ANALYSIS OF THE EFFECT OF CONJUGATED LINOLEIC ACID ON MARKERS OF OXIDATIVE STRESS AND INFLAMMATION IN OBESE INDIVIDUALS

By

Austin G. Phillips

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CERTIFICATE OF APPROVAL

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This is to certify that the Master's thesis of

Austin G. Phillips

has been approved

_____
Philippe Thuillier, PhD

_____
Thomas Becker, MD

_____
Betty Izumi, PhD MPH

_____
Dawn Peters, PhD

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Abbreviations

8-OH-dG – 8 hydroxy 2'-deoxyguanosine

BMI – Body Mass Index

C9T11 – cis-9 trans-11 isomer of conjugated linoleic acid

cDNA – complementary deoxyribonucleic acid

CLA – conjugated linoleic acid

CRP – C-reactive protein

DEXA – dual-energy X-ray absorptiometry

DMSO – dimethyl sulfoxide

DNA – deoxyribonucleic acid

FPG – formamidopyrimidine glycosylase

GCRC – General Clinical Research Center

IL-6 – interleukin-6

LBM – lean body mass

LDLR – low-density lipoprotein receptor

NIH – National Institutes of Health

Nrf2 – nuclear factor erythroid 2-related factor

PPAR – peroxisome proliferator activated receptor

RNA – ribonucleic acid

RT-PCR – Real Time Polymerase Chain Reaction

SGC-7901 – human gastric cancer cell line

SNP – single nucleotide polymorphism

SOD1 – superoxide dismutase 1

T10C12 – trans-10 cis-12 isomer of conjugated linoleic acid

TNF – tumor necrosis factor

Abstract

In recent years, obesity has become a significant worldwide problem, and has been shown to be associated with chronic diseases such as diabetes, hypertension, and cancer. The mechanisms by which obesity is linked to these diseases are not fully understood. Studies have shown that conjugated linoleic acid (CLA) is effective at reducing obesity and preventing cancer in mice, and that it increases lean body mass in humans. Furthermore, research has linked inflammation, oxidative stress, and increased activity of oxidative stress response genes to cancer. In this randomized clinical trial, we examine a possible link between CLA and both oxidative stress and inflammation. I examined forty-eight healthy, obese individuals for twelve weeks to assess the effects of three different doses of CLA (placebo, 3.2 g/day, and 6.4 g/day) on several factors. These factors include DNA damage due to oxidative stress, expression of the oxidative stress response genes Nrf2 and SOD1, serum C-reactive protein (CRP) levels, and serum interleukin-6 (IL-6) levels. I used Comet assays to measure DNA damage, Real-Time Polymerase Chain Reactions to measure Nrf2 expression and SOD1 expression, and clinical laboratory analyses to measure serum CRP levels and serum IL-6 levels. Increases in CLA dosage were significantly associated with increases in CRP levels ($p = 0.007$) and IL-6 levels ($p = 0.02$). Age confounded the association among CLA dose and IL-6 levels, but not CRP levels. In conclusion, we found that supplementation with CLA for twelve weeks increases the levels of CRP in obese, but otherwise healthy individuals, but further

research needs to be conducted to assess the mechanism by which CLA affects inflammation, and subsequently, obesity.

Background

Obesity has become a significant problem in recent years, both in the United States and abroad. At present, 67% of American adults are either overweight or obese.[1,2] Obesity has been linked to a variety of diseases, such as type 2 diabetes, heart disease, joint disease, hypertension, stroke, cancer, and oxidative stress.[3-5] This, in turn, could decrease the average life expectancy and increase medical costs. In 2008, $147 billion were spent on obesity-related medical costs.[6]

Currently, many methods are available for classifying obesity, the quickest and most commonly used of which is body mass index (BMI). BMI is calculated by the formula [weight (kg)/height (m^2)], and anyone with a BMI above 30 is considered obese.[7] Diet and physical activity levels have been shown to influence a person's weight.[7] The American Heart Association recommends that individuals seeking to reduce their body weight restrict their intake of saturated fat, trans fat, and sugar.[8] In addition to these, other factors can raise a person's risk of becoming obese. Previous research has linked both hormones and genetic composition to the propensity of obesity. The hormones leptin and ghrelin help regulate energy metabolism. Certain forms of peroxisome proliferator-activated receptor (PPAR) genes have also been implicated in obesity and physical fitness. PPARδ (PPARD) and PPARγ coactivator 1α (PPARG1CA) have been associated with changes in physical fitness levels.[9]

Compared to healthy individuals, obese individuals have been shown to have elevated levels of oxidative stress. Oxidative stress describes an imbalance of pro-

and anti-oxidants, and causes a buildup of excess reactive oxygen molecules (known as free radicals) that may result in DNA damage in the body.[10] In addition to obesity, oxidative stress has been associated with several other chronic conditions, including type 2 diabetes, and cardiovascular disease. Several genes can be used to assess oxidative stress levels. Among them, superoxide dismutase (SOD1) and transcription factor Nrf2 are involved in the removal of free radicals. In contrast, interleukin-6 (IL-6) and C-reactive protein (CRP) are proinflammatory genes, and thus, elevated levels of any of these are indicators of oxidative stress and inflammation.[11-14]

