD include a “replacement effect” such that the amount of added lean beef in the MED5.5 could have replaced foods that have been shown to improve NO production such as nuts, EVOO and antioxidant rich fruits; or it could simply reflect the increased amount of antioxidant rich foods on our version of the AAD as it was designed to meet nutrient guidelines. It is often the case that our average American control diet is more nutritionally sound than our participant’s habitual diets.

The null effect on augmentation index (AI) despite a decrease in PWV could be a result of the dissociation often seen between PWV and AI (220). AI is influenced by several factors
including heart rate, sex, height, and age and as a result does not always correlate well with PWV. Moreover, the measure of AI depends upon the timing of the reflected waves as well as the intensity of the reflected waves. Since reflection time was reduced, as indicated by the reduction in PWV, yet AI was not, it could be speculated that the changes occurred in the larger central arteries.

Strengths and Limitations

Despite the robust benefits of a highly controlled randomized cross-over feeding trial, our study is not without limitations. It is possible that the reduction in certain antioxidant rich foods (e.g. EVOO, fruits and vegetables) as a result of the increase in lean beef could explain the lack of a treatment difference between the MED5.5 and AAD. Future studies should ensure that servings of these components remain consistent across all MED diets, and instead, alter levels of cereals and grains. In addition, the AAD provided in this study was likely higher in potassium and calcium rich foods than are typically consumed in central Pennsylvania such that the quantities of these nutrients may have contributed to the improvements in central pressure following the AAD. In the future, the collection of participant’s dietary data prior to the start of the controlled feeding period may provide greater insight regarding the observed changes in AAD. In an effort to replicate a traditional Med style diet many of the menu items may be challenging to prepare and not easily adaptable into the lifestyle of many Americans. However, the fact that our results are consistent with other studies of the Med diet demonstrate our ability to replicate the dietary pattern in an American population with high levels of dietary compliance and a low dropout rate (<15%). Our population was also notably healthy at study start with healthy average brachial blood pressures. Given the observed beneficial observed, we feel this is a testament to the vascular health benefits of a Med dietary pattern.
Conclusion

In conclusion, the consumption of a Med style dietary pattern containing 0.5 to 5.5 oz./day of lean beef significantly reduces brachial systolic and diastolic blood pressure when compared to an average American diet containing 2.5 oz/day of beef. Greater improvements in central systolic and diastolic pressure and arterial stiffness are also possible following consumption of Med diet with lean beef in amounts up to 2.5 oz/day when compared to an average American diet. These findings suggest that current recommendations to limit red meat intake need to be refined in the context of type and amount specifically recommended. The promotion of a healthy dietary pattern, such as the Med dietary pattern, which includes lean beef may be more translatable to an American population. Future guidelines should consider recommendations that target that the majority of the population, many of whom wish to consume a healthy dietary pattern that includes some lean red meat.
Chapter 5: The Effects of Lean Beef as Part of a Mediterranean-Style Dietary Pattern on Cholesterol Efflux: A Randomized Controlled Trial

ABSTRACT

**Background:** Despite the robust cardiovascular benefits associated with a Mediterranean (Med) diet, there are inconsistencies regarding its effect on HDL-C. In response to emerging evidence about the role of HDL function in cardiovascular risk, assessing cholesterol efflux capacity will increase our understanding about how a Med dietary pattern exerts beneficial effects.

**Objective:** To gain a better understanding of HDL function by evaluating cholesterol efflux capacity following consumption of a traditional Med dietary pattern with varying quantities of lean beef compared to an Average American control diet (AAD).

**Design:** In a randomized, 4-period, crossover, controlled feeding trial a Med diet (CHO 42%, PRO 17%, FAT 41%, SFA 8%, MUFA 26%, PUFA 8%) with different quantities of lean beef (0.5, 2.5 and 5.5 oz/d) was compared to an Average American diet (AAD; CHO 52%, PRO 15%, FAT 33%, SFA 12%, MUFA 13%, PUFA 8%) in generally healthy males and females (n=28). Plasma cholesterol efflux capacity from J774 macrophages was measured at baseline and after each treatment period.

**Results:** There were no between treatment effects for global or transporter-specific cholesterol efflux to apoB-depleted serum. Compared to baseline values, global and ABCA1 cholesterol efflux were significantly reduced following the AAD diet (p<0.05 for both); whereas non-ABCA1 cholesterol efflux was significantly decreased from baseline following the MED0.5 diet. The combined effects of the three Med diets versus AAD, on ABCA1 cholesterol efflux were significantly higher following a Med style diet (3.2 vs. 3.6; p=0.012, respectively).
**Conclusions:** In our study, a Med diet with varying quantities of lean beef did not improve cholesterol efflux capacity, however, it is notable that only the AAD elicited significant reductions in cholesterol efflux. This finding suggests that the Med dietary pattern, and possibly the polyphenolic content of the extra virgin olive oil, preserved HDL functionality despite the reduction in absolute HDL cholesterol. Increasing quantities of lean beef as part of a Med diet did not influence these results.

**Keywords:** High-density lipoprotein, HDL function, cholesterol efflux, Mediterranean diet, cardiovascular disease.
INTRODUCTION

Current dietary guidelines for CVD reduction recognize the importance of healthy dietary patterns, especially plant-based dietary patterns, versus single nutrients, with the primary goal being to decrease saturated fat (SFA) intake. It is well established that reducing dietary saturated fat decreases LDL-C, a major CVD risk factor (221). Moreover, reducing SFA also decreases HDL-C (222). However, a recent focus on the relationship between HDL-C and CVD risk has provided greater understanding about the importance of HDL function versus absolute HDL concentration (160, 223). For example, large pharmacological trials designed to increase HDL concentrations have not demonstrated protection from CVD events (224, 225). Thus, the reduction in CVD risk associated with healthy low fat diets does not appear to be influenced by concomitant reductions in HDL-C (225).

New insight into the role of HDL affirms it has several anti-atherogenic functions that may influence CVD risk, which, in contrast to previous views, are not dependent upon increases in HDL-C concentrations but rather improvements in HDL functionality. HDL function is assessed by cholesterol efflux capacity, which is an assessment of HDL’s transport of cholesterol from peripheral cells to the liver for recycling or excretion (161). Cholesterol efflux, independent of HDL concentration, has been shown to be inversely related to coronary artery disease (165) and incident CVD events (166, 167) in several studies. Recognizing the strong relationship of diet to both HDL-C and CVD risk has prompted an increase in dietary interventions designed to assess the role of HDL functionality, yet results have been contradictory.

In a postprandial study of the effects of different dietary fats on cholesterol efflux in healthy young women (n=6), Sakr et al. (226) reported that when compared with beef tallow or sunflower oil, high oleic sunflower oil enhanced cholesterol efflux 4 (P<0.05) and 8 hours
following a high fat load. Sunflower oil [67% polyunsaturated fatty acids (PUFA)] had no effect, whereas a mixed oil, containing 49% of PUFA and 39% of monounsaturated fatty acids (MUFA) increased efflux after 8 hours. Following consumption of beef tallow, (50% SFA and 45% MUFA) a decrease in efflux was observed followed by an increase at the 8-hour time point. The authors speculate the response was due to poor digestibility and delayed absorption of MUFA (226). Similar improvements in cholesterol efflux from a high MUFA oil were observed in another study of healthy women (n=12). This was a controlled feeding trial with four 7-week isocaloric diets containing 30% of total calories as fat, with 15.6% of the fat being provided by one of the following: MUFA (olive oil), n-3 PUFA (rapeseed oil), n-6 PUFA (sunflower oil) and SFA (milk fat); however this study used cultured fibroblasts incubated with HDL₃ (227). In contrast, Montoya et al. (228) conducted a similar dietary intervention in healthy men and women in Spain (n=41) for 5 weeks and found an increased capacity of serum to promote the efflux of cholesterol from cells in culture following the n-3 PUFA-enriched diet.

To date, the largest and most rigorous study to assess HDL functionality as part of a dietary intervention was conducted on a sub-sample (n=296) of the PREDIMED study. This landmark randomized controlled trial comparing two Mediterranean diets (Med diet), one supplemented with mixed nuts and the other with extra-virgin olive oil (EVOO), to a reduced fat diet in a Spanish population (n=7447) reported a 30% risk lower risk of major cardiovascular events over a period of 5 years with a Med diet. In the ancillary study by Hernaez et al., (168) the authors found that both Med diet groups improved cholesterol efflux compared to baseline (p=0.018 and p=0.013 for the EVOO and mixed nuts group, respectively); no change was observed for the reduced fat control diet. Of note, both Med diet groups also experienced a trend in the reduction of HDL-C concentration. In an earlier study by Hernaez et al. (169), the
investigators reported comparable increases in cholesterol efflux capacity following 3 weeks consumption of EVOO, which they attributed to the high polyphenol content. Similar to the previous study of the Med diet (168) there were no changes reported in HDL-C concentration.

