Selected measures of health in women fed conjugated linoleic acid-enriched products from organic, pasture-fed cattle

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Selected measures of health in women fed conjugated linoleic acid-enriched products from organic, pasture-fed cattle

by

Andrew William Brown

A thesis submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Major: Biochemistry

Program of Study Committee:
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Iowa State University
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Any opinions, findings, conclusions, or recommendations expressed in this publication are those of the author and do not necessarily reflect the view of the U.S. Department of Agriculture.
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Chapter I: General Introduction

Introduction

The production and sale of beef and dairy products claiming to be organic (that is, produced with the intention of ecological responsibility) have grown steadily since the early 1990s. In the decade between 1995 to 2005, organic beef farming experienced over a nine fold increase in organic beef cattle from about 4,000 head to over 36,000 head, while organic dairy farming witnessed a seven fold increase in organic dairy cattle from around 12,000 head to over 87,000 head (1). However, a food that is certified organic does not guarantee any differences in nutrient composition as compared with conventional foods. In contrast, the practice of pasturing beef and dairy cattle (that is, letting them graze on pastures for nearly all of their food intake) has been shown to cause noteworthy changes in the nutrient composition of the resulting products, particularly in the fatty acid composition: increasing the length of pasturing and decreasing the consumption of prepared feeds increases the presence of trans fatty acids, the amount of polyunsaturated fatty acids, and the ω3:ω6 fatty acid ratio (2). The most pronounced difference between beef and dairy products from grain-fed versus pasture-fed cattle is the content of a series of diunsaturated fatty acids known as conjugated linoleic acids (2).

Conjugated linoleic acid (CLA) has been both positively and inversely correlated with
changes in the risk factors associated with cardiovascular diseases, diabetes, liver necrosis, body composition, and a number of other conditions to be reviewed in the following chapter. Many of the studies on CLA have been conducted using supplements with isomeric compositions that do not mimic those found in the natural food supply, that contain super-dietary concentrations, or are provided to free-living humans, conditions that do not account for a number of other potentially confounding factors such as isomer specific effects, natural consumption patterns, or differences in caloric intake. To date, no studies have compared the effects of products from pasture-fed cattle versus similar products from feedlot cattle, and therefore certainly none have compared products naturally high in CLA from pasture-fed sources versus naturally CLA deficient products from feedlot sources.

The boom in consumers choosing “natural” and “organic” products on the auspices of purported improved health means that research must be conducted to determine the actual effects these products have on human beings. Further, the increasing number of advertisements for CLA supplements appearing on shelves, and the popular use of CLA for marketing by pasture-fed beef and dairy producers creates a demand for scientific literature elucidating the effects these products have on consumers. Mass media may be considered the largest source of nutritional information for the average consumer, and the information is frequently reported not by knowledgeable scientists or dietitians, but journalists. In one example, Time Magazine ran a story entitled “The Grass-Fed Revolution,” in which conventional agriculture was described as detrimental to health while grass-fed beef and dairy
products were praised(3). Sadly, none of the information presented in the story amounted to any scientific evidence that grass-fed products were healthier than conventional products because no scientific evidence exists to date to establish that link. Rather, present evidence indicates that beef and dairy products from pastured cattle have what is generally considered to be improved fatty acid characteristics, such as the previously mentioned increase in polyunsaturated fatty acids, improved $\omega 3: \omega 6$ fatty acid ratio, and increase in CLA content; it is then assumed that these changes will result in improved health, potentially resulting in spurious correlations.

The scientific evidence for the effects of CLA on health, unlike products from pasture-fed animals, is much more well researched. However, the scientific literature overall is inconclusive. The varied responses to CLA supplementation range from improved health, to maintaining status quo, to even impairing health. With no evidence comparing beef and dairy products from pasture-fed versus grain-fed cattle, and a body of controversial evidence on the effects of CLA, the present study was designed to look at the effects that products naturally rich in CLA have on female volunteers as compared with products naturally deficient in CLA.

**Organization of thesis**

After this general introduction, a comprehensive review of the present literature on CLA is to follow in Chapter II. The literature review will focus on aspects of CLA related to the study that will be presented in Chapter III, including the history of CLA as it exists in scientific literature, early research on and the discovery of CLA,
methods of production, purported health effects, and theoretical mechanisms of physiological action. Chapter III will present original research conducted at Iowa State University on the effects of products naturally rich in CLA from beef and dairy products from pasture-fed cattle against those from grain-fed cattle. The study will be presented in journal format, modeled after the American Journal of Clinical Nutrition format. Chapter IV will include a general discussion of CLA and the presented research, as well as general conclusions about CLA and the presented research, after which recommendations for future research will be discussed. In the appendix, a study that examines the safety of the use of sucralose to help maintain participant adherence to experimental protocols will be presented. This thesis will then conclude by acknowledging the many individuals that contributed to make this work possible, followed by recognition of the materials referred to throughout the thesis, as denoted by numbers between parentheses.
Chapter II: Review of Literature

Introduction

Health and nutrition research related to lipid consumption has focused on a variety of topics, from total lipid intake and macronutrient composition of diets, to the consumption of individual fatty acids. While total lipid intake and dietary macronutrient consumption is still a prominent focus of research, lipid nutrition has evolved to focus on the impacts of certain classifications of fatty acids, such as saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs), polyunsaturated fatty acids (PUFAs), omega-3 (ω-3) fatty acids, and omega-6 (ω-6) fatty acids, to name a few. Of late, a variety of research has been conducted on a fairly small and relatively specific grouping of fatty acids known as conjugated linoleic acids (CLA).

CLA refers to a group of octadecadienoic acids in which the two double bonds are separated by only one single bond. The structure of the bonds lends the word conjugated to the series name, whereas linoleic acid is actually a misnomer: linoleic acid is c9,c12-octadecadienoic acid. The name, however, is now well indoctrinated into scientific lexicon and recognized in the field of lipid nutrition, misnomer or not.

In this review of literature, the production of CLA, the potential health benefits of CLA consumption, and the mechanisms of CLA’s actions will be considered.
CLA Production

Mechanisms in Animals

Dietary CLA can be derived from a number of animals, but the most proficient producers, and certainly the most relevant to human nutrition, are ruminant animals. Rumen hydrogenation of unsaturated fatty acids has been reported and researched for decades. In 1954, Hartman and colleagues found fatty acids containing trans double bonds in considerable quantities (3.5-11.2% of fatty acids present) in ruminant tissues, whereas non-ruminant tissues contained only trace amounts (4). The pastures on which the ruminant animals were grazing did not contain an appreciable amount of trans fatty acids, so the difference between the animals' dietary lipid composition and physiological lipid composition was theorized to be the result of previously reported rumen biohydrogenation (4). Other studies in the 1950s elucidated that not only does the rumen hydrogenate linolenic acid (c9,c12,c15 octadecatrienoic acid), the most common dietary fatty acid of pastured ruminants, to linoleic acid, but that hydrogenation can proceed to full saturation (5). Furthermore, Shorland and colleagues showed that the fatty acid composition of clover fields on which sheep were grazing did not match the composition of the sheeps' rumen contents. Concerned that this may merely reflect the catabolism of the lipids, linolenic acid was added to an artificial rumen; subsequently, SFAs, MUFAs, conjugated and non-conjugated dienes and trienes, and trans double bonds were detected, all of which are evidence of rumen biohydrogenation (5). These alterations
partially explained the discrepancy that existed between the fatty acid content of the diets of ruminants and the fatty acid profiles of their milk and meat.

One of the earliest mechanisms to explain the presence of conjugated dienes in butter was theorized by Bartlet and Chapman in 1961, in which linoleic acid was thought to isomerize to a cis-trans conjugated diene before either being absorbed or being hydrogenated to a trans monoene; the trans bond formed in the isomerization step was thought to be the rate limiting step(6). Research on one particular microbe, *Butyrinvibrio fibrisolvens*, led to the confirmation of Bartlet's and Chapman's hypothesis of biohydrogenation(7). Presently, it is thought that PUFAs are toxic to many rumen microbes, and that hydrogenation is, in effect, a way of detoxifying the rumen(8)(9). The production of CLA in the rumen is a sign of toxic conditions for the rumen microbes, which would decrease cellulose metabolism and rumen growth performance if conditions became overbearing(9).

Rumen biohydrogenation is responsible, directly or indirectly, for the production of many isomers of CLA, such as the c9,t11, t7,c9, and c7,t9 isomers commonly found in beef(10) and dairy(11) products, as well as the production of 14 other naturally produced trace isomers(12). However, the isomeric profiles of ruminant milk and meat products do not match the isomeric profile expected from rumen biohydrogenation alone, which is evidenced by a large discrepancy in the fatty acid profiles of duodenal contents and milk from lactating dairy cows(13). While the
duodenal contents contain a low amount of CLA, the milk shows a much higher content, and a vastly different CLA isomeric profile dominated by c9,t11 CLA. Other mechanisms must therefore exist to convert absorbed biohydrogenation products into CLA.

One of the most abundant biohydrogenation products from the rumen are trans octadecenoic fatty acids, and particularly the t11 isomer, known as trans vaccenic acid (tVA)(7). The endogenous desaturation of tVA by a Δ9 desaturase, known as steroyl-CoA desaturase (SCD), results in the production of c9,t11 CLA(13). The production of tVA in large amounts via biohydrogenation results in an overabundance of this byproduct, which is then desaturated in extrarumenic tissues; however, not all of the tVA is desaturated, resulting in tVA in milk and meat. For instance, Backderf and Brown reported that butter contains approximately 4% of its fatty acid content as tVA(14). Humans, too, have SCD, which endogenously converts consumed tVA to CLA(15), and thus the final CLA content in a human after consuming ruminant products is not a direct reflection of the CLA consumed.

Effects of Ruminant Diet on CLA Production

Ruminant animals fed on pasture have a higher proportion of their fatty acids as PUFAs as compared with grain-fed animals. In particular, ruminant animals grazing on pastures consume much more linolenic acid, as well as a rich supply of linoleic and oleic acids. Thus, more partial hydrogenation results in the rumen when
compared to the standard grain diet, and, further, an increase in grazing period prior to slaughter results in an increase in CLA content in the meat: one study showed a linear CLA content increase of 150% in muscle tissue and 250% in subcutaneous adipose tissue when grazing periods were varied from 0 days of grazing to 158 days of grazing prior to slaughter (16). The effect of grass feeding has been confirmed by at least one other study (17).

Other alterations in the ruminant diet may also lead to increased concentrations of CLA in the products from these animals. Supplementing grain diets with different oils, for instance, can cause a change in CLA content of the meat. Sunflower enriched diets result in approximately a three fold increase in CLA (2), linseed enrichment results in a two fold increase (2), and supplementation with a 2:1 mix of sunflower oil and fish oil results in almost a ten fold increase (18). The resulting CLA has been reported as being natural in some literature, though whether cattle consuming fish oil and flax is natural is debated.

Most of the CLA produced naturally through biohydrogenation and subsequent desaturation is in the form of the $c_9,t_{11}$ isomer. Increasing the amount of some oils in the diet, such as fish oil, results in an increase in the $t_{10},c_{12}$ isomer of CLA, which has been implicated as a product of rumen dysfunction (12); caution must therefore be used when attempting to use cattle as CLA mills.
Commercial CLA Preparations

The amount of dietary fats and oils a ruminant converts to CLA is a rather small percentage, accounting for up to only a few percent of the total lipid in ruminant products (2)(16)(18). Because of the interest in CLA, commercial methods have been developed in which polyunsaturated oils are converted to CLA mixtures through a series of acid and alkali reactions. In early research, the composition of the CLAs created through these processes resulted in unnatural ratios of a number of CLA isomers. Christie and colleagues reported that some commercial CLA supplements contain all trans CLAs, all cis CLAs, and, in one sample, found the positional isomers of 8,10 : 9,11 : 10,12 : 11,13 in a ratio of 14:30:31:24, though the geometric isomerization of each positional isomer was unknown (that is, the 8,10 positional isomer could have contained c8,c10, c8,t10, t8,c10, and/or t8,t10) (19). Most analyses simply assumed the two major peaks were the two most common CLA isomers in commercial preparations: c9,t11 and t10,c12 (19). Further advances in the reaction and purification steps allowed for large scale production of purified mixed isomer CLAs, as well as specific isomeric ratios of CLA, including the alkali isomerization of various oils, urea crystallization for purity, or interesterification into triacylglycerols (TAG) (20).

The bioavailability of these commercially synthesized CLAs has been in question because the responses to mixtures of CLAs administered in different food matrices or pill supplements to humans have varied vastly from animal models, which will be
reviewed later. The synthetic production of CLA is generally used to produce free fatty acids, specific esters, or TAG, all of which do not mimic the natural composition of lipids consumed in the diet. As an example, TAG generally contain their most unsaturated fatty acids on the SN$_2$ position of the glycerol backbone, while the SN$_1$ and SN$_3$ positions contain the most saturated fatty acids. Gastric and pancreatic lipases, therefore, react differently to TAG that contain a different pattern of saturation.

**Effects of CLA on Markers of Health**

Studies have been conducted on the effects of CLA on markers of health in cell models, tissue cultures, animal models, and humans, with varying results. Some variation in results can be accounted for by noting the differences in isomers used in each study, or in the appropriateness of the model being applied to humans. Variation also exists in whether the CLA preparation is fed *ad-libitum*, as part of a controlled diet, or as a supplement. Furthermore, some researchers have concluded that because their research shows no biological effects in their sample populations that CLA has no effect in the total population, regardless of trends toward significance or the inappropriateness of extrapolating from one population to another. The compounding of these confounding factors results in a very muddled view of the effects of CLA on various markers of health.

One of the first studies attributed to studying the effects of CLA was conducted by
Pariza and others on pan-fried hamburger(21). Concerns over mutagenic properties of fried hamburger led the investigators to test extracts of the hamburgers using the Ames test and rats, in which they found that CLA actually decreased mutagenesis. This report is often considered the first on the potential health benefits of CLA, and much research has ensued in the following decades.