Steps that individuals take to lose weight and reduce obesity include modifying their diets and consuming commercial supplements. For this reason, one common approach taken in obesity research is to study dietary factors associated with obesity and obesity-related diseases. Conjugated linoleic acid (CLA) is a fatty acid naturally found in food, and has recently been used in weight-loss supplements.[15] CLA has been linked to obesity reduction, cancer prevention, cholesterol reduction, and insulin sensitivity increase.[15] CLA has been shown to be effective in thwarting obesity and preventing cancer in mice.[16] Many isomers of CLA exist, though more is known about the trans-10, cis-12 (t10c12) and cis-9, trans-11 (c9t11) isomers. The c9t11 form has been shown to inhibit SGC-7901 cells, which are implicated in metastasis of gastric cancer.[17] Interestingly, it has been noted that, when together, the effects of these two isoforms may negate each other. For instance, t10c12 CLA prevents the accumulation of triglycerides, while c9t11 CLA promotes its

accumulation.[16] An increase in lipid peroxidation has been associated with the t10c12 isomer, but not the c9t11 isomer.[14]

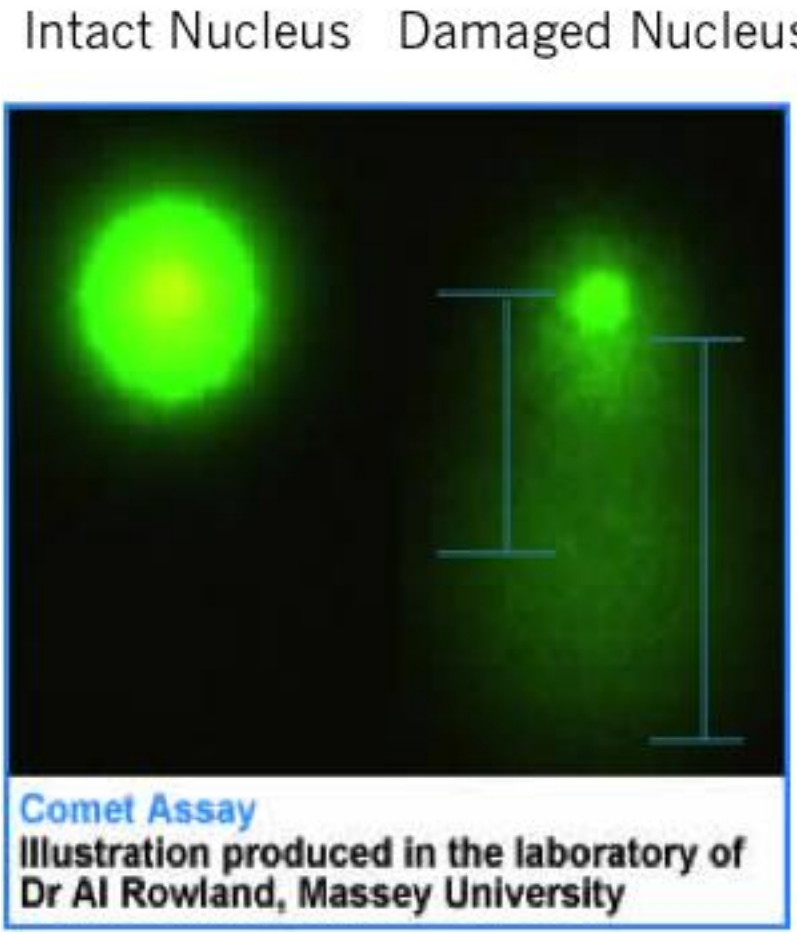
Studies have also illustrated that specific genes moderate the effects of these two isomers. Glucose and lipid metabolism of these isomers are partially mediated by PPAR-gamma.[18] Adiponectin gene expression, which is regulated by PPAR, increases as a result of t10c12 CLA, as does low-density lipoprotein receptor (LDLR) expression. C9t11 CLA has no effect on the expression of either of these transcription factors.

It has been shown that CLA modulates PPAR-gamma by regulating adiponectin gene expression and binding to genes to activate PPAR-gamma.[19] Maggiora's study showed that CLA inhibited cellular growth in several human tumor cell lines. However, these findings have not been shown in vivo.

Previous research in human umbilical endothelial cells has suggested that CLA can decrease the amount of DNA damage.[20] To date, the effect of CLA on DNA damage has not been examined in obese humans.

Several methods are available for assessing DNA damage. Because of reliability and ease, comet assays are a commonly used method to measure DNA damage in individual cells.[21] Using gel electrophoresis, damaged DNA is separated from intact DNA, which creates a tail. A longer tail corresponds to greater DNA damage. The two measurements used to assess the tail are tail moment and tail length. Tail moment describes the distance between the center of the nucleus and the center of the tail, while tail length describes the distance between the edge of the nucleus and the edge of the tail. Figure 1 shows Comet assays for intact and damaged DNA.[22]

Figure 1: Comet Assays of Intact and Damaged DNA Nuclei



Total DNA damage is the summation of oxidative and relative DNA damage. Oxidative DNA damage occurs as a result of 8-hydroxy 2'-deoxyguanosine (8-OH-dG) binding to the double-strand DNA. Assessing its presence requires treating the DNA strand with formamidopyrimidine glycosylase (FPG) at a neutral pH. This treatment breaks the DNA strand where 8-OH-dG is present, and converts these double-strand breaks to single-strand breaks. This process results in smaller fragments that will migrate faster and further during the Comet assay. Figures 2 and 3 describe the process of treating DNA with FPG.