Dietary fats, and specifically EVOO, appear to have a beneficial impact on cholesterol efflux, however the influence of a dietary pattern remains largely unknown. Inconsistent findings in the literature, due to the multiple mechanisms that exist for the efflux of cellular cholesterol, make the study of HDL functionality challenging. Although there is some evidence for the benefits of a Med diet, which could be attributed to the high EVOO content, little is known about the impact of altering the source of dietary fat on HDL functionality. Inconsistencies in the research may be related to the use of cells that do not reflect the conditions present in the atherosclerotic intima, which is rich in macrophages that contain the ABCA1 transporter as the major exporter of cholesterol to HDL (229). Therefore, the present study measured global and transporter specific (ABCA1) cholesterol efflux capacity from J774 macrophages to human apo-B depleted serum.

We previously showed that a Mediterranean dietary pattern with varying quantities of lean beef (0.5 oz./day, 2.5 oz./day and 5.5 oz./day) improved several cardiovascular risk markers, including LDL-C and apoB, however, all three Med diets elicited a reduction in HDL-C. Thus, the objective of the current study was to assess HDL functionality in response to a traditional Med diet (0.5 oz./day) compared to a Med diet containing lean beef at quantities equivalent to (2.5 oz./day) and above (5.5 oz./day) those of current U.S. consumption in a sample of 28 generally healthy males and females.
METHODS

Experimental Design

This was a 4-period, randomized, crossover, controlled-feeding study designed to investigate the effects of varying quantities of lean beef daily (0.5 oz, 2.5 oz, and 5.5 oz) on cardiometabolic risk factors. Daily macronutrient intakes of the three Med diets (MED0.5, MED2.5 and MED5.5) were identical: 42% of total energy as carbohydrate, 17% protein and 41% total fat, 8% SFA and 26% MUFA. The three diets differed primarily in the amounts of lean beef with further adjustments required to maintain the energy and macronutrient composition. The Average American diet (AAD) had a macronutrient composition representative of current U.S. intake (52% of total calories as carbohydrate, 15% protein, 33% total fat, 12% SFA and 13% MUFA). All of the meals and snacks were provided to participants. Participants received each 4-week test diet in random order with a 1-2 week compliance break between diet periods in which participants could resume their self-selected, habitual diets. At baseline (start of study) and at the end of each diet period, participants completed a series of clinical and physical assessments. On test mornings, participants arrived in the fasted state (12 h with only water, 48 h without alcohol, and 12 h without vigorous exercise) to the Clinical Research Center. Trained research staff measured their height, weight, and blood pressure and obtained a fasting blood sample (~30 mL on each day). Whole blood was drawn into either serum separator tubes and allowed to clot or into EDTA-containing tubes. Blood was centrifuged at 1500 × g at 4°C for 15 min, and aliquots of serum and plasma were stored in a −80°C freezer and not thawed until analyses were conducted.
Study Population

Detailed methods and participant characteristics have been described previously (Chapter 3). This analysis includes participants (n=28) from the Penn State site only. Briefly, men and women (ages 30–65 y) with a BMI (in kg/m²) of 20–35 and HDL cholesterol >15th percentile of U.S. population (men > 34 mg/dL, women > 41 mg/dL) were eligible for the study. Exclusion criteria included the following: tobacco use; use of prescription cholesterol-lowering medications; vegetarian diet; weight gain or loss of ≥10% within the previous 6 mo; alcohol consumption of ≥14 drinks/wk (i.e., ≥196 g ethanol/wk); chronic illness; refusal to stop vitamin or mineral, lipid-lowering, or other supplements; and pregnant, lactating, or wanting to become pregnant before or during the study. Each participant signed a written informed consent to participate. The study protocol was approved by the Institutional Review Board of The Pennsylvania State University. This trial was registered at clinicaltrials.gov as NCT02723617.

ABCA1-mediated Cholesterol Efflux Capacity

ABCA1-dependent cholesterol efflux capacity of serum HDL samples was determined as described in detail elsewhere (230-233). Polyethylene glycol was used to precipitate apoB-containing lipoproteins from serum samples to isolate the HDL fraction as previously described (234). In brief, global and ABCA1-mediated cholesterol efflux were measured using J774 mouse macrophage cells in the presence and absence of cAMP. Cells were pre-incubated with 3H cholesterol for 24 h and allowed to equilibrate overnight with or without cAMP for the assessment of global cholesterol efflux and ATP-binding cassette transporter (ABC) A1 efflux, respectively. After washing, the cells were incubated for 4 h with the serum HDL samples (apoB-depleted serum) added at a concentration of 2.8% (vol:vol). Cholesterol efflux is
expressed as the radiolabel released as a percentage of 3H cholesterol within cells before addition of serum. All efflux values were corrected by subtracting the small amount of radioactive cholesterol released from cells incubated with serum-free medium. The global cholesterol efflux from cAMP treated J774 cells includes cholesterol efflux mediated by ABCA1, SR-BI, ABCG1, passive diffusion or other still unknown carriers (231). ABCA1-independent efflux was measured from the untreated cells. ABCA1-dependent efflux from J774 cells was determined as the difference in efflux from cAMP-treated and untreated cells. All study samples from the same subject were run within the same assay. Intra-assay coefficients of variation for both global and ABCA1 cholesterol efflux is 8%.

**Statistical Analysis**

Statistical analyses were performed by using SAS (version 9.4; SAS Institute). Using PROC UNIVARIATE the residuals for each variable were used to assess normality as well as visual inspection of distributions (histograms and stem and leaf plots), skewness value, and Shapiro-Wilk p-value. Treatment effects were tested per protocol (participants completing the entire study were included in the analysis) and with the use of a linear mixed model. In addition, a linear mixed model was used for subgroup analyses to investigate whether participant baseline characteristics (i.e., BMI) modified the effects of treatment on outcome variables. Baseline characteristics were stratified into categories on the basis of established cutoffs (i.e., BMI <25 or ≥25). For this secondary analysis, treatment, group (BMI), and treatment-by-group were considered as fixed effects and subject as the random effect. The Bonferroni correction was used to adjust for multiple comparisons. Pearson correlations were used to evaluate associations between baseline variables. Significance was set at P < 0.05.
RESULTS

Participants (n = 28; 14 females, 14 males) were healthy middle-aged (45 ± 2.2 y) individuals with a BMI of 25 ± 0.7 kg/m², near optimal LDL-C (105 ± 6 mg/dL) and normal to high HDL-C (55 ± 1.9 mg/dL) concentrations. Baseline characteristics of our sample population including measures of apoA1-containing HDL subpopulations and global and transporter-specific cholesterol efflux are presented in Table 5-1.

Table 5-1. Baseline characteristics of study participants (n=28).

<table>
<thead>
<tr>
<th></th>
<th>Mean ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>45 ± 2.2</td>
</tr>
<tr>
<td>Males:Females</td>
<td>14:14</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>25 ± 0.7</td>
</tr>
<tr>
<td>TC (mg/dL)</td>
<td>186 ± 8.2</td>
</tr>
<tr>
<td>LDL-C (mg/dL)</td>
<td>105 ± 6.1</td>
</tr>
<tr>
<td>HDL-C (mg/dL)</td>
<td>55 ± 1.9</td>
</tr>
<tr>
<td>TG (mg/dL)</td>
<td>101 ± 12.7</td>
</tr>
<tr>
<td>Glucose (mg/dL)</td>
<td>98 ± 1.8</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>115 ± 2.3</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>79 ± 1.3</td>
</tr>
<tr>
<td>APOA1</td>
<td>150 ± 3.4</td>
</tr>
<tr>
<td>APOB</td>
<td>91 ± 5.1</td>
</tr>
<tr>
<td>Plasma HDL Subspecies</td>
<td></td>
</tr>
<tr>
<td>HDLP3</td>
<td>35.1 ± 0.9</td>
</tr>
<tr>
<td>HLP3</td>
<td>8.6 ± 0.6</td>
</tr>
<tr>
<td>HMP3</td>
<td>11.5 ± 0.9</td>
</tr>
<tr>
<td>HSP3</td>
<td>15.0 ± 1.1</td>
</tr>
<tr>
<td>HZ3</td>
<td>9.5 ± 0.1</td>
</tr>
<tr>
<td>NHC3</td>
<td>60.8 ± 2.2</td>
</tr>
<tr>
<td>Cholesterol efflux to serum, %</td>
<td></td>
</tr>
<tr>
<td>Global</td>
<td>10.1 ± 0.3</td>
</tr>
<tr>
<td>ABCA1</td>
<td>4.0 ± 0.2</td>
</tr>
<tr>
<td>Non-ABCA1</td>
<td>6.1 ± 0.2</td>
</tr>
</tbody>
</table>
Cholesterol efflux