**Body Composition**

Obesity and overweightness have become increasing concerns world wide in developing and developed countries alike. The CDC reports that the National Health and Nutrition Examination Survey (NHANES) from 1976 to 2004 has shown an increase in obesity from 15.0% to 32.9% of adults in the United States(22). Obesity has been implicated in approximately 112,000 excess deaths as compared with normal weight individuals(23), and the co-morbidities associated with obesity include diabetes, hypertension, and a host of other problems(22). Anything that can be associated with a reduction in obesity, therefore, is researched heavily, and CLA is no exception.

Animal studies have shown alterations in body composition, such as a decrease in body fat mass and an increase in lean mass, associated with CLA supplementation in mice(24)(25), hamsters(26)(27), and rats(28)(29)(30). Differing effects of individual CLA isomers were demonstrated in animal models, with evidence that the t10,c12 isomer is the bioactive isomer in relation to body composition(27). However,
the results are not uniform in different animal genetic models: genetically diabetic and obese Zucker rats did not respond to CLA while their non-obese counterparts did respond (29). Results such as these spurred human research on the effect of CLA supplementation on human body composition.

As with the Zucker rats, BMI class may or may not play a factor in the ability of CLA supplementation to result in body compositional changes in humans. Using dual-energy x-ray absorptiometry (DXA), Laso and colleagues showed that consumption of 3 g/d of a 50:50 mixture of $c_9, t_{11}$ and $t_{10}, c_{12}$ CLA isomers resulted in a 2% decrease in total body fat mass with a 3% decrease in trunk fat mass in a 12 week trial in overweight subjects ($25<BMI<30$), while the obese subjects ($30<BMI<35$) showed no such differences (31). Consistent with these results, a group of men and women, most of whom had a BMI of normal to overweight ($18<BMI<30$), lost 3.8% of their body fat mass after CLA supplementation (32). In another study, Steck and colleagues reported that supplementation with 6.4 g/d CLA resulted in an increase in lean mass of 0.64 kg over a 12 week period in obese subjects, but again no changes were recorded in the fat mass of the obese participants. They also saw no effects with only 3.2 g/d CLA (33). In yet another group of obese participants, who were subjected to a weight loss regimen and then supplemented with 3.4 g/d of CLA or a placebo for one year to test total- and fat-mass regain, no differences were seen between the two treatments (34). Consistent with the other obesity studies, abdominally obese men, either supplemented with mixed CLA isomers or purified
t10,c12 CLA, showed no differences in abdominal adiposity, body weight, or body fat mass as compared with a placebo (35). However, contrary to Laso and others' results, supplementing middle aged males with a BMI in the range of normal to overweight (18<BMI<30) with 1.4 g/d c9,t11 incorporated into dairy products by modified feeding resulted in no change in body weight or BMI (18). Thus, no consistent results related to CLA supplementation are seen in individuals of different BMI classes.

Type and amount of exercise in conjunction with CLA inconsistently alters the results of supplementation. In one study, 20 participants exercised in a gym for 90 min three times per week and were either given a placebo or 0.6 g/d of CLA in capsule form. The participants supplemented with CLA showed decreased adiposity as compared with the placebo group, but neither group had a difference in body weight (36). CLA supplementation of 3.6 g/d, both in conjunction with directed exercise or with supplementation alone, improved body composition and decreased body weight in young females (37). In other studies, participants engaged in strength-training receiving 6 g/d of CLA supplementation against a placebo did not show differences in any markers of body composition (38). In free living humans, the alterations in body composition seen with CLA supplementation were determined not to be the result of the amount of exercise in which the individuals were engaged (39) (40). In adult males and females who reported being physically active on their own volition, 3.9 g/d of CLA supplementation resulted in no change in body composition
or distribution as measured by DXA (41). It does not appear, then, that exercising in conjunction with CLA supplementation has a definitive synergistic effect on body composition.

The most convincing evidence to date that CLA effects body composition is a meta-analysis of 18 double-blind, placebo-controlled studies that indicated CLA consumption results in a dose-dependent response, with an approximate efficacy equation of -24 g of body fat each week for each gram of CLA consumed; the analysis also reported an approximate linear response for 6 months followed by a decline in response until body fat stabilizes after 2 years (42). This would imply that chronic CLA supplementation may, at best, have a modest effect on body composition.

Glucose Intolerance and Insulin Resistance

Insulin, in a generic description, is the hormone primarily responsible for the postprandial distribution and storage of energy-containing macronutrients. Impairment of insulin sensitivity can result from a host of maladies related to energy homeostasis. Research investigating the effects of CLA supplementation on animal models of insulin resistance have shown mixed results. Obese mice showed impaired insulin sensitivity upon supplementation with CLA (43), as did normal male mice (44) and hamsters (26). However, in obese rats, CLA was shown to decrease circulating glucose and insulin concentrations, both markers of improved glucose
tolerance(30)(45).

In humans, inconsistent results are demonstrated as well. One variable to explain the inconsistencies is sex, where insulin sensitivity seems to be improved in females as compared to males. Supplementation with 3.6 g/d mixed isomers of CLA resulted in an improvement in glucose tolerance in a group of young females(37), while 5.5 g/d mixed isomers resulted in no differences in markers of glucose tolerance in young men(46). In non-obese men and women who exercised regularly, only women had lower mean insulin concentrations in an oral glucose tolerance test, whereas the men showed no change(41). Supplementation of 1.4 g/d of c9,t11 CLA in middle-aged men did not alter circulating glucose or insulin concentrations(18), nor did supplementation with three different doses of either c9,t11 or t10,c12 isomers of CLA result in differences in a number of insulin resistance measurements in another group of healthy men(47). In contrast to the trend of men being unaffected by CLA, abnormally obese men supplemented with 3.4 g/d of the t10,c12 CLA isomer actually increased insulin resistance; however, with 3.4 g/d of mixed isomer supplementation, no change was seen in insulin sensitivity(35). In a mixed-sex group of nine overweight, non-diabetic subjects, insulin sensitivity was decreased after supplementation with 4 g/d of mixed isomers of CLA(48), which, at least superficially, appears to conflict with the other literature mentioned. Though the trend would seem to be that females are more responsive to the insulin sensitizing capabilities of CLA supplementation, very few studies have directly compared the
effects between the two sexes, and so the stated trend may be considered speculative; further, there are conflicting reports regarding isomeric effects, and there is also a potential interaction with body mass classification.

Often, insulin resistance brings thoughts of diabetes to mind. Very little research has been conducted in diabetic subjects, however. In the only study to date to investigate the effects of CLA on glucose metabolism in subjects with type II diabetes mellitus, Moloney and colleagues reported an increase in fasting glucose concentrations by over 6% after treatment with 3.0 g/d 50:50 mixed isomer CLA supplementation against a placebo. In addition, they demonstrated that supplementation resulted in impairment of several insulin resistance indices (49). With such gloomy results, it may be prudent not to pursue CLA research in diabetic subjects until its effects are further understood.

Coronary Heart Disease Risk

The American Heart Association reports that the alterable risk factors for cardiovascular diseases (CVD) are elevated serum cholesterol concentrations, increased blood pressure, smoking, physical inactivity, obesity, and diabetes (50). Because there is no known reason that CLA supplementation should decrease smoking or increase physical activity upon supplementation, these two risk factors will not be addressed here within. Alterations in body composition and insulin resistance have already been addressed separately, so only cholesterol
Circulating cholesterol concentrations have been associated with CVD(51). Lipoproteins, the circulating particles that are primarily comprised of apolipoproteins, cholesterol, TAG, and phospholipid, have been differentially correlated with CVD depending on the density class to which they belong. The lower density lipoproteins, which contain apolipoprotein B, or ApoB, have been positively associated with CVD(51). ApoB-containing lipoproteins include very low-density lipoproteins (VLDL; density <1.006 g/mL), intermediate-density lipoproteins (IDL; density >1.006 and <1.019 g/mL) and low-density lipoproteins (LDL; density >1.019 and <1.063 g/mL). Of VLDL, IDL, and LDL, IDL has recently been recognized as one of the most atherogenic lipoprotein classes, though elevated VLDL and LDL concentrations have still been named as culprits(52). High-density lipoproteins (HDL), on the other hand, are generally considered anti-atherogenic, and decrease the risk of CVD(51). Between two subclasses of HDL (HDLII; density >1.063 and <1.125 g/mL. HDLIII; density >1.125 and <1.21), the HDLII fraction is considered more beneficial in preventing atherogenesis(53). If CLA can increase HDL while decreasing ApoB containing lipoproteins, and in particular increase HDLII and decrease IDL, then theoretically CLA supplementation would decrease the incidence of cardiovascular diseases.

Male Sprague Dawley rats fed differing concentrations of CLA had decreased total concentrations and blood pressure will be addressed here.
cholesterol (TC) concentrations and an improved HDL:TC ratio; even though HDL was significantly decreased, the improved ratio of HDL:TC indicates that an even greater decrease in LDL occurred as well(54). Similar results were seen in an atherosclerosis-prone mouse model(55)(56), thus inspiring human research.

Some human research was able to reproduce the beneficial effects shown in some animal models. In diabetic individuals, a 50:50 mix of CLA isomers supplemented at 3.0 g/d resulted in an 8% increase in HDL cholesterol, primarily in the HDLII fraction, and decreased the LDL to HDL ratio, both considered improvements in blood lipid profiles(49). Other subjects showed improved lipid profiles, as well, throughout two years of CLA supplementation including a decrease in TC and LDL(57).

Not all studies reported that CLA improves CVD risk factors, though. In fact, a study in overweight and obese men showed that the placebo resulted in a more significant decrease in TC and TC:HDL than did CLA(58). Another study demonstrated a decrease in both HDL and TC concentrations, but not LDL, indicating the majority of the TC decrease was the result of the decrease in HDL, theoretically leading to an increased risk of CVD(59). One suggestion for the difference in results is a dose-dependent and isomer-specific response that was noted in healthy men supplemented with individual isomer capsules for 8 weeks: t10,c12 CLA increased TC, primarily through the raising of LDL, whereas c9,t11 CLA did not show the same results(47).
To make matters less definitive, a number of studies in humans have shown no changes in circulating cholesterol concentrations. Supplementation with 3.6 g/d with or without exercise did not change circulating TAG, TC, HDL, or LDL concentrations in young females(37). In a group of participants with LDL phenotype B (high concentrations of small, dense LDL particles), CLA supplementation did not affect LDL or HDL concentrations(60). Other research has shown no effects of CLA on blood lipids, either(41)(61). In summary, the variables dictating the differences in cholesterol response to CLA supplementation have not been fully elucidated.

Besides cholesterol concentrations, blood pressure and arterial elasticity are other markers of arterial health. Increases in blood pressure are often observed at the onset of obesity, and the Zucker diabetic obese rat is no exception. Upon supplementation with CLA, however, the obesity-related high blood pressure was attenuated in these rats(45). Human studies, again, result in varying responses. Raff and colleagues demonstrated that supplementation with 4.7 g/d of a 50:50 mixture of CLA isomers resulted in no change in arterial health(62), while another study on supplementing CLA and calcium to pregnant women decreased pregnancy-induced hypertension(63). Japanese, overweight, male volunteers did not show any alterations in blood pressure with supplementation(64). Therefore, it appears that how CLA supplementation may decrease blood pressure has yet to be determined.
Liver health and function

A report of CLA causing hepatomegaly in animal models (65) has led to concern over CLA supplementation and liver function in humans. Two correlates of liver integrity that can be measured from blood samples are aspartate aminotransferase (AST) and alanine aminotransferase (ALT) (66). These two enzymes function primarily in the liver and are only released into the circulation upon hepatocyte lysis. Thus, an increase in circulating AST and ALT activity generally indicates an increase in liver necrosis. Furthermore, chronic liver diseases may be comorbidities associated with increased BMI, insulin resistance, and dyslipidemia (66), all of which have been correlated to CLA supplementation.

Human studies on the effects of CLA on liver function as measured by AST and ALT have not shown anything concerning to date. Studies 6 weeks (61), 12 weeks (67), and 12 months (68) in duration have resulted in no changes in AST and ALT activities between CLA supplementation and controls, indicating that CLA does not seem to affect liver integrity in acute or chronic CLA supplementation.

Mechanism of action

The questionable safety of CLA has resulted in multitudes of research regarding the mechanisms in which CLA may cause its effects. For instance, if CLA does indeed result in a change in body composition, does CLA cause altered lipid metabolism or
simply affect the desire to consume energy? What are the causes for the increases and decreases in insulin sensitivity? And on what does CLA act to cause such changes?

Because CLA is a fatty acid, it is appropriate that much of the research to define the mechanisms of CLA’s action focus on lipid related genes. Peroxisome proliferator-activated receptor gamma (PPARγ) is an adipocyte-specific transcription factor that dimerizes with the retinoid x receptor (RXR), which together, when activated, can upregulate or suppress certain target genes related to lipid metabolism and energy homeostasis(69). Of interest are the upregulation of adipocyte fatty acid binding protein (aP2), which is responsible for intracellular binding of fatty acids; acyl-CoA synthetase (ACS), which creates a fatty acyl-Coenzyme A thioester; fatty acid transporter protein (FATP), which transports fatty acids across the cellular membrane; leptin, which is a hormone generally associated with suppressing appetite; adiponectin, which can increase hepatic insulin sensitivity, among other effects; and lipoprotein lipase (LPL), the enzyme that hydrolyzes TAG from circulating lipoprotein molecules(69). The regulatory activities of PPARγ are activated by certain fatty acids, including CLA, as well as fatty acid metabolites such as eicosanoids(48)(69). Recently, Zhou and others found that CLA supplementation in an obese rat model upregulated the mRNA expression of PPARγ and some of its target genes, such as aP2, FATP, ACS, and adiponectin(30). In another set of studies conducted on human preadipocytes, an isomer specific response was noted;
in particular, c9,t11 CLA increased PPARγ expression, whereas the t10,c12 isomer decreased PPARγ expression(70). Furthermore, t10,c12 CLA was able to result in delipidation of adipocytes, whereas the c9,t11 isomer was not(71).