Figure 2: DNA Strand Prior to FPG Treatment. FPG targets the sites where 8-OH-dG is bound to the double-strand DNA to convert these double-strand breaks to single-strand breaks.

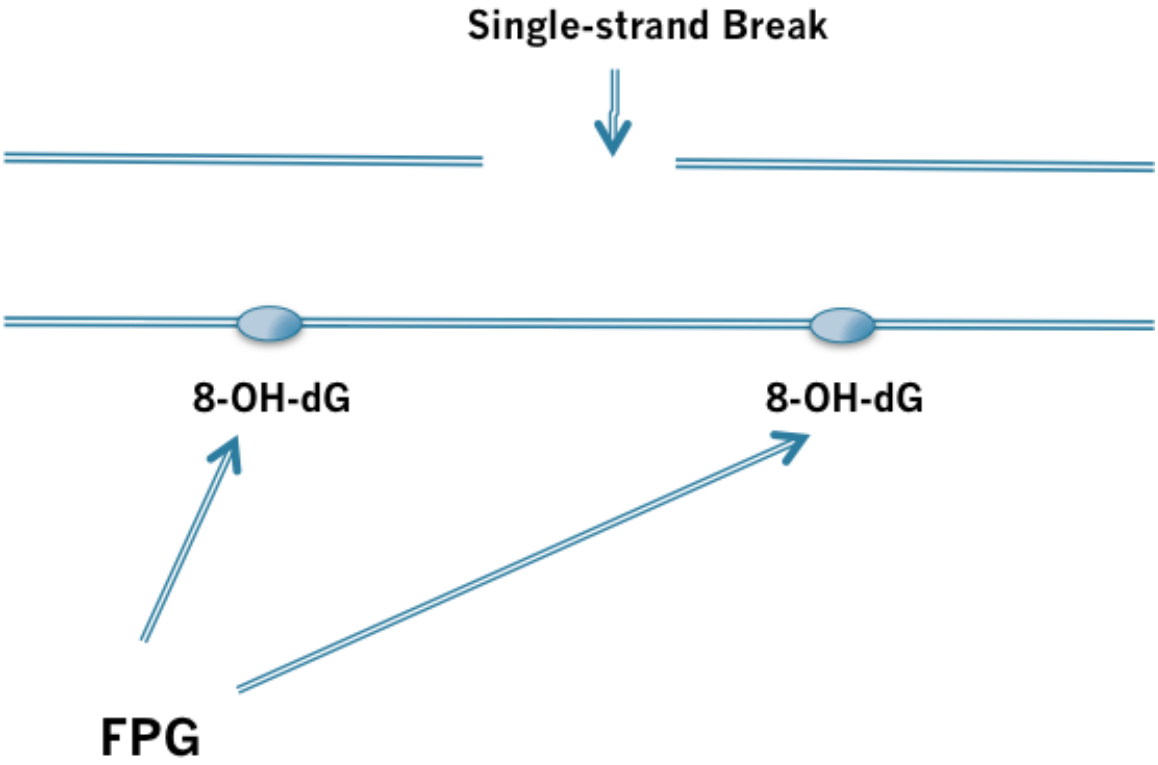
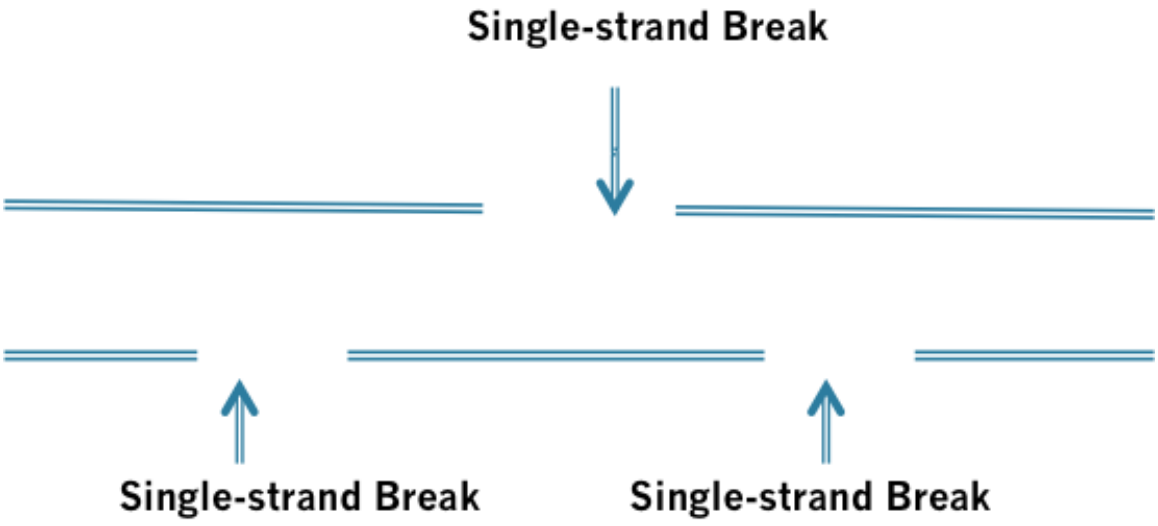


Figure 3: After treatment with FPG, the DNA strand is unwound and the double-strand breaks caused by 8-OH-dG are converted to single-strand fragments. In the Comet assay, these fragments will migrate further away from the DNA nucleus, thus generating a longer tail.