There were no between treatment effects for global or transporter-specific cholesterol efflux to apoB-depleted serum. Results are presented in Table 5-2. Compared to baseline values global and ABCA1 cholesterol efflux were significantly reduced following the AAD diet (p<0.05 for both); whereas non-ABCA1 cholesterol efflux was significantly decreased from baseline following the MED0.5 diet (Figure 5-1). In an investigation into the combined effects of the three Med diets compared to AAD a significant effect of treatment was observed. Compared to AAD, ABCA1 cholesterol efflux was significantly higher following a Med style diet (3.2 vs. 3.6; p=0.012, respectively). Previous investigations from our lab and others have reported differential effects on cholesterol efflux capacity based on baseline BMI values. In a subgroup analyses of our sample, we did not observe any influence of baseline BMI on global or transporter specific cholesterol efflux (data not shown).

Table 5-2. Effects of Med diets with lean beef on global and transporter specific cholesterol efflux.

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>AAD</th>
<th>MED0.5</th>
<th>MED2.5</th>
<th>MED5.5</th>
<th>Treat effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABCA1 efflux</td>
<td>3.98 ± 0.24</td>
<td>3.24 ± 0.23</td>
<td>3.64 ± 0.21</td>
<td>3.65 ± 0.29</td>
<td>3.64 ± 0.26</td>
<td>P=0.08</td>
</tr>
<tr>
<td>Global efflux (+cAMP)</td>
<td>10.12 ± 0.32</td>
<td>9.27 ± 0.31</td>
<td>9.46 ± 0.27</td>
<td>9.69 ± 0.39</td>
<td>9.71 ± 0.33</td>
<td>p=0.33</td>
</tr>
<tr>
<td>Non-ABCA1 efflux (-cAMP)</td>
<td>6.13 ± 0.16</td>
<td>6.03 ± 0.16</td>
<td>5.82 ± 0.13</td>
<td>6.04 ± 0.16</td>
<td>6.07 ± 0.14</td>
<td>p=0.15</td>
</tr>
</tbody>
</table>
**Figure 5-1.** Changes in ABCA1, global and non-ABCA1 cholesterol efflux following 4 weeks of a Med diet with varying quantities of lean beef compared to an average American control diet.

Bars represent means and error bars represent standard errors of the means; *indicates significant change from baseline ($P<0.01$); different letters are significantly different; $P \leq 0.01$

**Baseline correlations**

Correlations for baseline measures of HDL-C, apoA1, HDL subspecies, cholesterol efflux, and BMI are presented in **Table 5-3**. HDL-C and apoA1 were highly correlated (0.57; $P = 0.001$), and were similarly associated with total HDL particle number and non-ABCA1 cholesterol efflux ($P < 0.05$ for all), however only apoA1 was significantly correlated with global cholesterol efflux ($p < 0.01$) and neither were correlated with ABCA1 cholesterol efflux ($P > 0.05$ for both). Large HDL particles were negatively associated with BMI ($p < 0.05$) and positively associated with mean HDL particle size, total HDL and small HDL particles ($p < 0.01$). Similar but negative associations were seen for small HDL particles including a negative association.
with medium HDL particles (p<0.001). BMI was inversely correlated with mean HDL size and large HDL particles (P <0.05 for both).

**Table 5-3.** Pearson correlations between total HDL cholesterol, total HDL particles, HDL subspecies, cholesterol efflux and BMI at baseline.

<table>
<thead>
<tr>
<th></th>
<th>HDL apoA1</th>
<th>Mean HDL size</th>
<th>Total HDL particles</th>
<th>Large HDL</th>
<th>Medium HDL</th>
<th>Small HDL</th>
<th>Global</th>
<th>ABCA1</th>
<th>Non-ABCA1</th>
</tr>
</thead>
<tbody>
<tr>
<td>apoA-I</td>
<td>0.57**</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean HDL size</td>
<td>0.70***</td>
<td>0.16</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total HDL particles</td>
<td>0.46*</td>
<td>0.47*</td>
<td>-0.15</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Large HDL</td>
<td>0.84***</td>
<td>0.29</td>
<td>0.93***</td>
<td>0.03</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Medium HDL</td>
<td>-0.02</td>
<td>-0.06</td>
<td>-0.06</td>
<td>0.24</td>
<td>-0.04</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Small HDL</td>
<td>-0.20</td>
<td>0.05</td>
<td>-0.49**</td>
<td>0.21</td>
<td>-0.39*</td>
<td>-0.66***</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Global</td>
<td>0.23</td>
<td>0.57**</td>
<td>-0.04</td>
<td>0.35</td>
<td>-0.03</td>
<td>-0.10</td>
<td>0.04</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ABCA1</td>
<td>-0.11</td>
<td>0.10</td>
<td>-0.29</td>
<td>0.20</td>
<td>-0.33</td>
<td>0.00</td>
<td>0.09</td>
<td>0.77***</td>
<td></td>
</tr>
<tr>
<td>Non-ABCA1</td>
<td>0.51**</td>
<td>0.79***</td>
<td>0.19</td>
<td>0.31</td>
<td>0.31</td>
<td>-0.01</td>
<td>-0.14</td>
<td>0.67***</td>
<td>0.13</td>
</tr>
</tbody>
</table>
| BMI              | -0.31     | 0.01          | -0.51**             | 0.14      | -0.44*     | 0.20      | 0.21   | -0.03 | 0.03      | -0.03
DISCUSSION

This is the first study, to our knowledge, to evaluate the effects of a Med diet with lean beef on HDL function as assessed by global and transporter-specific cholesterol efflux. Although observational studies suggest an association between HDL-C concentration and decreased risk of CVD, evidence from Mendelian randomization studies (224) challenge the view that increasing HDL-C reduces cardiovascular risk. Pharmacological trials (235, 236) also show no benefit of increasing HDL-C concentration on CVD events or mortality. Despite a 25% increase in HDL-C after 2 years of niacin therapy in the AIM-HIGH study and nearly a 30% HDL-C increase following an intervention with the HDL-C-“boosting” drug, dalcetrapib (dal-OUTCOMES), there was no significant reduction in major cardiovascular outcomes (235, 237). The current view is that HDL-mediated cholesterol efflux from macrophage foam cells (238) or the flux of cholesterol from macrophages to the liver (239) correlates better with atherosclerosis than HDL-C concentration. Consequently, the measurement of cholesterol efflux capacity should be recognized as a potential target for the development of novel therapies intended to prevent and reverse atherosclerosis regardless of the effect on HDL-C. Moreover, it is important to recognize the role of HDL structure and function, such as particle size (240), particle concentration (241), and cholesterol efflux capacity (242) as potential mediating factors in cardiovascular risk protection.

Mutharasan et al., (243) found that both large and medium HDL particles were positively correlated with cholesterol efflux capacity whereas small HDL particles were inversely correlated. A similar association was reported by El Khoudary et al. (244) in a sample of 46 pre- and post-menopausal women in which they found a positive correlation between large and medium HDL particles and cholesterol efflux capacity; no association was observed for small
HDL particles. An assessment of 2,924 adults in the Dallas Heart Study reported a positive linear correlation of cholesterol efflux capacity with both HDL particle concentration and size (242). In contrast, there are small scale studies that have shown that small HDL particles have greater efficiency in promoting cholesterol efflux capacity (245). In a mechanistic study by Du et al. (185) using various HDL acceptors of defined size and composition (i.e. HDL\textsubscript{2} and HDL\textsubscript{3} subfractions) isolated from human plasma and reconstituted HDL the authors found smaller acceptors mediated greater efflux capacity. These findings suggest that the number of HDL particles of a certain size rather than the concentration of total HDL may be the mechanism that regulates cholesterol efflux capacity (243). However, additional research on the roles of each are warranted.