To determine the organismal ramifications of the purported changes in PPARγ expression, adipokines, which are bioactive adipocyte secretions, were measured in several models. Circulating leptin concentrations are negatively associated with energy intake: the higher the circulating leptin, generally the less likely the organism is to consume energy. Like most adipokines, leptin is generally expressed in higher concentrations with the more adipose tissue present in the organism. One would then expect that with the depletion of adipose tissue seen in some studies, and the isomer specific delipidation of adipocytes previously described, that leptin concentrations would decrease upon CLA supplementation. In contrast, adipocyte models show an increase in leptin expression(71), while leptin was not altered upon mixed isomer CLA supplementation in a normal male mouse model(44). Likewise, a number of studies have found no differences in leptin concentrations in humans upon CLA supplementation, either as a mix of isomers or as purified t10,c12 CLA(35)(37)(72). It would seem that, on this brief evaluation, CLA's organismal physiological effects are not the result of alterations in leptin secretion, potentially indicating that changes in body composition related to CLA supplementation are not necessarily the result of altered energy homeostatic signaling.
To examine other physiologic changes related to CLA supplementation, adipokine-related insulin sensitivity was investigated. Insulin resistance can be the result of a number of metabolic and hormonal changes besides the classic view of glucose and insulin concentrations. One such related hormonal change is from the adipokine adiponectin. Unlike leptin and other adipokines, circulating adiponectin concentrations, are inversely proportional to body fat mass\(^{(73)}\). With the observation that adiponectin improves hepatic insulin sensitivity\(^{(73)}\), it may be an important hormone to study in regard to CLA supplementation.

An animal model of male mice showed insulin resistance upon feeding with 1.5% energy as mixed CLA isomers. The insulin resistance was purportedly caused by adiponectin depletion, and reversed upon removal of CLA from the diet. Administering rosiglitazone, a PPAR\(\gamma\) agonist, to upregulate adiponectin production prevented depletion of adipose tissue and also prevented insulin resistance\(^{(44)}\). However, one study in diabetic rats demonstrated improved insulin sensitivity with a concomitant increase in circulating adiponectin, which may be one explanation for the differences between mice and rats in insulin sensitivity related to CLA\(^{(45)}\). In fact, another study where insulin sensitivity was improved in rats supplemented with CLA showed that both common CLA isomers, separately or together, increased the expression of adiponectin receptors R1 and R2 in the liver\(^{(74)}\). These data strongly suggest a role of adiponectin and PPAR\(\gamma\) as a mechanism of action for the physiologic effects of CLA. The question then becomes whether humans are more
like rats, with CLA improving adiponectin and insulin sensitivity, or more like mice, with CLA decreasing both adiponectin and glucose tolerance, which only rigorous research may answer.

With a body of controversial evidence suggesting or refuting the notion that CLA can increase or decrease adiposity, only two studies to date reported adiponectin concentrations related to CLA supplementation in humans\(^{(75)}\)(\(^{(76)}\)). After 6 months of CLA supplementation of 3.4 g/d of a 50:50 mixture of the two most prominent isomers of CLA, Syvertsen and colleagues measured no alterations in circulating adiponectin concentrations. Using the euglycemic hyperinsulinemic clamp method of determining insulin resistance, which is considered the gold standard, the researchers found no significant differences in insulin sensitivity. They also noted no differences in BMI or body fat percentage\(^{(75)}\). In another study, Risérus and colleagues compared a mix of CLA isomers or the \(t10,c12\) isomer alone against a control, and also found no differences in adiponectin concentrations, though a decrease in insulin sensitivity was observed\(^{(76)}\). Because adiponectin concentrations are correlated with body composition, the demonstrated lack of change in concentrations presented in these two studies does not preclude adiponectin as important in studies where body compositional changes are observed, but so far the research does not seem to strongly implicate adiponectin as an important mechanism by which CLA acts.
Circulating lipids are primarily cleared by uptake of lipoprotein particles by the liver or the hydrolysis of TAG in the blood. With the upregulation of PPARγ, a concomitant increase in LPL has been noted, which would result in clearance of circulating lipids into the adipocyte\(^{(69)}\). Downregulation of LPL, then, would aid in the adipocyte delipidation shown in at least one model\(^{(71)}\). A different PPAR, PPAR\(\alpha\), may also be implicated in circulating cholesterol concentrations, which are affected by addition of cholesterol to, and clearance from, the blood. Cell models using human hepatoma (HepG2) cells demonstrated an upregulation of the LDL receptor in response to treatment with \(t_{10},c_{12}\) CLA, which could result in improved LDL clearing, and would therefore decrease circulating cholesterol and TAG as shown in some models\(^{(77)}\).

Eicosanoids, which are other agonists of PPARγ, are synthesized from the two essential fatty acids in humans: linoleic and linolenic acids. Thus, CLA’s effects on essential fatty acid metabolism could be a concern. However, at a supplementation of 1.2 g/d of either \(c_{9},t_{11}\) or \(t_{10},c_{12}\) CLA for 7d, Turpeinen and colleagues did not observe any alterations in linoleic or linolenic acid metabolism, indicating that CLA may not interfere with the metabolism of the essential fatty acids\(^{(78)}\).

Still yet another mechanistic concern is that there is a growing body of evidence to suggest that CLA may have sex-specific effects, as indicated before in the review of insulin resistance research. One potential explanation for these differences could be
CLA’s effects on estrogen. In particular, cell culture work has indicated that CLA inhibits estrogen response elements and partially regulates estrogen activity through estrogen receptor dephosphorylation(79). This reinforces the notion that CLA may effect males and females in different ways(41).

Conclusions

CLA supplementation in humans has resulted in inconsistent and often antagonistic conclusions from one experiment to the next. While there are trends for alterations in body fat mass, adiposity, circulating cholesterol concentrations, insulin resistance, and other markers of health, no definitive comments can be made on CLA supplementation. One theory for the variation in responses to CLA supplementation is the matrix in which the supplement is given. Some studies reviewed here incorporated CLAs by artificially adding them to foods and pills, in free fatty acid form or in TAG form; other studies have severely altered the eating patterns of cattle to produce beef and dairy products rich in CLA. Furthermore, there is clear evidence for isomer-specific differences in human response, as well as differences in sex, body composition, and insulin sensitivity at the onset of supplementation.

A natural supplement has yet to be tested. As reviewed, ruminant animals are capable of producing CLA from certain dietary lipids, but as the practice of grain-feeding cattle has increased, the CLA content has generally decreased. Some studies have tried to alter the dietary rations of cattle in a manner to promote CLA
production and publish under the notion that this is natural incorporation; however, organizations such as the American Grassfed Association do not consider feeding cattle fish oils and flax seed natural(80). Furthermore, these oils may result in rumen dysfunction(12). With this in mind, we have proceeded to conduct a research study on humans in which the CLA supplement is in a natural food matrix, produced by pasturing cattle, with the placebo products from grain-fed cattle. The following chapter is the description of the study in a journal article format.
Chapter III: Presentation of Research

Abstract

Background: Conjugated linoleic acid (CLA) purportedly alters body composition, glucose tolerance, hepatic function, lipoprotein distributions, and other markers of health. However, results of research are often inconclusive or contradictory, and presently no studies have investigated the effects of natural incorporation of CLA from pasture-fed cattle.

Objective: The present study investigated the effects of two diets high in beef and dairy products, one diet comprised of products from a pasture-fed system that were naturally enriched with CLA and one diet comprised of commercial products of grain-fed origin that were naturally deficient in CLA, on insulin resistance, body composition, circulating lipids, and other selected disease risk factors.

Design: Eighteen healthy women age 20-39 y consumed one of two diets for 56 d. Balanced, nutritionally complete diets comprised of 31% energy from lipid, 13% protein, and 54% carbohydrates were administered with the primary difference being CLA content (CLA group: 1.17 g/d; Control group: 0.35 g/d).

Results: The CLA diet did not result in any differences in insulin sensitivity, body composition, circulating blood lipids, nor other measured disease risk factors as compared with the Control diet.

Conclusion: A diet naturally enriched with a 3.5 fold increase in CLA by incorporation of beef and dairy products from pasture-fed cattle did not result in
measurable improvements in selected measures of health in premenopausal women as compared with a similar diet with beef and dairy products from grain-fed cattle.

Introduction

Various isomers of conjugated octadecadienoic acid, also known as conjugated linoleic acid (CLA), have been reported to both improve and impair markers of health in humans. Experimental results have reported improving(49)(57) and impairing(58)(47) circulating lipids, increasing(37)(41) and decreasing(48) insulin sensitivity, depleting(31) or not altering fat mass(35), and increasing(68) or not changing(67) indicators of liver necrosis. Still other studies have shown no effects in some or all measured risk factors(18)(37)(60).

Some of the variation in results may be explained by the variation in the studies themselves. Different experiments on male or female subjects caused differing results in terms of insulin resistance: CLA appears to improve insulin sensitivity in women(37)(41), whereas men remain unchanged(18)(46) or worsen(48). Body composition prior to CLA supplementation also may play a factor: experiments have resulted in improved body composition in non-obese participants(31)(32) but no change in obese subjects(31)(34). Still other research indicates differences in CLA isomer composition as a source of varying results, with the t10,c12 isomer showing some effects and the c9,t11 isomer showing others(47).

Most published studies have used CLA supplementation in the form of commercially
available pills provided to free living adults. However, the discovery of the potential health benefits of CLA are generally attributed to Pariza and colleagues who showed CLA from pan-fried hamburgers had anti-mutagenic properties (21). Thus, we have found it appropriate to return to the roots of CLA research: the natural incorporation of CLA in food.

CLAs are most commonly found in ruminant products because of the partial biohydrogenation that occurs in the rumen (5) and the subsequent desaturation of trans-vaccenic acid to c9,t11-CLA (14). Feeding patterns and practices of ruminant livestock are known to alter the fatty acid composition (including CLA content) of products derived from these animals. In particular, bovine consuming quickly growing pasture tend to produce the highest natural concentrations of CLA commonly available to Americans, with CLA content increasing with longer grazing periods (16). Supplementing ruminants' diets with oils has a more marked effect on CLA concentrations in bovine-derived products, such as sunflower oil resulting in a three fold increase in CLA (2), linseed enrichment causing a two fold increase (2), and a 2:1 mix of sunflower oil and fish oil resulting in about a ten fold increase (18). Whether the endogenous enrichment is the result of oils or pasture, the increase in CLA is predominantly in the c9,t11 isomer (18) (16) (2).

To the best of our knowledge, only one study to date has investigated the effects of feeding dairy products endogenously enriched in CLA to humans (18). However, the enrichment was through supplementing dairy cattle with fish and sunflower oils,
which are unnatural to the cattle’s diet. Whether or not oil supplementation constitutes natural enrichment with CLA is, therefore, debatable, especially among farmers who believe in strict pasture feeding. In the spirit of determining the effects of CLA naturally incorporated into foods, our objective is to compare the effects of beef and dairy products from pasture-fed cattle, which are naturally enriched with CLA, against beef and dairy products from grain-fed cattle, which are naturally deficient in CLA, in terms of insulin resistance, body composition, circulating blood lipids, and other disease risk factors in healthy female subjects.

Materials and Methods

Human participants

Research was approved by the Iowa State University Institutional Review Board, and all women screened signed an informed consent document.

Women between the ages of 20 and 40, with a body mass index (BMI) between 19 and 30 (normal and overweight), not pregnant or nursing, non-smoking, non- to moderate-drinkers, and who considered themselves to be in good health were invited to be screened for participation. Screening included determination of height, weight, and BMI, completion of a medical history questionnaire, and a fasting blood sample analyzed by Laboratory Corporation of America (LabCorp; Omaha, NE) for serum triacylglycerols (TAG), total cholesterol (TC), and high-density lipoprotein cholesterol (HDL). Over 7000 women within the age limits were contacted, of which 56 women thought they fit the criteria and agreed to be screened, and 19 women fit
the previously stated criteria, as well as having serum cholesterol concentrations near or above the median for their age as determined by The Third National Health and Nutrition Examination Survey (NHANES III). One participant discontinued participation during the first weekend as a result of an injury unrelated to the study. The characteristics at screening of the women who completed participation in the study are summarized in Table 1.

Experimental procedures
Diets were formulated using Nutritionist Pro (version 2.3; First DataBank, Inc.; San Bruno, CA) by a registered dietitian. Diets were formulated to average 2000 kcal/d with 35% of calories coming from lipid, 15% from protein, and 50% from carbohydrate, which approximates the average composition consumed by females age 20-40 y in the United States(81).

Beef with elevated concentrations of CLA was obtained by selecting from a group of organic, pasture-fed steers harvested after a period of rapid pasture growth. The control ground beef was obtained from a feedlot, grain-fed steer. Beef was ground into 85% lean ground beef.

Dairy products high in CLA were obtained from organic, pasture-fed dairy cattle. Ice cream was obtained from Pastureland Creamery (Woodward, IA), cheese was obtained from Farmers All Natural Creamery (Wellman, IA), and butter was generously donated by PastureLand Cooperative (Minneapolis, MN). Commercial
products from grain-fed cattle were analyzed to find a control product similar to the treatment products in macronutrient composition and energy density. Haagen-Dazs vanilla ice cream (Oakland, CA), FastCo cheddar cheese (Boone, IA), and Land-O-Lakes butter (Saint Paul, MN) were chosen for the Control products.

All meals were prepared in Iowa State University’s Human Metabolic Unit (HMU). Participants consumed 3 meals/d during weekdays in the HMU; lunch on Sunday was also served in the HMU. All of Saturday's meals, as well as breakfast and dinner on Sundays, were prepared in the HMU and sent home with reheating instructions. Participants were asked to consume no foods or beverages other than those provided, with the exceptions of water, tea, coffee, and no-calorie soft drinks sweetened with Splenda® (McNeil Nutritionals; Ft. Washington, PA) for the duration of the 56 d intervention. Splenda was included as a manner in which to increase participant compliance, and was determined to be fairly inert in terms of measured outcomes for the present study, as outlined in Appendix A.