Given CLA's connection to obesity, cancer, and oxidative stress, it is possible that CLA can alter the association among these conditions. We seek to investigate whether CLA can reduce the amount of DNA damage due to oxidative stress in obese adults. In addition, we plan on examining whether CLA can reduce inflammation and the expression of oxidative stress response genes, and in turn, reduce an individual's risk of complication due to obesity.

Significance

Obesity has become a significant problem in recent years, both in the United States and abroad. At present, 67% of American adults are either overweight or obese.[1,2] Obesity has been linked to a variety of diseases, such as type 2 Diabetes, gallbladder disease, hypertension, stroke, metabolic syndrome, and cancer.[3] This, in turn, could decrease the average life expectancy and increase medical costs. In 2008, $147 billion were spent on obesity-related medical costs, while the total NIH budget was only $28.8 billion.[6,23] Although the NIH budget has increased to $30.68 billion, the medical costs related to obesity are rising at a much faster rate.[24,25]

Although obesity has been linked to these diseases, the mechanisms by which they are linked are not fully known. This hinders the ability to reduce disease risk in obese individuals. Similarly, CLA has been shown to be associated with obesity and chemoprevention, but this mechanism is not fully understood either. Animal studies have shown that CLA has a positive effect on obesity, but this influence has not been well characterized in human populations. This evidence suggests that an

intervention targeting oxidative stress could be useful in reducing the rates of obesity and obesity-related diseases.

Currently, CLA is available for use as a supplement in the United States, and may also be enriched in animal products.[26,27] Because of this increased popularity, it is important to understand the effects of CLA on humans.

Preliminary Studies

Preliminary analyses in this population reveal that high (6.4 g/day) doses of CLA significantly increase lean body mass (LBM), compared to placebo.[28] Additionally, laboratory analyses showed that taking high doses of CLA for twelve weeks increased C-reactive protein (CRP), IL-6, white blood cell (WBC) count, and alkaline phosphatase, while hemoglobin, hematocrit, and sodium levels decreased. Steck et al. also indicate that although LBM increased, CLA supplementation had no effect on body fat mass, weight, or BMI.

Specific Aims

Currently, 67% of American adults are either overweight or obese. Obesity has been linked to type 2 diabetes, hypertension, stroke, metabolic syndrome, cancer, and oxidative stress.[1-4] Diet and exercise have been shown to be effective at obesity reduction and prevention. CLA is a fatty acid found in food and commercially available supplements, and has been associated with obesity reduction, inflammation, oxidative stress, cholesterol reduction, and increased insulin sensitivity.[14,15] Previous research has suggested that CLA could decrease oxidative stress and promote weight loss.[29] Given CLA's connection to obesity, inflammation, and oxidative stress, it is possible that CLA can alter the association between these

conditions. We seek to investigate how supplementation with CLA affects the amount of DNA damage due to oxidative stress in obese individuals. In addition, we plan to examine how CLA affects the expression of oxidative stress response genes and levels of proinflammatory markers in obese individuals. Participants were randomized to receive either a placebo (8 g safflower oil/day), 3.2 g/day CLA, or 6.4 g/day CLA for twelve weeks. Outcome measurements were obtained at baseline, week six, and week 12 of the trial.

This project aims to accomplish the following:

Specific Aim 1: Assess the effect that CLA supplementation has on DNA damage in obese, but otherwise healthy, adults. Our hypothesis is that individuals receiving CLA will have less damage than those receiving placebo, and that as the dose of CLA increases, the amount of DNA damage present will decrease. This will involve comparing the amount of DNA damage due to oxidative stress among the three CLA dose groups. Comet assays will be used to measure single strand breaks in DNA and DNA adduct levels (e.g., 8-OH-dG) in lymphocytes isolated from blood samples. We will use the tail moment measurements obtained from these assays to evaluate the change in oxidative stress-induced DNA damage from baseline to week 6 of treatment. In addition, we will analyze the overall effect of CLA on DNA damage and whether age and/or sex confound the association.

Specific Aim 2: Assess the effect that CLA supplementation has on expression of genes involved in the oxidative stress response in obese, but otherwise healthy, adults. Our hypothesis is that individuals receiving CLA will have reduced expression of oxidative stress response genes than those receiving placebo, and that

as the dose of CLA increases, the expression of these genes will decrease. This will involve comparing the changes in expression of two oxidative stress response genes (Nrf2 and SOD1) among the three CLA dose groups. We will use data from Real-Time Polymerase Chain Reactions (RT-PCR) to assess the changes in RNA and evaluate the differences in genes expressed among the three dose groups from baseline to week 12 of treatment. In addition, we will analyze the overall effect of CLA on the genes expressed and whether age and/or sex confound the association.

Specific Aim 3: Assess the effect that CLA supplementation has on inflammation in obese, but otherwise healthy, adults. Our hypothesis is that individuals receiving CLA will have less inflammation than those receiving placebo, and that as the dose of CLA increases, the serum levels of these inflammatory markers will decrease. This will involve comparing the changes in serum levels of two proinflammatory markers (CRP and IL-6) among the three CLA dose groups. We will use data from clinical laboratory analyses to assess and compare the differences in serum levels of each marker among the three groups from baseline to week 12 of treatment. In addition, we will analyze the overall effect of CLA on the serum levels of these markers and whether age and/or sex confound the association.