In the current study, baseline ABCA1 efflux was not associated with large or small (or lipid-poor) HDL. Whereas, baseline non-ABCA1 efflux was associated with HDL-C (0.51) and apoA1 (0.79), (P <0.01 for both). In another study of a Med diet, Hernaez et al. (168) demonstrated that consumption of a traditional Med diet for one year increased both cholesterol efflux capacity and large HDL particles relative to baseline (168). An investigation into the role of extra virgin olive oil (EVOO), a major component of the Med diet, on cholesterol efflux in a sample of 26 healthy volunteers found that following 12 weeks consumption of 25ml/d of EVOO there was an increased capacity of serum to mediate cholesterol efflux from THP-1 macrophages by 9.8% compared with baseline (246). Helal et al. (246) further evaluated the effect of EVOO on the capacity of HDL to accept cholesterol in an ex vivo model. EVOO consumption enhanced the HDL-mediated cholesterol efflux capacity by 12% (p<0.05). The improved capacity of HDL to mediate cholesterol efflux is thought to reflect an improvement in HDL function (246). These
results indicate that consumption of EVOO increases macrophage cholesterol efflux as well as increasing HDL mediated cholesterol efflux.

The benefits of EVOO have been attributed largely to the polyphenol content, which has been shown to enhance the expression of genes related to HDL metabolism and function (247). In a randomized controlled trial of 47 healthy male volunteers, consumption of a polyphenol rich olive oil for 3 weeks significantly improved cholesterol efflux capacity (+3.05%) compared to a reduction of 2.34% in the low polyphenol olive oil group (p=0.042) (169). Several comparable dietary interventions, however, have reported no treatment effects of MUFA rich diets on cholesterol efflux capacity despite significant reductions in atherogenic lipoproteins (228, 248). For example, the investigation by Blanco-Molina et al., (248) compared the effects of a reduced fat NCEP Step 1 diet (28% FAT, 9% SFA, 14% MUFA, 5% PUFA, and 0.027 mg cholesterol/kJ) versus a MUFA rich (from unrefined virgin olive oil) diet (39% FAT, 9 % SFA, 25% MUFA, 5% PUFA, and 0.027 mg cholesterol/kJ) on cholesterol efflux to whole serum using a rat hepatoma cell line. Following 24 days of consumption the authors found reductions in cardiovascular risk markers (apoB, the TC:HDL ratio, and the apoB:apoA1 ratio) following the MUFA rich diet as compared to the NCEP Step 1 diet; however, no between treatment differences were observed for cholesterol efflux (248). Of note, when dietary cholesterol (0.068 mg cholesterol/kJ) was added to each of the diets, only the NCEP Step 1 diet with cholesterol elicited a significant increase in cholesterol efflux compared to the NCEP Step 1 diet. This suggests that dietary cholesterol may mediate the effect of diet on cholesterol efflux capacity.

These findings were supported by Escola-Gil et al., (249) in a study with mice fed a low-fat, low-cholesterol diet, a high fat, high cholesterol diet, or a high-SFA and low-cholesterol diet. The authors reported that when compared to both a high fat, low cholesterol diet and a low
fat, low cholesterol diet, only the high fat, high cholesterol diet increased cholesterol efflux from macrophages. The observed upregulation in the expression of liver ABCG5 and ABCG8 provides evidence of a potential mechanism by which dietary cholesterol may influence cholesterol efflux capacity. Although we did not find any between treatment effects on global or transporter-specific cholesterol efflux; the AAD diet did yield significant reductions from baseline in global and ABCA1 cholesterol efflux, which was not observed with the Med diets. Therefore, it is possible that the high MUFA content (from EVOO) of the three Med diets preserved HDL functionality, despite the low cholesterol content. Clearly, questions remain about the relationship of efflux capacity with diet and cardiovascular risk. Therefore, future studies are needed to evaluate functional and biological characteristics of HDL in response to different diets.

**Strengths and Limitations**

Currently, a major limitation in evaluating HDL functionality in the present study and others is the lack of standard assessment methods. It is important to recognize that cholesterol efflux assays may provide discrepant results that reflects the methodology employed (250), which can vary as a function of: cell line, use of whole serum, apo-B depleted serum, or isolated HDL particles, and global or transporter specific outcomes. Another limiting factor of the present study was the small sample size. Our study was powered based on the primary outcome LDL-C, which likely limited our ability to see an effect. Notably, when the three Med diet groups were combined we were able to see an effect of treatment on cholesterol efflux, which suggests the effects of diet may have reached significance with a larger sample size. Moreover, our participants were healthy with normal (to high) HDL-C concentrations, which may have limited the influence of diet over a 4-week period. The strengths of our study include the highly
controlled design and employment of novel techniques for assessing the effect of a Med diet with varying quantities of lean beef on cardiovascular risk in a U.S. population.

**Conclusion**

In conclusion, our study adds to the current evidence base about the benefits of a Med diet on CVD risk. While we did not observe an improvement in cholesterol efflux capacity, it is notable that only the AAD elicited significant reductions in cholesterol efflux. Furthermore, when the effects of the Med diets were combined a significant treatment effect compared to AAD was observed. These findings suggest that the Med dietary pattern, and possibly the polyphenolic content of the EVOO, prevented a reduction in HDL functionality despite the reduction in absolute HDL-C. Additionally, there were no differences in cholesterol efflux capacity among the three Med diets, which suggests that the increase in lean beef in the treatment diets did not attenuate the effects observed with a traditional Med diet. It is evident that the study of HDL biology and function is complex and additional studies using standardized methods for assessing HDL function are needed.
Chapter 6: Research Summary and Future Directions

The studies conducted in this dissertation were designed to investigate the effects of lean beef as part of a Mediterranean style dietary pattern on CVD risk factors. Specifically, these studies evaluated the effects of three quantities of lean beef (0.5 oz., 2.5 oz., and 5.5 oz. per day) as part of Mediterranean compared to an average American control diet on the following CVD risk factors: 1) lipids, lipoproteins, apolipoproteins and lipid subspecies, 2) vascular health and arterial stiffness as assessed by brachial and central systolic and diastolic pressures, pulse wave analysis and pulse wave velocity, and 3) the biological and functional properties of HDL that extend beyond HDL-C concentrations. This chapter summarizes the results of these studies and proposes additional research based on these findings.

Our findings demonstrate that under controlled feeding conditions, implementation of a Med dietary pattern in a U.S. population does confer similar cardioprotective benefits to those reported among individuals native to the Mediterranean regions. When compared to an average American diet, a Med diet with up to 2.5 oz./day of lean red meat elicited significantly greater improvements in cardiovascular risk factors, specifically lipid measures and vascular health. Previous research suggests the benefits may be attributed to the polyphenol rich EVOO or the antioxidants and bioactive components of the nuts, vegetables and fruits. Based on our findings, we support the hypothesis that it is the synergistic effect of the dietary pattern as whole. As such, at quantities of lean beef as high as 5.5 oz./day the removal of nutrient rich food components may have contributed to the lack of differentiation between the MED5.5 and the AAD. Since the responses were similar across each of the three Med diets it also is possible that our AAD was higher in nutrient quality as compared to our participants baseline (habitual) diets.
Collecting baseline dietary information would have proven useful in allowing us to discern this possibility and assist future researchers in understanding the metabolic changes that occur from baseline.

Reductions in LDL-C concentration were consistent with the reductions in LDL particle number and apoB. Although we did observe significant reductions from baseline in the large buoyant LDL particles for each of the 3 Med diets and all diets decreased IDL, we also observed a significant reduction in LDL particle number following each of the MED diet groups with no change following the AAD. When similar results have been found in studies replacing dietary SFA with MUFA, one potential mechanism that has been proposed is an increase in LDL clearance. For example, results from animal and tissue culture studies have shown that SFA decrease the activity of the LDL receptor while MUFA increase the activity of the LDL receptor. It also has been hypothesized that part of the LDL-lowering effect is due to reduced production of VLDL because this is the major metabolic precursor particle for LDL. Based on our findings it is possible that decreased LDL production, in response to the increase in MUFA content of the diet, was the primary mechanism for the LDL-lowering effect. Future kinetic studies should be conducted to further examine these mechanisms. Although we observed no change in small, dense LDL there is growing evidence to support total LDL particle number, rather than absolute concentration or size as being a better predictor of CVD outcomes. Experimental studies have reported that hepatic lipase and lipoprotein lipase are involved in the generation of remnant lipoproteins and small, dense LDL. Measurement of hepatic lipase and lipoprotein lipase activities would provide insight about the influence of dietary pattern on small, dense LDL production.
Many questions remain with respect to the biological and functional role of HDL. The multiple mechanisms of action as well as a limited ability to measure these properties make the study of HDL complex. Although our study was not powered to detect diet-induced changes in HDL function, we did observe a treatment difference between the Med diet and AAD when all three MED groups were combined. Moving forward, one of the main challenges in evaluating the effects of dietary interventions on increasing efflux will be measuring their effects. There is a need for a validated and widely available assay of HDL function or cholesterol efflux via RCT in addition to assessing HDL concentration. Once standard assessment methods are established future studies can be powered based on both established CVD risk markers as well as on additional HDL-related functionality measures.