**Blood sample collection**

Participants were asked to fast for at least 10 h prior to day 0 and again before day 56. An intravenous catheter (IV) was inserted into the arm of each participant by registered nurses. Fasting serum samples were collected with BD Vacutainer Serum Separator tubes for analysis by LabCorp, and fasting plasma samples were collected in BD Vacutainer EDTA tubes (BD; Franklin Lakes, NJ). An oral glucose tolerance test (OGTT) was conducted by administering a 75 g glucose drink (SUN-
DEX No. TG30010P, Fisher Scientific; Pittsburgh, PA) that was to be consumed within five minutes of being administered. Blood was again drawn via IV and transferred to EDTA treated tubes at 30, 60, 90, 120, 150, and 180 minutes after completion of the glucose drink.

Serum was allowed to coagulate at room temperature for 20-40 minutes, per LabCorp's instructions. EDTA tubes were kept on ice until centrifugation. Blood samples were centrifuged at 3000 x g for 15 minutes at 4°C. An aliquot of plasma was treated with 50 µl of 5500 KIU/mL aprotinin (Sigma; St. Louis, MO) and stored in a glass tube for analysis of glucagon. All other plasma samples were aliquoted into plastic microfuge tubes and stored at -20°C until analyzed.

Lipoproteins were isolated from 3 mL of fasting plasma sample by a sequential flotation ultracentrifugation method modeled after Havel et al(82). Briefly, plasma was transferred to a 4 mL polycarbonate tube and layered with a solution equal in density to the infranatant for a total volume of 3.9 mL. The sample was centrifuged at 109,000 x g for 20 hours at 18°C in a Beckman Ti 50.4 rotor in a L8-M ultracentrifuge (Beckman; Palo Alto, CA); 1.8 mL of supernatant were transferred to a 2 mL Corning Cryogenic Vial (Corning, Inc; Lowell, MA). The infranatant was then adjusted to the next density by addition of 1.5 mL of a more dense solution, layered with 0.3 mL of the same density as was adjusted to, and the centrifugation procedure was repeated. The obtained fractions were of the following densities in g/mL: very low-density lipoproteins (VLDL) < 1.006 < intermediate-density lipoproteins.
(IDL) < 1.019 < low-density lipoproteins (LDL) < 1.063 < high-density lipoprotein subfraction II (HDLII) < 1.125 < high-density lipoprotein subfraction III (HDLIII) < 1.21.

**Analysis of selected markers of health**

Concentrations of TC were determined from each lipoprotein fraction by colorimetric assay (Kit C7510, Pointe Scientific; Canton, MI). Glucose was colorimetrically quantified from the OGTT samples (Kit G7521, Pointe Scientific). Insulin and glucagon were measured from the OGTT samples by radioimmunoassay (HI-11K and GL-32K, respectively, Linco Research; St. Charles, MO), as were fasting concentrations of adiponectin (HADP-61HK, Linco Research).

LabCorp results were analyzed for differences in aspartate aminotransferase (AST), alanine aminotransferase (ALT), and TAG, as well as used as a quality control for the in-lab analysis of lipoprotein fractions.

Body composition was determined using a Hologic Delphi W dual-energy X-ray absorptometer (DXA) (Hologic Inc.; Bedford, MA).

**Diet analysis**

An extra set of meals was collected daily for each treatment including all provided foods and liquids. Daily total provided intake was determined gravimetrically. Samples were stored at 4°C until homogenized using a Waring industrial blender with a cooling coil (Waring Commercial 4L Laboratory Blender, 4L 2610T coil; New
Hartford, CT) set to 4°C by a cryogenic cooler (Caron Model 2050; Marietta, OH). Homogenate was frozen at -20°C until analyzed.

Daily dry matter intake was determined by lyophilizing 5 replicates of 5 g aliquots of daily intake homogenates. Protein was approximated using a micro-Kjeldahl procedure to determine nitrogen content(83). Briefly, 60 ± 3 mg of the dry matter samples were added to Kjeldahl tubes in duplicate; a Kjeltab (Thompson and Capper Ltd; UK) and sulfuric acid were added to each tube. Samples were digested for 4 h, and titrated to determine total nitrogen. Protein was approximated using a factor of 6.25 times the nitrogen content.

Total lipids were extracted from 2 g samples of daily homogenates in triplicate by a modified Folch procedure, using 2:1 chloroform:methanol (v:v)(84) under nitrogen to prevent oxidation; lipid content was then determined gravimetrically.

Fatty acid methyl esters (FAME) were prepared from lipid extracts using sodium methoxide as a methanolic base(85). Samples were analyzed on a Varian 3350 gas chromatograph with a flame ionizing detector equipped with a Varian 8200CX autosampler (Varian; Palo Alto, CA) and a Supelco 2560 fused silica capillary column (100 m x 0.25 mm x 0.2 μm film thickness, Sigma) under the following column conditions: initial column temperature was set to 70°C and held for 2 min; increased to 165°C at 19°C/min and held for 20 min; increased to 192°C at 0.6°C/min and immediately increased to 230°C at 50°C/min and held for 15 min for a
total run time of 87.76 min. FAMEs were identified by retention times compared with purified lipid standards (NuCheck; Elysian, MN).

Trimethylsilyl derivatives of dietary sterols were prepared from the non-saponifiable fraction of the total lipid extract. Briefly, 5α-cholestanee was added as an internal standard and the lipid extract was saponified by addition of 11% w/v potassium hydroxide in a 55% v/v ethanol/deionized water solution. The non-saponifiable fraction was isolated and derivatized with N,O-Bis(trimethylsilyl)trifluoroacetamide with 1% trimethylchlorosilane (Thermo Scientific Pierce; Rockford, IL). Sterol derivatives were analyzed on the gas chromatograph described above equipped with a 30m Supelco column with an initial column temperature of 150°C increased to 200°C for 20 min followed by an increase to 250°C for 30 min. Standards were prepared and analyzed the same way, and cholesterol derivatives and 5α-cholestane in the samples were identified by retention times compared with derivatized standards.

**Statistical analysis**

All statistics were computed using SAS (version 9.1, SAS Institute Inc.; Cary, NC). Screening cholesterol concentrations were used to pair individuals with similar cholesterol, randomize the pairs to treatments, and pairing removed for all further analyses. Change scores were calculated by subtracting the day 0 values from the day 56 values, where appropriate. Change scores and baseline values were compared between treatments; change scores were also analyzed with the student's
t-test against zero to determine if values significantly changed from the beginning to the end of the study. Timecourse data generated for plasma glucose, insulin, and glucagon concentrations from the OGTT were analyzed for differences in fasting values, within treatment changes, and differences between treatment change scores at each timepoint. Curves were compared using the GLM procedure with repeated measures, or as change scores for the areas under the curves (AUCs). Statistical significance was considered p < 0.05.

Results

Diet compositions
The compositions of the analyzed experimental diets were reasonably close to the target composition (Table 2). Though the experimental and target compositions differed slightly in each macronutrient, the values obtained indicate a fairly similar dietary composition between the administered Control and CLA diets that resulted in no significant differences in calculated intake of total energy, carbohydrate, or protein (all p > 0.1), and a small (~2%), though statistically significant, difference in lipid percentage. Cholesterol intake was similar between the two diets.

Fatty acid profiles of the diets were similar in saturated fatty acid (SFA), monounsaturated fatty acid (MUFA), and polyunsaturated fatty acid (PUFA) content, but with a significant difference in total CLA content and in specific isomers of CLA (Table 3). In the CLA diet, 1.17% of the identified FAME were CLA isomers, predominantly the c9,t11 isomer; the control diet contained 0.35% of the identified
Blood lipids

Changes in cholesterol concentrations in individual lipoprotein fractions, as well as the sum of the cholesterol fractions, were analyzed (Figure 1). No differences in cholesterol concentrations were seen between treatments at day 0 in any fraction, nor in TC. After dietary intervention, the IDL fraction decreased significantly within treatment from day 0 in both the Control and CLA groups (-3.1 and -2.2 mg/dL, respectively), as did the HDLIII fraction (-8.3 and -7.8 mg/dL, respectively); the other fractions did not change significantly from day 0. Though the HDLIII fraction changed from day 0 to day 56 within the treatments, total HDL concentrations (HDLII + HDLIII) did not. No significant differences were observed in change scores between treatments in any fraction or in TC.

Concentrations of TAG in plasma were consistent throughout the study (Figure 2). No differences between total TAG concentrations between the two treatments were observed at day 0, nor did TAG concentrations change within treatments from day 0. Furthermore, changes in TAG concentrations from the beginning of the study to completion did not differ between treatments.

Body composition

Total body DXA scans were divided into left and right arms and legs, trunk and head. The analyses of the trunk values are shown in Figure 3. Trunk compositions did not
differ significantly at day 0 between treatments, nor did the change scores differ between the two treatments. However, within each treatment there was a significant decrease in trunk fat mass (Control: -1.0 kg; CLA: -0.9 kg), with no significant change in trunk lean mass. Both groups also showed a within treatment decrease in total trunk mass (Control: -1.0 kg; CLA: -1.2 kg). The changes in trunk fat and total mass resulted in a statistically significant 2% decrease in trunk fat mass percentage within each treatment, but this change did not differ between treatments.

In a separate analysis, the values obtained from the left and right legs and arms, as well as the trunk, were summed and analyzed as subtotal body composition values (Figure 4). Similar to the trunk composition, no differences between treatment groups before intervention were noted, nor were there any differences in the change scores between treatments. Also similar to the trunk composition, the subtotal fat mass decreased within each treatment from baseline (Control -1.8 kg; CLA -1.9 kg), there were no within treatment changes in subtotal lean mass, and there was a within treatment decrease in subtotal total mass (Control: -2.3 kg; CLA -1.8 kg). A statistically significant 2% decrease in fat mass was observed in the subtotal composition within each treatment, which did not differ significantly between treatments.

**OGTT**

Qualitatively, mean glucose concentrations across timepoints in both groups appeared to converge to much more similar curves after intervention as compared
with before, though no significant differences were observed between groups in the pre-treatment glucose timecourse data (Figure 5). Only the 30 min timepoint differed significantly within treatment from the beginning of the study to the end. No differences were observed between the change scores of the two treatment groups at each timepoint, nor did the curves differ significantly from pre- to post-treatment. Analysis of the AUCs revealed that the Control subjects averaged a significant increase of 2091 min*mg/dL, which is a 15% increase in the glucose AUC from the beginning of the study to the end; no significant change was observed within the CLA group, nor did the changes in glucose AUC differ between groups.

Glucagon and insulin curves (Figures 6 and 7) both showed no differences between groups on day 0 of the study. Neither curve changed significantly from day 0 within treatment, and the within treatment changes in the curves did not differ significantly between the treatments. Concomitantly, no changes were seen between or within groups when AUCs were calculated for the glucagon and insulin time course data.

Fasting adiponectin concentrations did not differ significantly at day 0 (Figure 8), nor did they differ within or between treatments during the course of the experiment.

Liver necrosis

Fasting AST and ALT activities are shown in Figures 9 and 10. Neither AST nor ALT activities differed between treatment groups at baseline. Activities did not change during the course of the study between or within groups.
**Discussion and Conclusions**

The focus of the present investigation was the comparison of the effects of naturally occurring CLA in beef and dairy products from pasture-fed cattle versus beef and dairy products naturally deficient in CLA from grain-fed cattle. An increase in CLA, however, cannot occur without either increasing total lipid intake or decreasing another fatty acid constituent of the diet. The fatty acid compositions of the administered diets reported in Table 3 indicate that the content of some fatty acids other than CLA differed significantly between the two diets. In particular, the lipid percentages of individual identified saturated and monounsaturated odd chain FAME were higher in the CLA diet than the Control diet. Little research has been done on odd chain fatty acids with regard to health, with relevant literature being dominated by disorders in propionate metabolism. Furthermore, the ratio of identified $\omega_3:\omega_6$ FAME was significantly different (1:9.4 Control; 1:5.8 CLA); particularly, arachidonic acid (C20:4-n6), a precursor of inflammatory prostaglandins and a constituent of endocannabinoids, was lower in the CLA diet and α-linolenic acid (C18:3-n3), an essential fatty acid related to decreased cardiovascular disease, was higher in the CLA diet. It is important to note, though, that the difference in CLA content between the two diets was one of the largest differences observed in fatty acid content, with only the magnitude difference of palmitate and oleate being larger; furthermore, the ~3.5 fold increase in CLA content between the Control and CLA diets was the largest relative change of all measured fatty acids. Therefore, even though confounding fatty acid differences are evident, CLA content remains one of the most striking
differences between the two diets.

The macronutrient compositions of the experimental diets were reasonably close to the target macronutrient composition (Table 2). The deviations from the target composition seen in the administered diets can be largely explained by experimental assumptions. Dry matter intake was divided into lipid, protein, and carbohydrate, of which only lipid and protein were measured. For the sake of approximation, all additional dry matter was assumed to be digestible carbohydrate, and was given the energy value of 4 kcal/g. Thus, carbohydrate and total energy intakes were lower, while protein and lipid intakes were higher, than the analyzed approximations. However, most of the carbohydrates in the diets came from foods common to the two diets; also, consistency in the measurements indicate that the assumptions are viable for comparison purposes within this experiment.

A number of factors may contribute to the variety of responses seen in human research on CLAs. Cell culture work related to breast cancer has shown that CLA inhibits estrogen response elements and partially regulates estrogen activity through receptor dephosphorylation(79), reinforcing the concept of gender differences in response to CLA supplementation. Where possible, the present study will be compared to studies on women. However, much CLA research has been conducted on mixed groups of men and women without separating out the gender effects, and many studies conducted on women focus on post-menopausal or lactating women, leaving few studies that have examined the effects of CLA supplementation on
healthy young females who are not pregnant or lactating.

The correlation of lipoprotein distribution with cardiovascular diseases has been well established (51). In particular, the ApoB containing lipoproteins, VLDL, IDL, and LDL, have been positively correlated with atherosclerosis, whereas HDL is negatively correlated with atherosclerosis, and in particular the HDLII fraction (53). Thus, we measured five lipoprotein fractions: VLDL, IDL, LDL, HDLII, and HDLIII. While no differences were seen between treatment groups, both groups on the study showed a decrease in IDL, which has been strongly implicated in coronary artery disease (52). Though a decrease was seen in the HDLIII fraction, no differences were seen in total HDL or in the HDLII fraction in both groups. It appears, then, that both groups exclusively may have a marginally decreased risk for cardiovascular diseases. In other studies of CLA supplementation on women, no differences were seen in TC, LDL or HDL between CLA and placebo treatments (37)(41)(61), in agreement with the results of the present study.