Research Question

Can CLA reduce either the amount of DNA damage due to oxidative stress or the amount of inflammation in obese individuals?

Methods

The study was conducted using biologic materials and data collected as part of a previously completed clinical trial of CLA in obese adults. The primary aim of this

trial is to determine if supplementation with CLA at low (3.2 g/day) and high (6.4 g/day) doses altered DNA damage due to oxidative stress, expression of oxidative stress response genes, and levels of proinflammatory markers in free-living obese (BMI between 30 and 35 kg/m^2) adults.

Study Subjects

Trial subjects were nonsmoking obese individuals between 19 and 51 years of age, and were otherwise healthy (that is, not diagnosed by a physician with another chronic condition). Subjects were recruited from the Durham, Chapel Hill, Carrboro, and surrounding areas of North Carolina. To obtain a sample representative of this community, subjects were recruited from the general population of Durham and Orange counties via posted flyers and other advertisements. Each subject received $200.00 for participating upon completion of the study. This amount was prorated as follows: $50 for each of the three visits to the General Clinical Research Center (GCRC) involving blood work, indirect calorimetry/dual-energy X-ray absorptiometry (DEXA), and an additional $50 for completion of the entire study.

Selection Criteria

Individuals were excluded from participation for reporting any of the following conditions: chronic illness, including history of cancer, cardiovascular disease, diabetes, or gastrointestinal disorder; anemia; HIV positive; significant abnormal clinical laboratory results (including unacceptable hematopoietic, hepatic, and renal function); food allergies or intolerances; current drug therapy for a diagnosed disease, including medications known to alter lipid metabolism; current use of

weight-lowering medications or diets; use of CLA-containing dietary supplements during the previous three months; consuming a medically prescribed diet that may interfere with the intervention; or current or planned pregnancy. Individuals who had no interest in participating in a clinical trial were also excluded. To determine eligibility, the study physician conducted a complete physical exam and reviewed clinical laboratory analyses for each potential subject.

Study Design

The Investigational Drug Services randomized subjects into one of three arms. Subjects received a six-week supply of pills on their first visit to the GCRC following completion of all study procedures provided they are deemed eligible at that time. Subjects were instructed to not start taking the pills until after they directly spoke with the study coordinator on the phone. On the day after their initial visit, the study coordinator called each subject to confirm his or her eligibility and instruct him or her on when to start taking the pills. The study coordinator documented when contact was made with the subject and the date the subject was given for starting the pills. All subjects received bottles of pills from the Investigational Drug Service and were instructed to take two pills four times per day. Placebo pills containing safflower oil looked identical to CLA pills composed of a 50:50 ratio of the C18:2 cis-9, trans-11 and the C18:2 trans-10, cis-12 isomers. The placebo group received bottles of placebo pills. The 3.2 g CLA group received one bottle of CLA pills and one bottle of placebo pills. The 6.4 g CLA group received bottles of CLA pills. For ease in remembering, subjects were instructed to take one pill from each bottle at each of three meals in the morning, afternoon, and evening and at bedtime. Additionally,

each participant received a magnet to place on their refrigerator as a reminder to take the pills throughout the day. Subjects were asked to bring their pill bottles at the 6-week and 12-week visits, so that compliance could be assessed based on the expected number of pills taken and the number of pills remaining in the bottles.

Measurement of Predictor Variables

Fat mass and lean body mass were measured at baseline and at week 12 via DEXA. GCRC nurses determined height and weight at each visit, and these values were used to calculate BMI (kg/m^2).

Measurement of Outcome Variables

We used existing samples from our clinical trial to determine the impact of CLA on two key markers of cancer risk: oxidative stress and DNA damage. We used the single cell gel electrophoresis (Comet Assay) to examine lymphocytes for oxidative stress-induced DNA damage.

Lymphocytes, isolated from participant blood samples at baseline, week 6, and week 12 of the trial and stored in liquid nitrogen are used to address the first part of Aim 1. By using three different variations of the Comet Assay for each sample, we were able to determine levels of 1) double-strand breaks [electrophoresis at neutral pH], 2) single-strand breaks (alkaline pH vs. neutral pH) and 3) oxidative DNA lesions (e.g., 8-OH-dG) using various DNA repair enzymes. This approach will thus allow us to evaluate the influence of CLA on the different types of nuclear DNA lesions induced by oxidative stress.

Venous blood samples were obtained from healthy obese participants (described in Table 1) in heparinized, EDTA-treated vacutainer tubes. A total of 3 samples per individual (baseline, 6 weeks after supplementation, and 12 weeks after supplementation) were collected. The peripheral blood mononuclear cells (lymphocytes) were isolated by the Ficoll technique, cryopreserved in DMSO and stored in liquid N_2. Lymphocytes were analyzed for DNA damage using the Comet Assay as described below.

Comet Assay Technique

The Comet assay offers a reliable and easy way to measure total and relative DNA damage. Total DNA damage is the sum of oxidative and relative DNA damage. Thus, to obtain the amount of oxidative DNA damage in each lymphocyte, the amount of relative DNA damage can be subtracted from the total DNA damage. The process of measuring DNA damage is described below.