This dissertation adds novel evidence to the existing dietary pattern and cardiovascular disease literature by employing a dose response approach to simultaneously investigate the role of lean beef and a Med dietary pattern on diet induced cardiovascular outcomes in human participants. Although it is well established that a Med dietary pattern confers cardiovascular benefits evidence of the role of lean red meat in the diet is unclear. Observational data have failed to distinguish the effects of lean red meat versus processed meats. As a result, lean red meat is inappropriately linked to the adverse cardiovascular outcomes that may be associated with higher fat and processed meats. The goal of dietary pattern research is to establish healthy dietary patterns that meet nutrient recommendations while also being adaptable at a societal level. Unlike the Mediterranean regions, beef is a popular food in the U.S. and provides several key nutrients. Just as different regions in the Mediterranean basin have their own diets the findings from this research suggest that the inclusion of lean beef represents another variant of a single entity, the Med dietary pattern. Alcohol, and red wine in particular, is a major component
of the dietary pattern and is thought to contribute to the increased HDL-C levels often associated with a Med dietary pattern, however in this trial alcohol was not recommended, which may have influenced the slight reductions we observed for HDL-C. Likewise, at the highest dose of lean beef (5.5 oz/d) the reduction or elimination of other major components of the diet such as fish, nuts, and legumes altered the dietary pattern in a way that more closely resembled a Western style diet. As such no differences were observed between the AAD and the MED5.5 for certain risk markers. Therefore, these findings do not justify the demonization of a single food (i.e. lean beef) but rather emphasize the importance of maintaining a healthy dietary pattern and specifically one that includes the major components of a Med dietary pattern. Further, this demonstrates that a Med dietary pattern is transferable to a U.S. population and can include lean beef at quantities that are consistent with average U.S. consumption (2.5 oz/d). However, additional research exploring the various disease states and mechanisms for which a Med diet exerts its protective effects are warranted.

Several population-based and prospective epidemiological studies have shown that in addition to coronary heart disease adherence to the Mediterranean diet might have a protective effect against obesity, diabetes, hypertension, several type of cancers, and, most recently, Alzheimer and Parkinson’s disease (251-259). Data from several randomized clinic trials have demonstrated a beneficial effect in the primary and secondary prevention of CVD, type 2 diabetes, atrial fibrillation, and breast cancer (260-262). The exact mechanism by which an increased adherence to the traditional Mediterranean diet exerts its favorable effects is not known. Many interrelated and overlapping factors have been hypothesized to play a role. Five mechanisms thought to mediate the positive health effects of a Med diet and promote longevity include the following: (a) lipid-lowering effect, (b) protection against oxidative stress,
inflammation, and platelet aggregation, (c) modification of hormones and growth factors involved in the pathogenesis of cancer, (d) inhibition of nutrient sensing pathways by specific amino acid restriction, and (e) gut microbiota-mediated production of metabolites influencing metabolic health (263). The evidence to support these mechanisms strongly indicates that nutrition is a key factor for the promotion of health and the prevention of the most common noncommunicable chronic diseases. Recent findings from animal and human translational studies are starting to shed light on the biological mechanisms that are mediating the beneficial effects of the traditional Mediterranean diet as well as other healthy dietary patterns (264). However, more mechanistic studies are needed to understand the interactions among diet induced modifications, and the key molecular pathways that promote cellular, tissue, and organ health during aging and disease prevention.

In support of the 2015-2020 Dietary Guidelines for Americans adopting a Med diet pattern and improving diet quality should be a key focus for bettering the lives of Americans. Diet quality has an enormous effect on chronic disease risk, in particular, cardiovascular diseases. The factors that contribute to poor food habits and promote disease include: (a) inadequate consumption of healthful foods; and (b) ingestion of foods created by suboptimal processing (for example, those rich in refined grains, starches, and sugars), foods prepared by modern methods (for example, high temperature commercial cooking), and foods containing additives such as trans fats and sodium (265). Based on the 2010 Global Burden of Disease analysis, the top five leading dietary contributors to death included low intakes of fruits, vegetables, whole grains, nuts and seeds and high intakes of sodium (266). In comparison, high intakes of sugar sweetened beverages and red meat had lower estimated mortality burdens. While reductions in these foods is important, such reductions would have a relatively small
effect on disease risk compared to a strategy that promotes the intakes of healthful dietary patterns.

Therefore, based on current and projected disease burdens, identifying and implementing effective interventions to improve diet quality needs to be a public health priority. Behavior change interventions that target individuals with personalized nutrition counseling have proven effective. However, these are typically limited in their sustainability because of restricted insurance coverage and high cost (267, 268). In comparison, population-based interventions can have a broader effect, lower cost, and improve sustainability (269, 270). Such interventions involve those implemented at the local, state, and national levels. Examples include education and mass media campaigns, food labeling and consumer information, food pricing and economic incentives, school and worksite interventions, and direct restrictions and mandates (270, 271). For example, with fruits and vegetables as a major component of a Med diet pattern, fiscal policy approaches that include changes in food pricing such as subsidies to increase healthful foods and taxes to decrease unhealthful foods could prove beneficial. To expand the evidence base for such efforts, academic institutions should prioritize research on optimal dietary targets and cost-effective policies and disseminate their findings by engaging with the community, advocacy groups and policy makers, and help to inform and evaluate industry efforts. The food industry, from agricultural producers to food manufacturers, retailers, and restaurants, must also commit to the provision of healthier foods. The food industry has made tremendous progress in reducing food-borne illness, increasing volume and production, improving convenience and stability, and reducing cost however, healthfulness must now be addressed with modern nutrition and policy science.
In summary, scientific advances, such as those presented in this dissertation provide a wealth of new evidence to identify several key dietary priorities for cardiometabolic health. Our results demonstrate that lean beef at intakes equivalent to the average consumption in the U.S. (2.5 oz./day lean beef), may be incorporated into a Med style diet with no differences in cardiovascular risk benefits compared to a traditional Med diet with 0.5 oz/day lean beef. These findings add to the growing evidence and consensus that improving food-based dietary patterns is the best means to reduce CVD, obesity and weight gain, and diabetes by replacing the previous emphases on isolated nutrients. Individual behavior-change efforts, health care system changes, novel technologies, and effective policy strategies are needed to complement and facilitate these individual food choices, which together will reduce cardiometabolic disease and economic burdens across the population.
Appendix

Appendix A: Informed Consent Form

CONSENT FOR RESEARCH
The Pennsylvania State University

Title of Project: The Dose-Response Effects of Lean Beef in a Mediterranean-Style Dietary Pattern on CVD Risk Factors

Principal Investigator: Penny Kris-Etherton, PhD
Address: 319 Chandlee Lab
Telephone Number: 814-863-8056
Subject’s Printed Name: _____________________________

We are asking you to be in a research study. This form gives you information about the research.

Whether or not you take part is up to you. You can choose not to take part. You can agree to take part and later change your mind. Your decision will not be held against you.

Please ask questions about anything that is unclear to you and take your time to make your choice.

1. Why is this research study being done?

We are asking you to participate in this research study to examine how the consumption of a Mediterranean-style dietary pattern that will provide three quantities of lean beef in a dose-dependent manner (0.5 oz. 2.5 oz. and 5.5 oz. per day) affects a broad range of metabolic responses that are important in the development of cardiovascular diseases.

This new study may provide important information about the health effects of different amounts of lean red meat in the diet and how lean red meat might impact risk factors for developing heart disease. Specifically, this study will examine the relationship between lean beef consumption and the levels of fat and cholesterol in your blood, markers of inflammation, blood pressure, and other blood markers of cardiovascular disease risk.
2. What will happen in this research study?