Circulating concentrations of TAG did not change in the present study, which agrees with several studies conducted in women with mixed CLA isomers from 2.1 to 3.6 g/d and from 6 weeks to 12 weeks in duration (37)(41)(61). Endogenous incorporation of CLA in dairy products supplemented to men did not result in differences in TAG concentrations either (18), indicating a fair amount of consistency in the literature.

The lack of effect of CLA supplementation on body composition shown in the present
study complements a similar absence of effect reported in a well controlled, isocaloric study on healthy females in a metabolic ward(86), as well as in a study of free living adult women(61). The absence of an effect is in stark contrast with other studies in which mixed isomers of CLA were supplemented at about 3.5 g/d to free-living women eating ad-libitum diets(37), nor does the present study support the conclusion that CLA decreases fat mass in a body-region specific manner(40). However, the observed 2% decrease in body fat mass in the CLA group in the present study fits the trend line demonstrated in Gaullier and colleagues' one year supplementation study(87), though the 2% decrease in body fat mass in the Control group does not. While one study reported differences in as little as 6 weeks(37), other studies have shown differences in 6 months(40) to 1 year(87), indicating the potential for CLA to have more of a chronic, rather than acute, effect on body composition, which is supported by a meta-analysis indicating a decrease in fat mass of 24 g/week for each gram of CLA supplemented(42). Such a decrease in fat mass would be too small to detect with significance in the present study.

Several reports have indicated that CLA supplementation results in improved insulin sensitivity or glucose tolerance in females(37)(41), without the corresponding effects in males(46). The present study does not support this trend, with no changes in glucose or insulin concentrations between the Control and CLA groups. Also, adiponectin, which increases hepatic insulin sensitivity(73), did not change between groups either. These results confirm other research that reports no alterations in adiponectin concentrations with(76) or without(88) concomitant changes in insulin
sensitivity. The c9,t11-CLA isomer, which was the most prominent isomer in the present study, upregulates peroxisome proliferator-activated receptor gamma (PPARγ), for which adiponectin is a target gene(70), so adiponectin concentrations would be expected to increase; in addition, adiponectin is inversely correlated with body fat mass(73), so the decrease in body fat mass should have resulted in a within treatment increase in circulating adiponectin concentrations. However, no change in adiponectin concentrations were observed, which coincides with no change in insulin sensitivity.

A report of CLA causing hepatomegaly in animal models(65) led to the desire to measure biomarkers of liver health, in particular AST and ALT. No changes in liver enzyme activity in the blood is an indication that liver integrity did not change during the course of the study within or between treatments, which agrees with the findings of other research(68)(67)(61).

While the results from the present study coincide with some other reports, the discrepancies with other studies can be explained in a number of ways: the present study used endogenously incorporated CLA, which is primarily comprised of the c9,t11-isomer, whereas many other studies use mixed isomer, commercially prepared supplements; the present study was an energy-controlled study, in which all foods were provided, whereas most other studies supply supplements to free living participants; the present study was conducted on healthy, non-pregnant, pre-menopausal, adult female participants, whereas many studies are conducted on
both sexes, males, or pregnant, lactating, or post-menopausal women; the present study was conducted over 8 weeks, whereas other studies have been shorter, or as long as two years. The fact that sex, isomer, source of supplementation, energy intake, and duration of supplementation, among other factors, all may influence the effects of CLA on biomarkers of health indicates the need for more research. Therefore, we can only conclude that consuming diets for 8 weeks that were high in beef and dairy products naturally enriched with CLA from pasture-fed cattle did not result in improvements in selected markers of health in healthy, pre-menopausal women when compared with similar foods from grain-fed cattle; extrapolation to other populations, supplementation methods, or isomer contents would not be prudent.
Figure 1. Cholesterol concentrations in five lipoprotein fractions

Lipoprotein fractions were classified by density and separated by sequential flotation from plasma samples taken on days 0 and 56 from the Control and CLA treatment groups. Cholesterol concentrations were quantified from each lipoprotein fraction; bars represent means. The fractions have the following densities: VLDL < 1.006 < IDL < 1.019 LDL < 1.063 < HDLII < 1.125 < HDLIII < 1.21. No between treatment differences were noted. The significance of within treatment changes from day 0 to day 56 are denoted by: + p=0.05; * p<0.05; ** p<0.01.
Figure 2. Total circulating TAG concentrations

Serum TAG concentrations were quantified on day 0 and day 56 for each treatment (Mean±SEM). No significant differences were noted between or within treatments at p<0.05.
Figure 3. DXA results for trunk compositions

Trunk lean, fat, and total masses for Control and CLA treatment groups at day 0 and day 56 were quantified by DXA (Mean±SEM). No significant differences between treatments were observed. Both treatment groups experienced a significant within treatment decrease in body fat (Control: -1.0 kg; CLA: -0.9 kg) and total (Control: -1.0 kg; CLA: -1.2 kg) masses. Within treatment differences are denoted by * (p<0.05).
Subtotal (full body excluding the head) lean, fat, and total masses for Control and CLA treatment groups at day 0 and day 56 were quantified by DXA (Mean±SEM). No significant differences between treatments were observed. Both treatment groups experienced a within treatment significant decrease in body fat (Control: -1.8 kg; CLA: -1.9 kg) and total (Control: -2.3 kg; CLA: -1.8 kg) masses. Within treatment differences are denoted by * (p<0.05).
Figure 5. Glucose concentrations during OGTT

Glucose concentrations were measured by colorimetric enzymatic assay from samples obtained before administering a 75g glucose drink (time=0) and every half hour after the drink was consumed for 180 min on days 0 and 56 for both treatment groups (Mean±SEM). Points are connected by a smoothed spline. No differences were seen between treatments. The AUC of the Control glucose concentration curve increased significantly from day 0 to day 56 by approximately 15%. Significant within treatment timepoint changes from day 0 to 56 were only observed at 30 min (* designates p<0.05).
Glucagon concentrations were measured by radioimmunoassay from samples obtained before administering a 75g glucose drink (time=0) and every half hour after the drink was consumed for 180 min on days 0 and 56 for both treatment groups (Mean±SEM). Points are connected by a smoothed spline. No significant differences were observed between or within treatments at p<0.05.
Figure 7. Insulin concentrations during OGTT

Insulin concentrations were measured by radioimmunoassay from samples obtained before administering a 75g glucose drink (time=0) and every half hour after the drink was consumed for 180 min on days 0 and 56 for both treatment groups (Mean±SEM). Points are connected by a smoothed spline. No significant differences were observed between or within treatments at p<0.05.
Fasting plasma adiponectin concentrations were quantified by radioimmunoassay on days 0 and 56 in Control and CLA treatment subjects (Mean±SEM). No significant differences were observed between or within treatments at a significance of p<0.05.
Serum AST activities were quantified as a surrogate marker of liver integrity on days 0 and 56 from the Control and CLA treatment groups (Mean±SEM). No significant differences were observed within or between groups at a significance of $p<0.05$. 
Serum ALT activities were quantified as a surrogate marker of liver integrity on days 0 and 56 from the Control and CLA treatment groups (Mean±SEM). No significant differences were observed within or between groups at a significance of p<0.05.
Table 1. Screening characteristics of selected female participants who agreed to participate

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Mean± SD</th>
<th>n=18</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Cholesterol (mg/dL)</td>
<td>185.4± 17.8</td>
<td></td>
</tr>
<tr>
<td>HDL-C (mg/dL)</td>
<td>70.3± 14.8</td>
<td></td>
</tr>
<tr>
<td>LDL-C (mg/dL)</td>
<td>94.5± 16.1</td>
<td></td>
</tr>
<tr>
<td>TAG (mg/dL)</td>
<td>102.6± 67.5</td>
<td></td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>24.3± 5.1</td>
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</tr>
<tr>
<td>Age (y)</td>
<td>24.1± 4.7</td>
<td></td>
</tr>
</tbody>
</table>

*n=18*
Table 2. Compositions of experimental diets

<table>
<thead>
<tr>
<th></th>
<th>Target</th>
<th>CLA$^1$</th>
<th>Control$^1$</th>
<th>p-value$^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy (kcal/d)</td>
<td>2000</td>
<td>2129 $\pm$ 34</td>
<td>2203 $\pm$ 30</td>
<td>0.1043</td>
</tr>
<tr>
<td>Lipid (% of energy)</td>
<td>35</td>
<td>30.5 $\pm$ 0.6</td>
<td>32.5 $\pm$ 0.6</td>
<td>0.0310</td>
</tr>
<tr>
<td>Protein (% of energy)</td>
<td>15</td>
<td>13.0 $\pm$ 0.3</td>
<td>12.6 $\pm$ 0.2</td>
<td>0.3581</td>
</tr>
<tr>
<td>Carbohydrate (% of energy)</td>
<td>50</td>
<td>56.5 $\pm$ 0.7</td>
<td>54.9 $\pm$ 0.7</td>
<td>0.1133</td>
</tr>
<tr>
<td>Cholesterol (mg/d)</td>
<td>300</td>
<td>270 $\pm$ 23</td>
<td>303 $\pm$ 24</td>
<td>0.3302</td>
</tr>
</tbody>
</table>

$^1$ x $\pm$ SEM  

$^2$ CLA vs Control
Table 3. Fatty acid compositions of experimental diets

<table>
<thead>
<tr>
<th>Category</th>
<th>Fatty Acid</th>
<th>CLA †</th>
<th>Control †</th>
<th>p-value $^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>SFA</td>
<td>Total</td>
<td>52.97 ± 0.66</td>
<td>53.56 ± 0.60</td>
<td>0.5085</td>
</tr>
<tr>
<td>4:0</td>
<td>0.51 ± 0.04</td>
<td>0.47 ± 0.04</td>
<td>0.5316</td>
<td></td>
</tr>
<tr>
<td>6:0</td>
<td>0.93 ± 0.05</td>
<td>0.88 ± 0.04</td>
<td>0.4260</td>
<td></td>
</tr>
<tr>
<td>8:0</td>
<td>0.68 ± 0.03</td>
<td>0.65 ± 0.03</td>
<td>0.4267</td>
<td></td>
</tr>
<tr>
<td>10:0</td>
<td>1.63 ± 0.04</td>
<td>1.58 ± 0.04</td>
<td>0.4273</td>
<td></td>
</tr>
<tr>
<td>11:0</td>
<td>0.19 ± 0.00</td>
<td>0.16 ± 0.01</td>
<td>0.0003</td>
<td></td>
</tr>
<tr>
<td>12:0</td>
<td>1.89 ± 0.04</td>
<td>1.87 ± 0.04</td>
<td>0.7410</td>
<td></td>
</tr>
<tr>
<td>13:0</td>
<td>0.10 ± 0.00</td>
<td>0.10 ± 0.00</td>
<td>0.9258</td>
<td></td>
</tr>
<tr>
<td>14:0</td>
<td>7.77 ± 0.18</td>
<td>7.34 ± 0.14</td>
<td>0.0687</td>
<td></td>
</tr>
<tr>
<td>15:0</td>
<td>1.03 ± 0.03</td>
<td>0.74 ± 0.01</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>16:0</td>
<td>27.10 ± 0.34</td>
<td>28.54 ± 0.30</td>
<td>0.0022</td>
<td></td>
</tr>
<tr>
<td>17:0</td>
<td>0.65 ± 0.01</td>
<td>0.59 ± 0.01</td>
<td>0.0001</td>
<td></td>
</tr>
<tr>
<td>18:0</td>
<td>10.28 ± 0.09</td>
<td>10.46 ± 0.11</td>
<td>0.1825</td>
<td></td>
</tr>
<tr>
<td>20:0</td>
<td>0.17 ± 0.00</td>
<td>0.15 ± 0.01</td>
<td>0.0025</td>
<td></td>
</tr>
<tr>
<td>24:0</td>
<td>0.05 ± 0.01</td>
<td>0.02 ± 0.01</td>
<td>0.0152</td>
<td></td>
</tr>
<tr>
<td>MUFA</td>
<td>Total</td>
<td>32.17 ± 0.21</td>
<td>32.77 ± 0.22</td>
<td>0.0549</td>
</tr>
<tr>
<td>13:1</td>
<td>0.11 ± 0.01</td>
<td>0.05 ± 0.01</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>14:1</td>
<td>1.45 ± 0.03</td>
<td>1.14 ± 0.02</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>15:1</td>
<td>0.21 ± 0.02</td>
<td>0.14 ± 0.01</td>
<td>0.0008</td>
<td></td>
</tr>
<tr>
<td>16:1</td>
<td>2.62 ± 0.06</td>
<td>2.57 ± 0.04</td>
<td>0.4261</td>
<td></td>
</tr>
<tr>
<td>17:1</td>
<td>0.27 ± 0.00</td>
<td>0.28 ± 0.00</td>
<td>0.0099</td>
<td></td>
</tr>
<tr>
<td>18:1</td>
<td>27.47 ± 0.17</td>
<td>28.48 ± 0.18</td>
<td>0.0001</td>
<td></td>
</tr>
<tr>
<td>20:1</td>
<td>0.05 ± 0.02</td>
<td>0.11 ± 0.02</td>
<td>0.0061</td>
<td></td>
</tr>
<tr>
<td>24:1</td>
<td>Tr</td>
<td>ND</td>
<td>0.3215</td>
<td></td>
</tr>
<tr>
<td>PUFA</td>
<td>Total</td>
<td>15.02 ± 0.79</td>
<td>13.83 ± 0.74</td>
<td>0.2763</td>
</tr>
<tr>
<td>18:2ω6</td>
<td>11.65 ± 0.74</td>
<td>11.87 ± 0.68</td>
<td>0.8728</td>
<td></td>
</tr>
<tr>
<td>18:3ω6</td>
<td>Tr</td>
<td>Tr</td>
<td>0.3566</td>
<td></td>
</tr>
<tr>
<td>20:2ω6</td>
<td>Tr</td>
<td>Tr</td>
<td>0.5027</td>
<td></td>
</tr>
<tr>
<td>20:3ω6</td>
<td>0.16 ± 0.02</td>
<td>0.17 ± 0.02</td>
<td>0.9043</td>
<td></td>
</tr>
<tr>
<td>20:4ω6</td>
<td>0.09 ± 0.01</td>
<td>0.14 ± 0.01</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>18:3ω3</td>
<td>1.90 ± 0.09</td>
<td>1.29 ± 0.09</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>20:3ω3</td>
<td>Tr</td>
<td>ND</td>
<td>0.3215</td>
<td></td>
</tr>
<tr>
<td>20:5ω3</td>
<td>0.04 ± 0.00</td>
<td>ND</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>22:6ω3</td>
<td>Tr</td>
<td>Tr</td>
<td>0.9031</td>
<td></td>
</tr>
<tr>
<td>CLA</td>
<td>Total</td>
<td>1.17 ± 0.04</td>
<td>0.35 ± 0.03</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>CLA c9t11</td>
<td>1.12 ± 0.04</td>
<td>0.35 ± 0.03</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>CLA (other)</td>
<td>0.05 ± 0.01</td>
<td>Tr</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>ω6:ω3</td>
<td>5.78 ± 0.27</td>
<td>9.39 ± 0.45</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
</tbody>
</table>

$^1 \pm$ SEM as g/100g identified FAME $^2$ CLA vs Control
Chapter IV: General Conclusions

Discussion

The study presented in Chapter III does not provide an optimistic view of products from pasture-fed as compared with grain-fed cattle. While the narrow focus of the experiment was to investigate the effects of CLA on markers of health, the other differences in fatty acid contents in the administered diets cannot be ignored, and thus this discussion will try to expand on the characteristics of the presented study in terms of the larger picture of diet, health, and agriculture.