An aliquot of each sample was subjected to neutral pH, alkaline pH, or incubated with DNA repair enzymes (provided by Dr. Stephen Lloyd) and then run under alkaline pH to examine for the different types of DNA lesions. Lymphocytes were analyzed by the Comet assay according to the method of Singh et al. with minor modifications.[30] Briefly, lymphocytes were embedded in 0.5% low-melting-point agarose at a final concentration of 1 x 10^5 cells/ml. The cell suspension (75 µL) was spread onto a glass slide that was previously coated with 1% normal melting point agarose. Cells were subjected to lysis to unwind the DNA and then electrophoresed at neutral pH or high pH. To detect oxidative base damage, each sample was lysed to unwind the DNA, pre-incubated with DNA repair enzymes for 45 min (FPG for

oxidized purines like 8-OH-dG) or 30 min (endonuclease III, for oxidized pyrimidines like thymine glycol) prior to embedding in agarose and electrophoresis under alkaline conditions. After electrophoresis, the slides were stained with propidium iodide, covered with a coverslip and analyzed using a fluorescence microscope. For each experiment, ten slides were processed together, including both a negative and positive control (cells treated with or without H_2O_2). A positive control was prepared by incubating a control lymphocyte sample (Control frozen) with 15 μM H_2O_2 for 5 min at 37°C. Each sample (including positive control) was tested in duplicate and fifty randomly selected cells/slide were examined on a Zeiss fluorescence microscope and the images analyzed for the extent of DNA damage (e.g., tail length, tail moment) using the Comet Assay III™ software (Perspective, Inc).

For determining double-strand breaks, cells were incubated at pH7. To determine single-strand break, cells were incubated at pH10. This converted single-breaks into double-strand break. A subtraction between the pH7 and pH10 runs allowed us to determine the extent of single-strand breaks within a sample. As described below the addition of selective detection of single-strand break and DNA base damage to the global Comet assay resulted in a very sensitive measure of DNA damage, which has never been reported in this population.

Levels of expression of oxidative stress and oxidative stress response genes

The effect of CLA on the expression of antioxidant response genes (SOD1, Nrf2) was determined by RT-PCR from total RNA previously isolated from patients' red blood cells. Total RNA was extracted with trireagent (MRC, Inc) and reverse transcribed to

generate complementary DNA (cDNA) for RT-PCR analysis. Samples were run on an ABS bioprism 7000 and processed with a TaqMan Assay (ABS, CA). The assay includes FAM probe and gene-specific primers, which create a 67bp amplicon. Relative RNA levels were calculated using a comparative cycle threshold (Ct) method and corrected to 18sRNA.

The effect of CLA on the expression of proinflammatory markers (IL-6, CRP) was determined by clinical laboratory analyses used to assess the serum levels of these markers in patients' red blood cells.

Statistical Analysis

The original randomized trial of CLA entered 16 subjects in each of three treatment groups (placebo, 3.2g CLA, 6.4g CLA), and the subjects provided blood samples at three time points (week 0, week 6, week 12). The primary outcomes are the change in oxidative-stress-induced DNA damage in lymphocytes (Comet assay) from baseline to week 6 of treatment, the changes in expression of oxidative stress response genes from baseline to week 12 of treatment, and the changes in levels of inflammatory markers from baseline to week 12 (highlighted in Table A1 of the appendix). Simple linear regression was used to determine the presence and strength of association between each outcome (the change in DNA damage, change in Nrf2 expression, change in SOD1 expression, change in serum CRP levels, and change in IL-6 levels) and the dose of CLA. Age and sex were separately entered into each model to examine their role as potential confounders. Each variable was entered separately to assess the individual effect, and then both variables were entered together to assess the overall effect.

Sample Size and Power

Since the sample size is fixed, power calculations were performed to assess the minimum difference in changes in serum levels of proinflammatory markers between the placebo and high CLA dose groups necessary to obtain 80% power at the $\alpha = 0.05$ level. Assuming standard deviations of 0.39 mg/L for the placebo group and 0.51 mg/L for the 6.4 g/day CLA group, the minimum difference in change in serum CRP levels that can be detected between the two dose groups with 80% power is 0.45 mg/L.[28] Assuming standard deviations of 1.15 mg/L for the placebo group and 0.83 mg/L for the 6.4 g/day CLA group, the minimum difference in change in serum IL-6 levels that can be detected between the two dose groups with 80% power is 0.99 mg/L.[28]

Human Subjects Protection

This project has been approved by the Oregon Health and Science University Institutional Review Board (IRB Number: IRB00009253).

Results

Study Subjects

Fifty-five participants were initially recruited to participate in this study. Three participants were deemed ineligible after the baseline visit. Three participants withdrew during the intervention period for personal reasons, and one withdrew due to pregnancy. Because of this, the final analyses were conducted on 48 participants. Of the seven participants that did not complete the study, one was in the placebo group, four were in the 3.2 g/day CLA group, and two were in the 6.4

g/day CLA group. Figure A1 in the appendix illustrates the selection and randomization process.