General Overview of the Study

Diet Design

If you agree to participate in this study, your participation will last approximately 22 weeks total, consisting of 4 diet treatment phases each lasting 4 weeks and separated by an approximate 2 week break. During each treatment phase, you will be provided with a balanced, precisely controlled Mediterranean style heart healthy diet (41% energy from fat, 42% carbohydrate, and 17% protein). Calorie levels will be estimated for weight maintenance, this is not a weight loss study therefore calories will be adjusted as needed to ensure that you do not lose or gain weight over the course of the study. All diet phases contain foods that are commonly found at a grocery store and differ only in the amount of lean beef provided. The four test diets are: 1) a Mediterranean diet (MED 0.5) providing 0.5 oz. per day of lean beef; 2) a matched MED diet (MED 2.5) that will have the same fatty acid profile as the MED diet, but will contain 2.5 oz. per day of lean beef; 3) a matched MED diet (MED 5.5), providing 5.5 oz. per day of lean beef (on a 2100 calorie diet); and 4) an Average American Diet (AAD). The three MED diets will contain similar foods with the exception of the amount of beef included and other protein equivalents. Each of the MED diets will include 7 oz. equivalents of protein, of which 0.5, 2.5 or 5.5 oz. will come from beef and the remainder will come from fish, poultry, pork, nuts, eggs, and legumes. All MED diets will provide 250 mg/day of omega 3 fatty acids (EPA and DHA) by varying the type of fish provided on each test diet. In addition, all MED diets will provide < 300 mg/day of cholesterol, and <2300 mg/day of sodium.

Study Design:

AAD: Average American Diet; MED 0.5: U.S. Mediterranean Diet (0.5 oz. per day of lean beef); MED 2.5: MED Diet with 2.5 oz. per day of lean beef; MED 5.5: MED Diet with 5.5 oz. per day of lean beef.

= Clinical visits for baseline and endpoint testing
**Procedures to be Followed**

**Screening Tests**

If you decide to participate in the study and are considered eligible after the telephone screening, you will be further screened during a visit to the Clinical Research Center (CRC) at Penn State to determine your eligibility to participate. This visit will consist of filling out standard forms (informed consent, medical history, personal information); questionnaires (i.e. attitudes toward food and eating); measuring height and weight so your body mass index (BMI) can be calculated; and measuring blood pressure (BP). If, after these measurements, it is determined you are still eligible to continue in the research, a blood sample will be taken from a forearm or hand vein and a complete blood count, including liver and kidney function and a blood fat panel will be performed (approximately 15 ml of blood or 1 tablespoon will be taken). You will feel a small pinch or discomfort when the needle is inserted. If the initial blood draw is unsuccessful it may need to be repeated, with your permission. If you take thyroid medicine, and do not have a current (within 6 months) lab test, we will draw 3.5 ml (0.2 Tbsp) more blood to conduct a thyroid test. If you are female, you will be given a urine pregnancy test. You will be contacted within 3-5 days with the results of the screening blood sample. A clinician at the CRC will review all of the screening data and if you are still eligible for the study, you will be contacted to schedule your start date and baseline data collection appointments. There will be no charge for the screening blood work or measurements and you will get these results. If you agree to participate in this study, you will agree to check with the study staff before participating in any other research studies; the study coordinator will let you know if it is alright to participate.

**Feeding Study**

If you agree to participate in the study you will agree to eat only those foods (3 meals and a snack every day) and beverages provided to you (some non-caloric beverages are allowed for free choice) during the feeding periods of the study. You will come to one of the Diet Centers on campus Monday through Friday for breakfast, lunch, or dinner (you choose which fits your schedule best), where meals will be prepared and provided for you. Your other two meals and a snack will be packed for you to take and eat at a place of convenience. On Fridays, you will be given a cooler that contains your remaining Friday meals and Saturday and Sunday meals and snacks. You will be required to appropriately refrigerate and store all foods provided to you for take-out.

You will be weighed regularly at your mealtime and you will provide the study staff with information about any non-study foods you may have eaten, any study foods not eaten and caffeine [limited to five (8oz), caffeine-containing beverages/day] and alcohol consumption (limited to 2 drinks/week). You are supposed to eat only the foods given to you and nothing else. You must eat all of the food given to you. If for some reason you fail to do this, it is important that you tell the study staff that you did not follow protocol so they can make a note of it in your records. The information you provide to the study
coordinators will be collected daily. It should only take a few minutes to complete this form each day. Your calorie intake may be adjusted over the course of the study in order to maintain your screening body weight. You understand that this is not a weight-loss study. The diets are designed to meet your calorie needs and keep your body weight constant. Calorie intake will be adjusted up or down as necessary to maintain your weight. Also, you understand that you must keep your exercise level constant throughout the whole study.

Baseline and Endpoint Testing

Blood sampling:

You cannot consume any food or drinks except water for 12 hours, and cannot drink alcohol during the 48 hours prior to having your blood taken. You also cannot engage in vigorous physical activity 12 hours prior to having your blood taken.

In addition to the blood taken at screening, blood samples also will be taken on two consecutive days at baseline and the end of each treatment phase for a total of 10 times. After a twelve hour fast (consumption of no food or drinks except water), a blood sample will be taken from your arm. If the initial blood draw is unsuccessful it may need to be repeated, with your permission. Your weight and blood pressure also will be recorded. Approximately 85 ml (about 5.5 tablespoons) of blood will be collected at each endpoint over two days (42 mls, or 2.8 Tbsp. each day). Therefore, over the 22-week study, blood will be taken 10 times with a total amount of ~ 425 mls of total blood taken. A typical American Red Cross blood donation is 1 pint (500 mls.) Blood samples will be frozen and analyzed at the end of the study (when all subjects have completed the study). The results of the study will only be available at the end of the entire study (which may take up to 3 years). Your blood may be tested for the following: blood fats (total cholesterol, LDL-cholesterol, HDL-cholesterol, triglycerides, LDL particle size and apolipoproteins), blood sugar (glucose, insulin), inflammatory markers and possibly how the blood fat is being metabolized (e.g. lipid hydroperoxides). NO personal information will be kept with any sample – only ID# will be assigned and only the Primary Investigator and the Study Coordinator will have access to the ID# assignments with the study files. If you are female, you will be asked to provide a urine sample at each blood draw. If you become pregnant during this time, you will be asked to leave the study.

Measures of vascular health

Aortic Blood Pressure and Augmentation Index: Pulse Wave Analysis

Following a 5 minute rest period, brachial artery systolic and diastolic blood pressure will be measured in your left arm using an automated blood pressure cuff. The cuff will reinflate and obtain a pulse wave form. Aortic (central) blood pressure and wave reflection characteristics (augmentation index) will then be derived from the pressure waveforms using a validated transfer function with a SphygmoCor System (AtCor Medical, Sydney,
Aortic Stiffness: Pulse Wave Velocity
Aortic stiffness will be assessed by calculating the pulse wave velocity (PWV) between the carotid and femoral arteries while in the supine position. A cuff will be placed on your thigh. The cuff will inflate during the test to record the pulse waveform in the femoral artery. A simultaneous measurement of the carotid artery pressure waveform will be obtained by an applanation tonometry sensor manually held in place above the carotid artery. PWV will subsequently be calculated by dividing the linear distance between the carotid and femoral sites by the transit time using the SphygmoCor system (AtCor Medical, Sydney Australia). This measurement will be performed in triplicate with one minute rest between each measurement.

Urine collection
At baseline (start of treatment phase 1 only) and at the end of each time point you will be asked to collect a 24-hour urine sample in the week prior to attending your clinic visit. You will be provided with a cooler containing a collection jug that will be used to collect urine (from approximately 7AM to 7AM). In addition, female participants of child bearing potential also will be asked to provide a urine sample for pregnancy testing at baseline and the end of each diet period.

Stool Sample Collection
At baseline (start of treatment phase 1 only) and at the end of each time point you will be asked to collect a stool sample (10-20g) in the week prior to attending your clinic visit. You will be provided with a stool sample kit and detailed instructions for collection of a clean sample. You will be asked to freeze the sample immediately and keep them frozen until your scheduled endpoint visit.

Compliance with Study Protocol

***Please note: Successful completion of this study depends on the total cooperation of the participants. If during the study, you cannot eat the food provided or comply with other study procedures (such as attending clinic visits), you will be asked to leave the study. Every effort will be made to give you a chance to comply with the study requirements, but if you do not follow the above study protocol you may be dropped from the study.

In addition, please advise us of any medical events (such as illness, injury, surgery etc) that arise during the course of the study. Depending on the event, we may require you to obtain a medical clearance before continuing with the study. Some medications may also interfere with our study outcomes so please inform us of any medication changes. ***
3. What are the risks and possible discomforts from being in this research study?