The discussion presented in Chapter III detailed a number of potential shortcomings of the study, including the duration of the study, the differences in fatty acid compositions besides CLA, and the small difference in macronutrient composition. The relatively short duration of the presented study limits the conclusions that can be drawn. As mentioned, there is a potential for more chronic effects of CLA on health, and 8 weeks may not be long enough to elucidate these effects. On the other hand, the improvements in the fatty acid profile of the CLA diet, such as the improved ω3:ω6 fatty acid ratio and decreases in oleic and palmitic acids, should have had synergistic effects with CLA if CLA indeed improves markers of health. However, the high CLA diet did not result in any measurable improvements in health, in spite of the other improvements to the fatty acid profiles. Another consideration is that the modest discrepancy between the macronutrient compositions of the two diets could
have been enough to alter the responses of the participants such that the CLA diet was ineffective. This is not likely, however, because the carbohydrate, protein, and total energy intake approximations did not differ between the two diets, indicating that the difference in lipid content did not result in lower energy intake, which further indicates that the difference in calories from lipids was divided among the other two macronutrients, effectively mitigating any direct substitution of one macronutrient for another. In addition, the composition of the CLA diet could almost be seen as biased to produce beneficial results: lower lipid content, a trend toward a decreased total energy intake with \( p \sim 0.1 \), as well as lower, though not statistically significant, cholesterol consumption, in addition to the increased content of CLA and overall improved fatty acid profile. Yet, no positive effects of CLA in this natural matrix were seen.

Whigam and colleagues have argued that many of the studies reporting no effects of CLA on body composition and other factors is the result of insufficient power\(^\text{(42)}\). In their meta-analysis, they indicate that a 12 week study would require 44 subjects on a CLA supplement and another 44 on a placebo to detect a significant decrease in body fat mass according to their regression equation. However, their meta-analysis was based primarily on \textit{ad-libitum}, pill-supplemented CLA research, which inherently results in a greater variability in responses and, further, includes other confounding factors, such as the uncontrolled energy intake of participants. While \textit{ad-libitum} studies are important for the clinical application of any treatment or supplement, the efficacy of CLA versus a placebo on body composition would almost certainly
depend on energy intake, food choices, food matrix, and a host of other factors uncontrolled in supplementation to free-living participants. In fact, the only study to date to be conducted in a metabolic ward in an attempt to control all confounding variables reports that CLA has no effects on women(86), which corresponds with the conclusions of the study presented in Chapter III. Thus, the clinical and experimental effects of CLA are likely vastly different.

Expanding beyond just the CLA content of the diets, advocates of pasturing cattle cite a number of studies that show improved fatty acid profiles of products from pasture-fed cattle(17)(16); the alleged improvement is based on other studies that indicate fatty acid saturation(89), ω3:ω6 ratios(90), CLA(42), and other fatty acids alter health. The conclusion that products from pasture-fed cattle improve health because of improved fatty acid profiles is thus at least once removed from the scientific evidence. In Chapter III, for the first time evidence was presented indicating consuming beef and dairy products from pasture-fed cattle does not result in improvements in select measures of health over beef and dairy products from grain-fed cattle. However, the lack of evidence of an effect was in a short term study, in energy-controlled dietary intake, and specifically in a group of young, healthy women. Thus, other research is necessary to more accurately determine the effects of CLA and products from pasture-fed cattle on human health.

**Future Research**

The conclusions of the study presented in Chapter III are limited in their scope. To
expand the scope of conclusions, other populations must be examined. In particular, the same study conducted on males, post-menopausal women, and individuals with relevant disorders such as diabetes and familial hypercholesterolemia, would be important in expanding the characterization of beef and dairy products naturally enriched in CLA in a controlled and restricted feeding setting.

Tricon and colleagues have investigated the effects of products from dairy cattle forced to produce higher concentrations of CLA by supplementing the cattle with oils(18). They claimed that the dairy products were simply enriched with c9,t11, but other research has indicated that subjecting the rumen to those conditions results in rumen dysfunction, and thus increased production of the t10,c12 isomer of CLA(12). Therefore, a study in which individuals were asked to substitute products of pasture-fed origin for their usual consumptions of conventional products in an ad-libitum manner would not only be a more natural way to determine the effects of higher concentrations of CLA in ruminant products in normal consumption patterns, but may also decrease the amount of the t10,c12 isomer of CLA in the products. The exclusion of t10,c12 CLA is of importance because of the potential for isomer-dependent effects of CLA on health(91). Again, these studies would have to be conducted on a variety of populations because of the evidence for the correlation of sex with the effects of CLA(41).

By conducting these two types of studies (that is, the controlled, isocaloric studies versus the ad-libitum supplementation studies), a wider array of conclusions may be
drawn. For instance, the effects of CLA supplementation of products from pasture-fed cattle could be analyzed to determine if sex effects the response to CLA. Also, determining the difference in controlled intake versus *ad-libitum* intake of the same products would provide insight into the mechanisms of action for the purported health effects of CLA; as an example, if differences exist between studies that supplement free-living humans and studies that control food intake, CLA may effect total energy consumption rather than effecting metabolism.

**Conclusions**

CLA research has yet to adequately define the variables that determine whether or not consumption of CLA results in improved or impaired biomarkers of health. Isomeric, sex, body compositional, supplementation method, and other effects may or may not play a role in the effects of CLA on health. Even less well defined than the effects CLA has on health are the effects that products from pasture-fed animals have on health. With the burgeoning pasture-fed industry, it is imperative to determine the human health effects, or lack there of, that these products possess. Thus, the conclusion of this thesis can only be that CLA does not uniformly effect various populations, and much more research must be conducted.
Appendix A: Acute effects of sucralose

Introduction

Empirical observations in Iowa State University’s Human Metabolic Unit (HMU) indicate that individuals on full feeding studies in which consumption patterns are entirely dictated by the study quickly become resentful of the lack of options for food, even when the menus are relatively varied. Thus, the longer the study, the more unrest, which is more likely to lead to non-compliance. Some of the unrest appears to be the result of seemingly stagnant eating patterns, while a larger portion appears to be the result of control; that is, the participants begin to resent not being able to select their own foods and beverages. However, to provide participants with options introduces unwanted variability, especially if the options are caloric in nature. This added variability must be weighed against the potential for non-compliance in determining if and what options should be made available to participants.

Much anecdotal evidence exists indicating that consumption of artificial sweeteners increases the desire to consume carbohydrates, increases hunger, causes exhaustion, impairs feelings of wellness, and causes a number of other maladies, with little scientific evidence to support these claims when sweeteners are consumed as directed. While it is not disputed that some individuals may have a sensitivity to these artificial sweeteners(92)(93), there is a lack of scientific evidence that these problems occur on a large scale(94). Further, the most popular research used to
combat the use of artificial sweeteners is either epidemiological or conducted in animal models, of which neither can make mechanistic or causal conclusions for the average human population. In a recent animal study, researchers at Purdue indicated that artificial sweeteners may alter the body's ability to determine the caloric values of food on the basis of taste alone(95). However, the study was conducted in rats using saccharin as the artificial sweetener. Rats prefer saccharin over sugar, and also prefer saccharin over sucralose, another artificial sweetener(96)(97), therefore making the conclusions of the Purdue research highly speculative. Further, a study in which groups of humans were supplemented with a mix of acesulfame K, aspartame, cyclamate, and saccharin, versus sucrose for 10 weeks resulted in the artificial sweetener group not altering food intake, whereas the sucrose supplement resulted in weight and body fat mass gain(98).

Of the most popular artificial sweeteners on the market today, sucralose is generally attributed the most promising record. Sucralose is stable at most temperatures and pHs, unlike its largest competitor aspartame, which allows for sucralose to be reliably used as a sweetening agent in acidic soft drinks, hot beverages such as tea and coffee, on breakfast cereals, and in baked goods(99). These characteristics make it an ideal choice for controlled calorie studies to provide variety to the participants. Aspartame, another popular artificial sweetener, is less stable, especially at elevated temperatures, making it a more limited choice. Further, anecdotal accounts from participants at the HMU gave the impression that aspartame carries more of a negative reputation among participants, which relates
well to controlled research (100).

Fasting glucose in type II diabetics over a 13 week period did not change in subjects supplemented with sucralose versus a control (101), which encourages its use in studies that include glucose measurements. However, to the best of our knowledge, no evidence to date exists on the effects of an acute oral dose of sucralose on glucose homeostasis, especially in normal humans. Further, because of the epidemiological and animal study data implicating artificial sweeteners as altering eating patterns, it is important to test the effects of sucralose on hunger: if the artificial sweetener makes a participant more or less hungry, compliance could be compromised. An ideal artificial sweetener for use in calorie-controlled studies would have no effects on energy homeostasis, on qualitative assessments of well-being, nor on hunger hormones. Thus, the present study aims to determine the effects of sucralose in the form of Splenda® (McNeil Nutrionals; Ft. Washington, PA) on glucose homeostasis and hunger in normal young females against water, sucrose, and sucrose combined with sucralose. Further, the effects of consuming these beverages prior to consumption of a standardized breakfast will be determined.

**Materials and Methods**

**Study design**

The study was designed as a complete factorial; the four treatments were 12 oz of water (Control), 50 g sucrose dissolved in 12 oz of water (Sucrose), 6 g of Splenda
dissolved in 12 oz of water (Splenda), and 50 g of sucrose plus 6 g of Splenda dissolved in 12 oz of water (Both). Treatments were based loosely on a typical single serving of soft drink. McNeil Nutritionals indicated that granular Splenda contains 6-8% sucralose by weight, and sucralose is 500-750 times sweeter than sucrose (100). Based on these assumptions, a range of Splenda drinks were designed, and an ad hoc blind taste panel was used to determine what Splenda content would approximate 50 g of sucrose (data not shown). The results of the taste panel indicated that the Splenda and Sucrose treatments, as defined above, were similar in sweetness, Control was much less sweet, and Both was much sweeter.

Participants were asked to not eat or drink anything except for water after 9 PM the night before the study. Participants arrived at the HMU at 7 AM. A Visual Analogue Scale (VAS) test was administered assessing hunger, tiredness, gastrointestinal well-being, and overall well-being. An intravenous catheter was inserted into each participant’s arm by a registered trained nurse and a fasting blood sample was drawn. Participants were then given a treatment beverage to consume within 5 min of administration. At 30 and 60 min after finishing the beverage, the participants were administered another VAS and another blood sample was drawn immediately thereafter.

At 60 min, a breakfast was administered consisting of scrambled eggs with cheese, orange juice, and buttered whole wheat toast. The breakfast was designed using
Nutritionist Pro (version 2.3, First DataBank, Inc.; San Bruno, CA) to contain 500 kcal, of which 15.7% were protein, 46.6% were carbohydrate, and 37.7% were lipid, which approximates the average composition consumed by females age 20-40 y in the United States(81). The participants were asked to consume the breakfast in 15 min or less. At 30, 60, 90, and 120 min after finishing consumption of the breakfast, another VAS was administered and another blood sample was taken. The catheter was then removed, concluding the participation for that day. The same protocols were repeated to administer each treatment to each participant on each of four separate days of participation, resulting in a four arm cross over design.

**Human Participants**

Research was approved by the Iowa State University Institutional Review Board. All individuals who were screened signed an informed consent document.

Women between the ages of 20 and 27, with a body mass index between 19 and 30, not pregnant or nursing, non-smoking, non- to moderate-drinkers, non-diabetic, who considered themselves in good health, were invited to be screened for participation. Screening included a measurement of height, weight, and a medical history questionnaire. The eight women who were closest in age and BMI were asked to participate. Participants had a mean BMI of $22.16 \pm 1.71 \text{ kg/m}^2$ and a mean age of $21.75 \pm 2.25 \text{ y (means \pm SD)}$.

One participant had an adverse event to the insertion of the IV and was unable to
complete one of the treatments; treatment sequence was thus rearranged to complete the Control, Sucrose, and Sucralose treatments. Other missing data points were the result of IV difficulties, a participant being unexpectedly called away during the last timepoint, or from sample loss related to equipment error. Of 224 samples expected to be collected (8 participants x 4 days x 7 timepoints), 209 were collected successfully. The missing data points were found to be unrelated to any one treatment.