The demographic characteristics of the study sample at baseline are in Table 1. Treatment groups did not differ in demographic characteristics or in weight, LBM, BMI, or physical activity at baseline. Study compliance was based on pill count, and compliance data is reported in table A14 in the appendix. As reported by Steck et al., no serious adverse effects were observed in this study.[28]

Table 1: Baseline Demographic Characteristics of Study Population

	Placebo (n=16)	CLA 3.2g/d (n=16)	CLA 6.4g/d (n=16)
Sex [n (%)]			
Male	4 (25.0)	5 (31.2)	4 (25.0)
Female	12 (75.0)	11 (68.8)	12 (75.0)
Race [n (%)]			
Caucasian	11 (68.8)	10 (62.5)	10 (62.5)
African American	5 (31.2)	5 (31.2)	6 (37.5)
Asian	0	1 (6.3)	0
Age (y) [mean ± SD]	34.9 ± 2.0	36.0 ± 2.0	35.4 ± 2.2
Height (cm) [mean ± SD]	166.7 ± 2.2	168.4 ± 1.7	167.0 ± 2.2
Weight (kg) [mean ± SD]	91.1 ± 2.5	93.0 ± 2.3	93.0 ± 2.5
BMI (kg/m^2) [mean ± SD]	32.7 ± 0.5	32.7 ± 0.4	32.9 ± 0.4
Body fat mass (kg) [mean ± SD]	35.6 ± 1.2	34.3 ± 1.3	34.5 ± 1.2
Lean body mass (kg) [mean ± SD]	50.7 ± 2.0	53.7 ± 2.0	53.5 ± 2.7

Effects of CLA on DNA Damage

Comet assays were used to examine lymphocytes for oxidative stress-induced DNA damage. As the dose of CLA increased, the amount of DNA damage due to oxidative stress also increased. However, this association was not significant, even after adjusting for both age at recruitment and sex (Table 2). The mean change in DNA damage from baseline to week 6 for each group is shown in Table 3.

Effects of CLA on Oxidative Stress Response Genes

The effect of CLA on the expression of antioxidant response genes (SOD, Nrf2) was determined by RT-PCR from total RNA previously isolated from patients' red blood cells. The changes in these genes were assessed by calculating the ratio of RNA levels of these genes at visit 3 to RNA levels at visit 1. As the dose of CLA increased, the amount of Nrf2 expressed also increased (Table 2). In contrast, the amount of SOD1 expressed decreased as the dose of CLA increased (Table 2). However, neither of these associations was significant, even after adjusting for age at recruitment and sex. The mean ratios of Nrf2 and SOD1 levels between visit 3 and visit 1 for each group is in Table 3.

Effects of CLA on Proinflammatory Markers

The effect of CLA on the expression of proinflammatory markers (IL-6, CRP) was determined by clinical laboratory analyses used to assess the serum levels of these markers in patients' red blood cells. For every 3.2 g/day increase in CLA dose increased, the serum CRP levels increased by 0.23 ($p < 0.01$). This association remained significant after individually adjusting for age and sex ($p = 0.02$ and $p = 0.01$, respectively), as well as after collectively adjusting for both ($p = 0.03$) (Table

2). For every 3.2 g/day increase in CLA dose, the serum IL-6 levels increase by 0.50 mg/L (p = 0.02). This association remained significant after individually adjusting for sex (p = 0.04). However, this association was no longer significant after individually adjusting for age, as well as after collectively adjusting for age and sex (Table 2). The mean changes of serum CRP and IL-6 levels for each group are in Table 3.

Table 2 summarizes the results from the simple linear regression analyses that were performed. This table shows the coefficients from each regression model, and these coefficients describe the direction and magnitude of the association between dose of CLA and each outcome. P-values for the associations between CLA and DNA damage, Nrf2 expression, SOD1 expression, serum CRP levels, and serum IL-6 levels are reported in tables A9-A13 in the appendix.

Table 2: Coefficients from Regression Analyses of CLA Dose Increase to Outcomes

Variables Adjusted For	DNA Damage/Oxidative Stress (95% CI)	Nrf2 (95% CI)	SOD1 (95% CI)	CRP (95% CI)	IL-6 (95% CI)
Unadjusted	6.19 (-3.16, 15.53)	0.25 (-0.06, 0.56)	-0.01 (-0.20, 0.17)	0.23* (0.07, 0.40)	0.50* (0.10, 0.91)
Age	4.91 (-4.04, 13.86)	0.25 (-0.06, 0.57)	-0.01 (-0.21, 0.18)	0.23* (0.06, 0.40)	0.50 (0.09, 0.91)
Sex	5.10 (-3.92, 14.12)	0.25 (-0.06, 0.56)	-0.01 (-0.20, 0.18)	0.23* (0.07, 0.40)	0.50* (0.09, 0.91)
Age and Sex	3.91 (-4.66, 12.47)	0.25 (-0.06, 0.56)	-0.01 (-0.21, 0.18)	0.23* (0.06, 0.40)	0.50 (0.08, 0.91)

*$p < 0.05$

Table 3: Mean Changes in Outcomes Between Groups

Dose Group	Mean Change in DNA Damage (SD)	Mean Change in Nrf2 Expression (SD)	Mean Change in SOD1 Expression (SD)	Mean Change in CRP Levels (SD)	Mean Change in IL-6 Levels (SD)
Placebo	-15.16 (7.86)	1.21 (0.10)	1.23 (0.09)	-0.16 (0.10)	-0.53 (0.31)
CLA 3.2 g/day	-8.01 (4.17)	1.15 (0.09)	1.39 (0.16)	0.15 (0.13)	0.32 (0.31)
CLA 6.4 g/day	-2.65 (6.24)	1.71 (0.33)	1.20 (0.90)	0.31 (0.06)	0.49 (0.21)