**Discomforts and Risks**

**Feeding Study**

The diets used in this study are nutritionally adequate, whole-food diets. Foods will be prepared according to accepted standards of sanitation and provisions are made to ensure the safety of foods provided for off-site consumption. However, it is possible that incorrect food handling during shipping, storage or preparation, if not detected, could result in food-borne illness. Every effort will be made to safeguard against this possibility. Feeding studies that require on-site eating of meals and strict adherence to the diets provided may interfere with social activities centered around eating such as dining in restaurants. While the menus will provide some variety in the diets, the number of food items will be more limited than that available in an average grocery store. The limited variety may become repetitious over the course of the study. In addition, you may experience a GI (stomach) upset from the change of diet, due to the increased/altered fat/fiber content. Symptoms also may include diarrhea or nausea but this will likely subside once you become accustomed to the new diet.

**Food Allergies**

You will be asked to report any food allergies during the telephone screen, however it is possible that an unknown food allergy may manifest during the study. This is most likely to occur within the first week of feeding since the same foods will be repeated each week. Each day you will be asked to complete a daily monitoring form so that we may track any adverse events, including potential food allergies, and identify the source as soon as possible. In addition, we ask that you please inform study staff immediately should any adverse events occur. If you should experience a severe reaction please seek immediate medical attention and discontinue consuming study foods.

**Blood Sampling**

Blood draws often cause mild pain, swelling or bleeding. There may be some bruising (blood under the surface of the skin), which will be minimized by pressing on the site after the needle is removed. There is also a slight chance of infection, dizziness or fainting. These risks will be minimized and most likely eliminated by having trained medical staff draw the blood in a clinical setting using sterile supplies. If dizziness or fainting occurs, the symptoms will be alleviated by having you lie flat with your feet raised. The medical staff will ask that you remain at the clinic until your blood pressure has been checked and they are sure that you are OK.

**Pulse Wave Analysis (PWA) and Pulse Wave Velocity**

There are no known risks associated with these measurements. The sensation of pressure from the blood pressure cuff or hand-held probe may be uncomfortable. There is a possibility for red blotching or mild bruising (petechiae) appearing on the skin above and below the location of the blood pressure cuff. Studies indicate that petechiae are rare (occurring in less than ½ of 1% of patients) and it is typically not uncomfortable and does not require treatment.
Urine Collection
Collecting urine over a 24-hour period while at home or work can be a disturbance to a participant’s normal schedule. In addition, some people may be uncomfortable collecting their own urine. You will have the option of choosing the most convenient 24-hour period during your last week of each dietary intervention to collect the samples.

Fecal Sample Collection
Some participants may experience a certain level of embarrassment or discomfort from being asked to collect stool samples. However, you will be provided with detailed instructions on how to collect the samples within the comfort of your own home, and at your convenience, to help reduce any concerns you may have.

Loss of Confidentiality
Your participation in this research is confidential. However, there is always a potential for loss of confidentiality despite our best efforts. To prevent this from occurring all records are coded with a unique ID number and no names are used. Records containing names or other identifying information are kept under lock at the PI’s research office. All records associated with your participation in the study will be subject to the usual confidentiality standards applicable to medical records. In the event of publication of this research, no personal identifying information will be disclosed.

4. What are the possible benefits from being in this research study?

4a. What are the possible benefits to you?
You will have a chance to learn the principles of good nutrition practices. You also will receive the results of your screening blood work and information about how your biomarkers for cardiovascular disease risk changed in response to the dietary treatments. On the basis of the findings from our previous research, we anticipate you also will experience beneficial effects of lean beef consumption in the context of a healthy Mediterranean style diet on your lipids, lipoproteins, and measures of vascular health. The final results of the study will not be available until all of the analysis is completed. This may take up to three years. However, no benefit from participation in this study is guaranteed.

4b. What are the possible benefits to others?
It is hoped that the proposed diet design will demonstrate that different quantities of lean beef can be included in a healthy Mediterranean-style dietary pattern for CVD risk reduction. This is important as current recommendations to limit beef consumption have prompted many consumers to either avoid or reduce consumption. Also, consuming lean beef in recommended amounts of protein equivalents in a heart-healthy dietary pattern may help promote healthy dietary practices that are consistent with current recommendations.
5. **What other options are available instead of being in this research study?**

Your participation in this study is voluntary. You may withdraw from this study at any time by notifying the investigators or other study personnel.

6. **How long will you take part in this research study?**

**Time Commitment for the Study**

Total time for study visits, after initial screening, is approximately 36 hours. Times may vary and females will require an additional 5 minutes for a urine pregnancy test at screening, baseline and the end of each diet treatment period. The following is an estimate of the amount of time participants will spend in study activities:

- **Screening appt:**
  - Day 1: Forms, BP, weight, height, blood draw – 45- 60 min
  - (Pregnancy testing: Females only - 5 min)

- **Baseline (Prior to treatment 1):**
  - Day 1: Blood draw, weight, PWA/PWV – 60 min
  - (Pregnancy testing: Females only - 5 min)
  - Day 2: Blood draw: 15 min
  - 24 hr urine collection (at home)
  - Fecal sample collection (at home)

- **End of treatments 1-4:**
  - Day 1: Blood draw, weight, PWA/PWV – 60 min
  - (Pregnancy testing: Females only - 5 min)
  - Day 2: Blood draw: 15 min
  - 24 hr urine collection (at home)
  - Fecal sample collection (at home)

- Eating at the clinic/filling out forms/measuring weight/picking up food ~ 20 min / 5 days per week for 16 weeks=1600 min or about 27 hrs

- At home:
  - Collecting/storing urine and fecal samples x 5 = 1.5 hrs

Total time commitment (with at home urine and fecal collections) is ~36 hrs.

7. **How will your privacy and confidentiality be protected if you decide to take part in this research study?**

Your participation in this research is confidential. Efforts will be made to limit the use and sharing of your personal research information to people who have a need to review this information. All records are coded with a unique ID number and no names are used. Records containing names or other identifying information are kept under lock at the PI’s research office. All records associated with your participation in the study will be subject to
the usual confidentiality standards applicable to medical records. In the event of publication of this research, no personal identifying information will be disclosed. Your blood specimens will be coded with your unique ID number and will be maintained until three years after the date from when the study is published, and then destroyed unless you give permission for use to keep your blood samples for future use (see end of document). At the end of the study (after all subjects have completed the study), you will be given your laboratory results without cost, and informed of the study results.

We will do our best to keep your participation in this research study confidential to the extent permitted by law. However, it is possible that other people may find out about your participation in this research study. For example, the following may review records related to this research:

- The Office for Human Research Protections in the U. S. Department of Health and Human Services
- The Penn State Institutional Review Board (a committee that reviews and approves research studies)
- The Penn State Office for Research Protections.

Some of these records could contain information that personally identifies you. Reasonable efforts will be made to keep the personal information in your research record private. However, absolute confidentiality cannot be guaranteed.

8. **What are the costs of taking part in this research study?**

8a. **What will you have to pay for if you take part in this research study?**

You will not bear any costs as a result of your participation in this study.

8b. **What happens if you are injured as a result of taking part in this research study?**

In the unlikely event you become injured as a result of your participation in this study, medical care is available. 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.

9. **Will you be paid to take part in this research study?**

For their time and participation in the study participants will receive monetary compensation of $500, prorated as follows and paid at the completion of their participation in the study:

- Completion of the 1st diet period = $100
- Completion of the 2nd diet period = $100
- Completion of the 3rd diet period = $125
- Completion of the 4th diet period = $175
The total of $500 will be paid at the completion of the participant's involvement with the study.

If you are not a Penn State employee you will be asked to provide your SSN for tax reporting purposes. Total payments within one calendar year that exceed $600 will require the University to annually report these payments to the IRS annually. This may require you to claim the compensation that you receive for participation in this study as taxable income.

10. Who is paying for this research study?

The funding for this study is provided by the National Cattleman's Beef Association (NCBA). However, the funding source will not be involved in data analysis. They will have the right to review all publications before submission however there are no contractual agreements that allow them to have influence on, or restrict, the publication of results.

11. What are your rights if you take part in this research study?

Taking part in this research study is voluntary.

- You do not have to be in this research.
- If you choose to be in this research, you have the right to stop at any time.
- If you decide not to be in this research or if you decide to stop at a later date, there will be no penalty or loss of benefits to which you are entitled.

You may be asked to leave the study at any time if you do not comply with the study protocol. During the course of the research you will be provided with any new information that may affect your health, welfare or your decision to continue participating in this research.