**VAS**

A line 100 mm in length was placed neutrally between two extremes in an effort to quantify subject feelings. Phrasing was intended to represent equally intense extremes at each end; further, phrases containing similar numbers of characters were constructed in an attempt to reduce anchoring based on the visual size of the phrase. Participants were asked to place a mark on the line between the two extremes that represented their feeling at that time. VAS scores were quantified by measuring from the left end of the mark to the line drawn by the participant to the nearest mm.

**Blood analysis**

Blood samples were kept on ice until plasma was separated by centrifugation at 3000 x g for 15 min at 4 °C. An aliquot of plasma was treated with 10 µl phenylmethylsulfonyl fluoride (PMSF, Sigma; St. Louis, MO) in methanol and 50 µl of 1 N hydrochloric acid for every 1 mL of plasma prior to storing in a plastic microfuge
tube for analysis of ghrelin active. Another aliquot was treated with 50 µl of 5500 KIU/mL aprotinin (Sigma) and stored in a glass tube for analysis of glucagon.

Unaltered aliquots of plasma were placed in plastic microfuge tubes for analysis of triacylglycerols (TAG), glucose, and insulin. Samples were stored at -20 ºC until analyzed. Glucose and TAG were assayed by colorimetric enzymatic assay (Kits G7521 and T7532, respectively, Pointe Scientific; Canton, MI). Ghrelin active, insulin, and glucagon concentrations were assayed by radioimmunoassay (GHRA-88HK, HI-11K and GL-32K, respectively, Linco Research; St. Charles, MO).

Statistics

The Mixed procedure of SAS (version 9.1, SAS Institute Inc.; Cary, NC) was used to account for missing data. Day of participation, subject, treatment drink, timepoint, and treatment sequence were used as classification variables, whereas treatment drink, timepoint, sequence of treatment, day, and the interaction of treatment and timepoint were fixed effects. Subject-within-sequence was used as a random effect. No significant differences existed among treatment sequences so response variables within timepoint were averaged by least squares means for each treatment across subjects for graphical representations. Individual timepoints were compared across treatments and against timepoint 0 (baseline) within treatment. Statistical significance was considered at p<0.05.

Results

Blood analytes
Glucose metabolism, as measured by glucose (Figure 11), insulin (Figure 12), and glucagon (Figure 13) concentrations, reflected the relative metabolic inertness of Splenda. Between the treatment drinks and breakfast, the Splenda treatment resulted in glucose concentrations slightly higher than the Control treatment but not different from baseline. While Sucrose resulted in significantly elevated glucose concentrations as compared with both baseline and Control, the combination treatment resulted in glucose concentrations similar to Splenda alone. As expected, after the breakfast was consumed, glucose concentrations surged in the Control and Splenda groups, which corresponds to the lower insulin concentrations prior to consumption of breakfast; that is, the fact that insulin was not stimulated by the beverage means that glucose clearance was not already stimulated, unlike the Sucrose and Both groups. However, within an hour after breakfast consumption, the glucose concentrations once again converged on a similar value, as had happened prior to breakfast across all groups. Ninety minutes after breakfast, the glucose concentrations were significantly different between the Sucrose and Splenda groups, even though the Splenda glucose concentrations did not differ from baseline or Control. All concentrations once again converged to a similar value after 120 min with the exception of the Both group.

Insulin concentrations increased significantly in the Both and Sucrose treatments above baseline and as compared with the Control after consumption of the beverage, as expected from the glucose content. The small but significant increase in insulin concentrations in the Splenda group as compared with the control 30 min
after the treatment drink was consumed (but not from baseline) may likely be caused by the maltodextrin used in Splenda to emulsify sucralose, though other explanations, such as a cephalo-pancreatic response, cannot be ruled out by these data. After consuming breakfast, the insulin concentrations of all groups increased above baseline, not differing from one another. No differences between treatments within timepoints were observed after the consumption of the breakfast.

Unlike glucose and insulin, glucagon concentrations do not present any clear differences among treatments. At 30 min after consuming the treatment, glucagon concentrations in the Splenda group were higher than the Control, but not different from baseline or any other treatments; at 60 min, glucagon concentrations from the Sucrose-group were significantly lower than the Control, though not different from baseline or any other treatment. Though the two differences exist at 30 and 60 min, no samples were different from baseline, indicating no great changes in concentrations. After consumption of breakfast, glucagon concentrations increased until they were greater than baseline for each treatment after 90 min; at 60 min after breakfast, those that consumed the Both treatment had glucagon concentrations greater than the Control and baseline, but not different from any other treatment.

TAG concentrations did not vary among treatments before breakfast (Figure 14). The only change before breakfast was the decrease in TAG concentrations below baseline within the Both group 60 min after drink consumption. The Both group remained lower than baseline until 90 min after breakfast, at which point
concentrations did not differ from baseline through 120 min. Also at 90 min after breakfast, the Sucrose group had lower TAG concentrations as compared with the Control group, though no treatments differed from baseline, nor did the Sucrose group differ from the other two treatments at that timepoint.

Ghrelin active concentrations decreased below baseline upon consumption of Both and Sucrose drinks, and concentrations were also significantly lower than those who consumed Splenda and Control drinks (Figure 15). This reflects the lack of effect of Splenda and the Control on the stimulation of the active form of ghrelin. After consumption of breakfast, the ghrelin active concentrations of the Splenda and Control groups declined in a manner similar to the decline seen in the Sucrose and Both groups upon consumption of the treatment drink. At 30 min, the Splenda group had higher ghrelin active than did the Sucrose or Both groups, but not different from the Control; after 60 min, the concentrations among groups converged through the end of the timecourse.

VAS
Feelings of gastrointestinal well-being (Figure 16) were similar throughout the entire timecourse among all treatments. Before consuming breakfast, no differences were noted against baseline nor among treatments. After breakfast, the stomach feeling generally improved, with all treatments increasing above baseline through 90 min, and the Control and Sucrose groups remaining improved through 120 min.
The measurement of hunger resulted in a difference among treatments at baseline (Figure 17). This difference, however, dissipated through 30 and 60 min after consuming the treatment drink. The Splenda and Control groups were significantly more hungry from baseline at 60 min, which corresponds with the ghrelin active data, and which is expected with no caloric consumption against the Sucrose and Both groups. After consuming breakfast, all participants remained less hungry than baseline through the end of the timecourse. Those consuming Sucrose remained fuller as compared with those consuming Both, but neither treatment was different from Control or Splenda.

Participants did not experience any immediate changes in feelings of consciousness as measured by how sleepy or awake they felt (Figure 18). Thirty min after participants consumed breakfast, those who consumed Splenda or Both felt more awake than did the individuals who consumed Sucrose, and the Splenda group felt more awake than before consuming the beverage. However, Splenda, Sucrose, nor Both deviated from the Control. At 60 min, participants converged on a similar feeling of alertness, though 90 and 120 min after breakfast the groups returned to the relative states they were in at 30 min after breakfast with the addition of Control being elevated above baseline.

Overall, participants’ perceptions of general well-being did not change much throughout the study (Figure 19). Those on the Sucrose treatment generally felt worse than those on the Splenda treatment, but when the significant difference at
baseline is accounted for, much of this effect is negated. However, besides baseline, only at 90 min did those on Sucrose feel significantly worse than those on the Control. Throughout the timecourse, those on the Sucrose and Splenda treatments improved their perception of overall well-being against baseline, though, again, the more unpleasant feelings at baseline of Sucrose makes this effect questionable for the Sucrose group. Those on Control or Both did not change throughout the timecourse against baseline.

Discussion

The present study was designed to determine if consumption of an approximate soft drink’s equivalent of Splenda would result in any noticeable metabolic changes or qualitative changes in feelings of well-being against an equivalent of water, sucrose, or a combination of sucrose and Splenda. Thus, the water acted as a negative control, sucrose as a positive control, and the combination to determine if any synergistic effects between sugar and sucralose exist. By administering the treatment drinks in a fasted state, the first hour of the timecourse resembled an oral glucose tolerance test; additionally, subsequent feeding of a breakfast resembles an average pre-meal beverage, such as a mid-afternoon soft drink prior to dinner.

Besides presenting a different aspect of glucose metabolism data within this study, the first data on the effects of artificial sweeteners on ghrelin in humans is presented and only the second to investigate the effects of an artificial sweetener on ghrelin at all(97). The other study is, in essence, the opposite of the presented study: instead
of supplementing the sweetener and measuring hormones, orexigenic neuropeptides were administered to rats and consumption patterns of a saccharin-water solution was monitored versus only a water solution. Regardless of the study design or the model, a direct relation between ghrelin and saccharin was not found there, nor were ghrelin concentrations and sucralose consumption related here.

Though the present study did not measure ad-libitum food intake of the participants, the responses indicated that participants' hunger was unaffected by Splenda when compared with water, both in terms of ghrelin as a stimulant of hunger and the VAS data indicating fullness. Therefore, no alterations in food intake would be expected. This extrapolation is supported by research on other artificial sweeteners over a longer term(98).

In general, those consuming the Splenda treatment responded in a manner similar to the Control, whereas Sucrose responded more similarly to Both. This cursory assessment indicates that Splenda should be a relatively safe option for controlled energy studies to provide a break from the inherent redundancy of such studies. However, the limitations of this study are that it was conducted in free living individuals with no lifestyle directions except pre-study fasting; the exposures to each treatment and the responses were acute; and the study was limited to young, healthy females. Thus, more research must be conducted to expand the claim that sucralose is safe for use in controlled experiments.
Plasma glucose concentrations were quantified colorimetrically (Mean±SEM). Baseline concentrations (BL) were quantified, followed by administration of a treatment drink. Drink completion time is marked by the 'Drink' dotted line. All treatment groups had increased glucose concentrations compared with the Control at 30 min, but concentrations converged at 60 min. After 60 min, breakfast was served; breakfast completion time is marked by the 'Bfast' dotted line. The Sucrose and Both treatments prevented the postprandial surge in glucose concentrations, whereas the Control and Splenda treatments experienced postprandial surges in glucose; again, concentrations converged after 60 min. Datapoints within a timepoint without common letters differ significantly; * denotes a datapoint differs significantly from baseline; ε indicates all datapoints within a timepoint are different from baseline. p<0.05 is considered significant.
Figure 12. Insulin concentrations

Plasma insulin concentrations were quantified by radioimmunoassay (Mean±SEM). Baseline concentrations (BL) were quantified, followed by administration of a treatment drink. Drink completion time is marked by the 'Drink' dotted line. Sucrose and Both treatments increased concentrations above baseline through 60 min, whereas Splenda and Control treatments did not, though Splenda increased insulin above Control at 30 min. After 60 min, breakfast was served; breakfast completion time is marked by the 'Bfast' dotted line. All treatments converged, and were all above baseline through 60 min after breakfast. Datapoints within a timepoint without common letters differ significantly; * denotes a datapoint differs significantly from baseline; ε indicates all datapoints within a timepoint are different from baseline. p<0.05 is considered significant.
Figure 13. Glucagon concentrations

Plasma glucagon concentrations were quantified by radioimmunoassay (Mean±SEM). Baseline concentrations (BL) were quantified, followed by administration of a treatment drink. Drink completion time is marked by the 'Drink' dotted line. Splenda treatment elevated glucagon concentrations above Control at 30 min, and Sucrose resulted in decreased glucagon as compared with Control at 60 min, though no treatments differed from baseline. After 60 min, breakfast was served; breakfast completion time is marked by the 'Bfast' dotted line. The Both treatment increased glucagon above Control and baseline concentrations at 60 min, but converged with the other treatments above baseline at 90 and 120 min. Datapoints within a timepoint without common letters differ significantly; * denotes a datapoint differs significantly from baseline; ε indicates all datapoints within a timepoint are different from baseline. p<0.05 is considered significant.
Plasma TAG concentrations were quantified colorimetrically (Mean±SEM). Baseline concentrations (BL) were quantified, followed by administration of a treatment drink. Drink completion time is marked by the 'Drink' dotted line. The Both treatment caused a decrease in TAG at 60 min below baseline, but did not differ from other treatments. After 60 min, breakfast was served; breakfast completion time is marked by the 'Bfast' dotted line. The Both treatment remained below baseline through 60 min; only at 90 min did two treatments differ, with Sucrose decreasing below Control. Datapoints within a timepoint without common letters differ significantly; * denotes a datapoint differs significantly from baseline. p<0.05 is considered significant.
Plasma ghrelin active concentrations were quantified by radioimmunoassay (Mean±SEM). Baseline concentrations (BL) were quantified, followed by administration of a treatment drink. Drink completion time is marked by the 'Drink' dotted line. Sucrose and Both treatments decreased below baseline, with only Sucrose decreasing below Control, at 30 min. At 60 min, Splenda and Control differed significantly from Sucrose and Both, but not each other, and vice versa. After 60 min, breakfast was served; breakfast completion time is marked by the 'Bfast' dotted line. Sucrose and Both treatments, and Splenda and Control treatments, remained similar at 30 min, after which all four treatments converged to similar concentrations. Datapoints within a timepoint without common letters differ significantly; * denotes a datapoint differs significantly from baseline. p<0.05 is considered significant.
Figure 16. VAS responses for gastrointestinal well-being

VAS scores to determine how the participants’ GI tracts felt were measured to the nearest millimeter (Mean±SEM). Baseline values (BL) were determined, followed by administration of a treatment drink. Drink completion time is marked by the 'Drink' dotted line. After 60 min, breakfast was served; breakfast completion time is marked by the 'Bfast' dotted line. No treatments differed from each other throughout the timecourse, and only differences from baseline were observed. * denotes a datapoint differs significantly from baseline; ε indicates all datapoints within a timepoint are different from baseline. p<0.05 is considered significant.
Figure 17. VAS responses for hunger

VAS scores to determine hunger were measured to the nearest millimeter (Mean±SEM). At baseline (BL), the Sucrose treatment group felt hungrier than the Control and Splenda groups. After BL, a treatment drink was administered; drink completion time is marked by the 'Drink' dotted line. For the next 60 min, all four treatment groups were not significantly more or less hungry than each other. After 60 min, breakfast was served; breakfast completion time is marked by the 'Bfast' dotted line. All participants became much fuller after consumption of breakfast as compared with baseline; the Sucrose treatment maintained fullness longer as compared with the Both group at 90 and 120 min. Datapoints within a timepoint without common letters differ significantly; * denotes a datapoint differs significantly from baseline; ε indicates all datapoints within a timepoint are different from baseline. p<0.05 is considered significant.
Figure 18. VAS responses for tiredness