12. If you have questions or concerns about this research study, whom should you call?

Please contact Dr. Kris-Etherton at (863-2923 or 863-8056) with any questions, complaints or concerns about the research. You can also call this number if you feel this study has harmed you. You may also contact the Office for Research Protections at (814) 865-1775, ORProtections@psu.edu if you:

- Have questions regarding your rights as a person in a research study.
- Have concerns or general questions about the research.
- You may also call this number if you cannot reach the research team or wish to talk to someone else about any concerns related to the research.
INFORMED CONSENT AND AUTHORIZATION TO TAKE PART IN RESEARCH

Signature of Person Obtaining Informed Consent
Your signature below means that you have explained the research to the subject or subject representative and have answered any questions he/she has about the research.

______________________________  _______  ____________
Signature of person who explained this research  Date  Time  Printed
Name

(Only approved investigators for this research may explain the research and obtain informed consent.)

Signature of Person Giving Informed Consent and Authorization
Before making the decision about being in this research you should have:

- Discussed this research study with an investigator,
- Read the information in this form, and
- Had the opportunity to ask any questions you may have.

Your signature below means that you have received this information, have asked the questions you currently have about the research and those questions have been answered. You will receive a copy of the signed and dated form to keep for future reference.

Signature of Subject

By signing this consent form, you indicate that you voluntarily choose to be in this research and agree to allow your information to be used and shared as described above.

___________________________  ____________  __________________
Signature of Subject  Date  Time  Printed Name

Optional part(s) of the study
In addition to the main part of the research study, there is another part of the research. You can be in the main part of the research without agreeing to be in this optional part.

Optional Storage of Blood Samples for Future Research
As part of this study, we are obtaining blood and stool samples from you. If you agree, the research team would like to store leftover samples of your blood and stool that are collected so that these samples may be studied in the future after this study is over. These future studies may provide additional information that will be helpful in understanding cardiovascular disease, but it is unlikely that these studies will have a direct benefit to you. Neither your doctor nor you will receive results of these future research tests, nor will the results be put in your health record. If you have any questions, you should contact Dr. Kris-Etherton at 814-863-2923.

Your leftover samples will be labeled with a code number and stored in Dr. Kris-Etherton’s locked laboratory. If you consent to the storage of leftover samples of your blood and stool for future research, the period for the use of the samples is unknown. If you agree to allow your samples to be kept for future research, you will be free to change your mind at any time. You should contact Dr. Kris-Etherton at 814-863-2923 and let her know you wish to withdraw your permission for your blood and/or stool to be used for future research. Should you choose not to allow for future testing of your samples they will be destroyed 3 years after publication of study results.

You should initial below to indicate your preferences regarding the optional storage of your leftover blood and stool samples for future research studies.

a. Your samples may be stored and used for future research studies to learn about, prevent, treat or cure cardiovascular disease and obesity and other health problems.
   ______ Yes ______ No

b. Your samples may be shared with other investigator/groups without any identifying information.
   ______ Yes ______ No

Signature of Person Obtaining Informed Consent

Your signature below means that you have explained the optional part(s) to the research to the subject or subject representative and have answered any questions he/she has about the research.

Signature of person who explained this research Date Time Printed Name

Signature of Subject

By signing below, you indicate that you have read the information written above and have indicated your choices for the optional part(s) of the research study.
Do we have permission to keep your personal information and contact you about your interest in participating in future studies for Dr. Kris-Etherton and her collaborators?

_____ Yes    _____ No    _____ Initials

**Person Explaining the Research:** Your signature below means that you have explained the optional part of the research to the participant/participant representative and have answered any questions he/she has about the research.

_____________________________     _____    ____    _____________
Signature of person who explained this optional research    Date    Time    Printed Name
# Appendix B: Sample Menu

<table>
<thead>
<tr>
<th>MED 0.5</th>
<th>MED 2.5</th>
<th>MED 5.5</th>
<th>AAD</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>BREAKFAST</strong></td>
<td><strong>BREAKFAST</strong></td>
<td><strong>BREAKFAST</strong></td>
<td><strong>BREAKFAST</strong></td>
</tr>
<tr>
<td>Whole grain bread</td>
<td>Whole grain bread</td>
<td>Whole grain bread</td>
<td>English muffin</td>
</tr>
<tr>
<td>w/strawberry</td>
<td>w/strawberry</td>
<td>w/strawberry</td>
<td></td>
</tr>
<tr>
<td>preserves</td>
<td>preserves</td>
<td>preserves</td>
<td></td>
</tr>
<tr>
<td>Egg beaters w/</td>
<td>Egg beaters w/</td>
<td>Egg beaters w/</td>
<td></td>
</tr>
<tr>
<td>spinach</td>
<td>spinach</td>
<td>spinach</td>
<td></td>
</tr>
<tr>
<td>Applesauce</td>
<td>Applesauce</td>
<td>Applesauce</td>
<td></td>
</tr>
<tr>
<td>Skim milk</td>
<td>Skim milk</td>
<td>Skim milk</td>
<td></td>
</tr>
<tr>
<td>Olive oil</td>
<td>Olive oil</td>
<td>Olive oil</td>
<td></td>
</tr>
<tr>
<td><strong>LUNCH</strong></td>
<td><strong>LUNCH</strong></td>
<td><strong>LUNCH</strong></td>
<td><strong>LUNCH</strong></td>
</tr>
<tr>
<td>Minestrone soup</td>
<td>Minestrone soup</td>
<td>Minestrone soup</td>
<td>Chicken salad</td>
</tr>
<tr>
<td>Falafel</td>
<td>Falafel</td>
<td>Falafel</td>
<td>sandwich on whole</td>
</tr>
<tr>
<td>Salad w/feta cheese</td>
<td>Salad w/feta cheese</td>
<td>Salad w/feta cheese</td>
<td>grain white bread</td>
</tr>
<tr>
<td>Tzatziki sauce</td>
<td>Tzatziki sauce</td>
<td>Tzatziki sauce</td>
<td>w/lettuce and</td>
</tr>
<tr>
<td>Pita bread</td>
<td>Pita bread</td>
<td>Pita bread</td>
<td>tomato</td>
</tr>
<tr>
<td>Sautéed zucchini</td>
<td>Sautéed zucchini</td>
<td>Sautéed zucchini</td>
<td>Baby carrots</td>
</tr>
<tr>
<td>and squash</td>
<td>and squash</td>
<td>and squash</td>
<td></td>
</tr>
<tr>
<td>Olive oil</td>
<td>Olive oil</td>
<td>Olive oil</td>
<td>Pretzels</td>
</tr>
<tr>
<td><strong>DINNER</strong></td>
<td><strong>DINNER</strong></td>
<td><strong>DINNER</strong></td>
<td><strong>DINNER</strong></td>
</tr>
<tr>
<td>Ratatouille</td>
<td>Ratatouille</td>
<td>Ratatouille</td>
<td>Ratatouille</td>
</tr>
<tr>
<td>Jasmine rice</td>
<td>Jasmine rice</td>
<td>Jasmine rice</td>
<td>Jasmine rice</td>
</tr>
<tr>
<td>Baked cod</td>
<td>Lean beef</td>
<td>Lean beef</td>
<td>Baked cod</td>
</tr>
<tr>
<td>Pita bread</td>
<td>Pita bread</td>
<td>Pita bread</td>
<td>Pita bread</td>
</tr>
<tr>
<td>Olive oil</td>
<td>Olive oil</td>
<td>Olive oil</td>
<td>Olive oil</td>
</tr>
<tr>
<td><strong>SNACK</strong></td>
<td><strong>SNACK</strong></td>
<td><strong>SNACK</strong></td>
<td><strong>SNACK</strong></td>
</tr>
<tr>
<td>Greek yogurt</td>
<td>Greek yogurt</td>
<td>Greek yogurt</td>
<td>Red pepper dip</td>
</tr>
<tr>
<td>Peaches</td>
<td>Peaches</td>
<td>Peaches</td>
<td></td>
</tr>
<tr>
<td>Almonds</td>
<td>Almonds</td>
<td>Almonds</td>
<td>Pita bread</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Cucumber</td>
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</table>
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PUBLICATIONS
Peer-Reviewed Journal Articles


Fleming JA, Hill AM, Kris-Etherton PM. The BOLD-X (Beef in an Optimal Lean Diet) Study: A 12 month Follow-Up Evaluation of Effects of Three Dietary Patterns on Weight Loss and Weight Maintenance in Metabolic Syndrome (Submitted).