VAS scores to determine degree of consciousness were measured to the nearest millimeter (Mean±SEM). Baseline values (BL) were determined, followed by administration of a treatment drink. Drink completion time is marked by the 'Drink' dotted line. After 60 min, breakfast was served; breakfast completion time is marked by the 'Bfast' dotted line. Those on the Sucrose treatment generally reported being sleepier than the Splenda and Both treatments after breakfast, though no treatment groups differed from the Control. Datapoints within a timepoint without common letters differ significantly; * denotes a datapoint differs significantly from baseline; ε indicates all datapoints within a timepoint are different from baseline. p<0.05 is considered significant.
Figure 19. VAS responses for overall well-being

VAS scores to determine overall feelings of wellbeing were measured to the nearest millimeter (Mean±SEM). Baseline values (BL) were determined, followed by administration of a treatment drink. Drink completion time is marked by the 'Drink' dotted line. After 60 min, breakfast was served; breakfast completion time is marked by the 'Bfast' dotted line. Those on the Sucrose treatment generally reported feeling less well than the Splenda treatment throughout the full timecourse; however, when the difference at baseline was taken into consideration, the Sucrose group reverted to being not different from the other treatments. Datapoints within a timepoint without common letters differ significantly; * denotes a datapoint differs significantly from baseline; ε indicates all datapoints within a timepoint are different from baseline. p<0.05 is considered significant.
Appendix B: Menus for the CLA study

Monday

Breakfast
- Pineapple juice 8 oz can
- Cheerios 1 self-serve bowl (19 g)
- Sugar 1 packet (1/10 oz)
- Skim milk 10 oz carton
- Bran muffin 1
- Raisins 1 0.5 oz box

Lunch
Taco
- Flour tortilla 1 (64 g)
- Ground beef 56 g
- Taco seasoning mix 1 tsp
- Cheddar cheese, shredded 42 g
- Tomato, diced 45 g
- Lettuce, shredded ~28 g
- Salsa 2 oz
- Tortilla chips 28 g
- Chocolate chip oatmeal cookies 2

Dinner
Pizza
- 7” Mama Mary’s crust 1
- Butter 5g
- Ground beef 28 g
- Pizza sauce 120 g
- Green pepper 10 g
- Onions 10 g
- Cheddar cheese, shredded 42 g
- Orange 1 (~131 g)
## Tuesday

### Breakfast
- Orange Juice: 8 oz or 250g
- Oatmeal: 35 g dry
- Raisins: 0.5 oz box
- Brown Sugar: 5 g
- Skim milk: 10 oz carton
- Raspberry muffin: 1

### Lunch
- Sloppy Joe
  - Ground beef: 28 g
  - Onion: 10 g
  - Green pepper: 10 g
  - Manwich mix: 20 g
  - Bun: 1
- Corn, frozen: 85 g
- Butter: 10 g
- Dill pickle: 40 g
- Peaches: 4 oz cup
- Grapes: 80 g
- Chocolate chip cookies: 2

### Dinner – set out chopped chives
- Meat loaf
  - Ground beef: 56 g
  - Oatmeal: 15 g
  - Onions, chopped: 10 g
  - Catsup: 1 Tbsp
- Mashed potato: 200 g
- Butter: 25 g
- Pineapple tidbits: 4 oz cup
- Premium vanilla ice cream: 66 g
Wednesday

Breakfast
Juicy Juice 1 box (6.75 oz)
Raisin Bran cereal 1 self-serve bowl (35 g)
Sugar 1 packet (1/10 oz)
Skim milk 10 oz carton
Banana 1 (~118 g)
Blueberry muffin 1

Lunch
Cheeseburger
Small bun 1
Butter 5 g
Ground beef 56 g
Cheddar cheese, sliced 42 g
Tomato, sliced 45 g
Lettuce, leaves ~28g
Tortilla chips 28g
Salsa 2 oz
Chocolate chip oatmeal cookies 2

Dinner
Spaghetti
Ground beef 56 g
Butter 5 g
Spaghetti sauce 112 g
Parmesan cheese 10 g
Spaghetti, cooked 160 g
Pillsbury crusty French roll 1
Butter 15 g
Looseleaf lettuce ~56 g
Italian dressing 15 g
Thursday

Breakfast
Orange juice 8 oz or 250g
Cream of wheat, dry 35 g
Brown sugar 5 g
Skim milk 10 oz carton
Apple cinnamon raisin muffin 1

Lunch
Calico bean casserole
Ground beef 28 g
Red kidney beans 75 g
Baked beans, vegetarian 75 g
Butter beans 75 g
Brown sugar 20 g
Molasses 7.5 g
Mustard, dry 1/8 tsp
Pillsbury crusty sourdough roll 1
Butter 10 g
Grapes 80 g
Peanut butter cookie 1

Dinner
Fajita beef on mashed potato
Ground beef 56 g
Onion, chopped 10 g
Green pepper 10 g
Fajita seasoning 1 tsp
Potato 200 g
Butter 25 g
Carrots 45 g
Looseleaf lettuce 56 g
Ranch dressing 15 g
Orange 1 (~131 g)
Premium vanilla ice cream 66 g
**Friday**

**Breakfast**
- Orange juice: 8 oz or 250g
- Corn Flakes cereal: 35 g
- Sugar: 1 packet (1/10 oz)
- Skim milk: 10 oz carton
- Bran muffin: 1
- Banana: 1 (~118 g)

**Lunch**
- Taco salad
  - Ground beef: 56 g
  - Taco seasoning: ½ tsp
  - Cheddar cheese, shredded: 28 g
  - Tomato, chopped: 85 g
  - Looseleaf lettuce: 100 g
  - Black olives: 15 g
  - Salsa: 2 oz
  - Tortilla chips: 28 g
  - Chocolate chip cookies: 2
  - Apple: 1 (~125 g)

**Dinner**
- Pizza
  - 7” Mama Mary’s Crust: 1
  - Butter: 5 g
  - Ground beef: 28 g
  - Green pepper: 10 g
  - Onions: 10 g
  - Pizza sauce: 120 g
  - Cheddar cheese: 42 g
  - Orange: 1 (~131 g)
  - Premium vanilla ice cream: 66 g
Saturday

Breakfast
Pineapple orange juice  8 oz can
Raisin bran cereal  1 self-serve bowl (35 g)
Sugar  1 packet (1/10 oz)
Skim milk  10 oz carton
Blueberry muffin  1

Lunch
Cheesy beef rice
Ground beef  28g
Onion  10g
Green pepper  10g
Cooked rice  65g
Butter  5g
Peas, cooked and drained  40g
Cheddar cheese, shredded  28 g
Bread stick  1
Butter  10 g
Carrots  30 g
Celery  30 g
Kiwi  1
Fruit cup  1

Dinner
Spaghetti
Ground beef  56 g
Butter  5 g
Spaghetti sauce  112 g
Parmesan cheese  10 g
Spaghetti, cooked  160 g
Pillsbury crusty sourdough roll  35 g
Butter  15 g
Looseleaf lettuce  ~56g
Italian dressing  15 g
Peanut butter cookies  2
Sunday

Breakfast
- Orange juice 8 oz carton
- Cheerios 1 self-serve bowl (19 g)
- Banana 1 (~118 g)
- Sugar 1 packet (1/10 oz)
- Skim milk 10 oz carton
- Apple cinnamon raisin muffin 1

Lunch
- Teriyaki noodle casserole
  - Ground beef 56 g
  - Onions 10 g
  - Butter 10 g
  - Teriyaki sauce 2 Tbsp
  - Egg noodles, cooked 160 g
- Pillsbury crusty sourdough roll 1
- Butter 15 g
- Carrots 30 g
- Apple 1 (~125 g)

Dinner
- Lasagna
  - Ground beef 28 g
  - Spaghetti sauce 120 g
  - Cottage cheese 56 g
  - Cheddar cheese, shredded 28 g
  - Lasagna noodles, dry 42 g
- Pillsbury wheat roll 35 g
- Butter 15 g
- Green beans 120 g
- Looseleaf lettuce ~56 g
  - Italian dressing 15 g
- Chocolate chip cookie 1
Appendix C: Recipes for the CLA study

Apple Cinnamon Raisin Muffins

140 g Brown Sugar
280 g Flour
11 g Baking Powder
2.5 g Nutmeg
5 g Ground Cinnamon
160 g Apple Juice
100 g Chopped Apple
80 g Raisins
160 g Butter
1 Egg

Preheat oven to 400 °F. Weigh large, empty bowl with rubber spatula.

Stir together brown sugar, flour, baking powder, and nutmeg in pre-weighed bowl. In medium bowl, combine egg, butter, and apple juice. Whisk until blended. In another bowl, toss together apples and cinnamon until evenly coated; stir in raisins. Pour egg mixture over dry ingredients, scraping with the rubber spatula to transfer as much as possible, and fold lightly 3-4 times with the spatula to partially combine. Add apple mixture and distribute evenly using as few strokes as possible.

Weigh bowl, spatula, and batter. Divide weight by 30. Weigh batter into muffin cups and place in muffin pan. Bake in middle of oven for 23-25 min or until tops are golden and spring back when lightly pressed. Let cool in pan 2 min; ease onto wire rack to cool 15-20 min. Package individually.
Blueberry Muffins

150 g White Granulated Sugar
315 g Flour
120 g Butter
11 g Baking Powder
1 Egg
120 g Skim Milk
300 g Blueberries

Preheat oven to 400 °F. Weigh large, empty bowl with rubber spatula.

In pre-weighed bowl, toss together flour, sugar and baking powder. In medium bowl, melt butter in microwave; whisk together egg, milk, and butter. Pour liquid ingredients over dry ingredients; scrape with rubber spatula to transfer as much as possible. Fold lightly 3-4 times with the rubber spatula (mixture should not be perfectly smooth and will be quite dry).

Weigh bowl, spatula, and batter. Divide weight by 27. Weigh batter into muffin cups and place in muffin pan. Add 10g of blueberries (about 5-6 large blueberries) to each muffin. Bake in middle of oven until tops are golden and spring back when lightly pressed (about 35 min). Let cool in pan 2 min; ease onto wire rack to cool for 15-20 min. Package individually.
Bran Muffins

300 g White Granulated Sugar
1 Tbl Baking Soda
½ tsp Salt
170 g All-Bran Cereal
255 g Butter
57 g 100% Bran Cereal
350 g Flour
490 g 1% Buttermilk
1 c Tap Water

Preheat oven to 350 °F. Weigh large, empty bowl with rubber spatula.

Pour boiling water over 100% bran cereal; set aside. Cream butter in pre-weighed bowl; gradually add sugar, flour, buttermilk, salt, baking soda, and all-bran cereal. Add bran/water mixture to other ingredients, scraping with rubber spatula to transfer as much as possible.

Weigh bowl, spatula, and batter; divide batter weight by 50. Weigh batter into muffin cups and place in muffin pan. Bake 15 min or until tops spring back when touched. Cool 2-3 min in pan. Ease onto wire rack and cool 15-20 min. Package individually.
Raspberry Orange Muffins

160 g Orange Juice
1 Egg
130 g White Granulated Sugar
150 g Raspberries
11 g Baking Powder
80 g Butter
280 g Flour

Preheat oven to 400 °F. Weigh large, empty bowl with rubber spatula.

Toss together flour, baking powder, and sugar in pre-weighed bowl. Melt butter in microwave; cool slightly. In medium bowl, combine orange juice, butter, and egg. Whisk until blended. Pour liquid over flour mixture, scraping to transfer as much as possible, and stir lightly to combine. Coarsely chop berries. Add berries and distribute evenly using as few strokes as possible.

Weigh bowl, spatula, and batter. Divide weight of batter by 24. Weigh batter into muffin cup and place in muffin pan. Bake in middle of oven 25 min or until tops are golden and spring back when lightly pressed. Let muffins cool in pans 2 min. Ease onto wire rack to cool for 15-20 min. Package individually.
**Chocolate Chip Cookies**

120 g Butter  
48 g Brown Sugar  
96 g White Granulated Sugar  
1 Egg  
½ tsp Vanilla Extract  
132 g Flour  
½ tsp Salt  
½ tsp Baking Soda  
252 Semi Sweet Chocolate Chips (~128 g)

Preheat oven to 375 °F. Weigh large, empty bowl with mixing utensil.

Cream butter in pre-weighed bowl; gradually add sugars. Beat in egg and vanilla. Combine dry ingredients and add to creamed mixture.

Weigh bowl, utensil, and batter. Divide weight of batter by 36. Place weighed dough on small squares of parchment paper on cookie sheets; add seven chocolate chips to the top of each one. Bake about 10 min. Cool on wire rack. Package individually.
Chocolate Chip Oatmeal Cookies

120 g Butter
110 g Brown Sugar
96 g White Granulated Sugar
1 Egg
½ tsp Vanilla Extract
125 g All Purpose Flour
½ tsp Salt
½ tsp Baking Soda
252 Semi Sweet Chocolate Chips (~128 g)
40 g Oatmeal

Preheat oven to 375 °F. Weigh large, empty bowl with mixing utensil.

Cream butter in pre-weighed bowl with mixing utensil; gradually add sugars. Beat in egg and vanilla. Combine dry ingredients, except for chocolate chips, and add to creamed mixture.

Peanut Butter Cookies

120 g Butter
110 g Brown Sugar
96 g White Granulated Sugar
1 Egg
250 g Peanut Butter
½ tsp Salt
½ tsp Baking Soda
½ tsp Vanilla
145 g Flour

Preheat oven to 375 °F. Weigh large, empty bowl with mixing utensil.

Beat butter until soft in pre-weighed bowl. Gradually add sugars. Beat in egg, peanut butter, salt, baking soda, and vanilla. Add flour; mix well.

Weigh bowl, utensil, and batter. Divide by 60. Form weighed amount of dough into a ball and place on a small square of parchment paper. Place on cookie sheet. Flatten in a grid pattern with a fork. Bake 10 to 12 min. Cool on wire rack. Package individually.
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