Discussion

Data from a previously conducted randomized, double-blind, placebo-controlled clinical trial were analyzed to examine the effects of two doses of CLA supplementation on DNA damage, antioxidant response genes, and serum levels of proinflammatory markers in obese, but otherwise healthy individuals. No substantial changes in DNA damage, Nrf2 expression, and SOD1 expression were observed during the 12-week intervention. Levels of CRP and IL-6 expressed increased significantly in the CLA dose groups, compared to placebo. This evidence suggests that short-term CLA supplementation results in an increase in expression of inflammatory markers.

Previous trials have examined the effects of CLA supplementation on CRP levels, and have found that CLA supplementation leads to an increase in serum CRP levels.[31] This study adds to the evidence that supports this conclusion.

certain studies have shown that age can affect IL-6 levels. A study of the role of IL-6 in cervical cancer risk among Eastern Chinese women showed that two

polymorphisms of IL-6 (rs2069837 and rs2069840) were associated with an increased risk of cervical cancer.[32] Furthermore, these single nucleotide polymorphisms (SNPs) were more prevalent in younger women (≤46 years) than older women. However, after adjusting for age at recruitment, there was no statistical difference in IL-6 levels among the three groups ($p = 0.06$). To date, the influence of age on this association has not been examined.

However, our data failed to show a significant association between CLA dose and both Nrf2 and SOD1 expression. One possible explanation for this is the choice of CLA isomers between studies. Many isomers of CLA exist, but the c9t11 and t10c12 have been more extensively studied. Findings from previous research suggest that the c9t11 and t10c12 isomers can have opposing effects, with t10c12 preventing triglyceride accumulation and c9t11 promoting it.[16] This suggests that the use of different isomers could result in differences in observed oxidative stress, expression of antioxidant response genes, and levels of proinflammatory markers. In a randomized, double-blind, placebo-controlled trial, Kim et al. administered a dose of 2.4 g/d of a CLA isomer mixture (equal amounts of t10c12 and c9t11) to healthy overweight/obese Korean adults for eight weeks, and failed to show an association between CLA supplementation and antioxidant metabolism.[33]

Furthermore, Risérus et al. found that the two isomers also have different effects on oxidative stress.[14,34] Their study involving overweight and obese Swedish male adults showed that supplementation with 3.4 g/day of t10c12 CLA augmented lipid peroxidation more than supplementation with either a placebo or 3.4 g/day of a CLA isomer mixture (equal amounts of t10c12 and c9t11).[14] In a subsequent study of

overweight and obese Swedish males, Risérus et al. examined the effects of supplementation with the CLA composition predominant in diet (83.3% c9t11, 7.3% t10c12), and found that this composition also augmented lipid peroxidation.[34] These data suggest that the effect of CLA supplementation on oxidative stress is attributable to the t10c12 isomer.

The t10c12 isomer could also account for the increase in the expression of proinflammatory markers. Chung et al. found that IL-6 secretion in human adipocytes increased after treatment with t10C12 CLA, but not after treatment with c9t11 CLA.

In addition to isomer-specific effects, other genes could explain the association between CLA supplementation and inflammatory markers. Results from past studies suggest that the PPARγ gene is involved in obesity and inflammation, and that CLA influences PPARγ expression.[18,19,35,36] A study by Brown et al. showed that expression of PPARγ in human preadipocytes decreased after treatment with t10c12 CLA, but increased after treatment with c9t11 CLA.[37] Other studies have also suggested that c9t11 CLA could be a PPARγ antagonist in human adipocytes, but that t10c12 CLA could either be a partial antagonist or weak agonist of PPARγ.[36,38] However, the mechanism by which CLA affects PPARγ expression is not fully understood. Herrmann et al. conducted a study in humans to examine the isomer-specific effects of CLA on PPARγ expression, and found that the influence of CLA on PPARγ depends on the PPARγ2 genotype.[18] They found that expression of the adiponectin gene and LDLR, which are regulated by PPAR, increase as a result of the t10c12 isomer, but not the c9t11 isomer. Though CLA has been shown to have

isomer-specific effects on PPARγ, further research is warranted to assess the mechanisms by which each isomer influences the PPARγ gene. Clearly, further studies using individual isoforms of CLA, c9t11 or t10c12, would shed light on each isoform's role on oxidative stress in the obese population.

Strengths and Potential Limitations

This study has several strengths and limitations. The randomized, double-blind, placebo-controlled clinical trial design allows us to examine the effects of two different doses of CLA, and compare these to the effect of taking a placebo, while eliminating several kinds of bias. However, the use of only one center means that we can only make comparisons on a regional level.

This study only examined obese participants, and as a result, baseline data is not available for the actions of CLA in nonobese individuals. Furthermore, the findings from this trial cannot be generalized to the nonobese population.

Although these results show a significant association between CLA dose and serum levels of proinflammatory markers, only two markers were analyzed. This is not sufficient to conclude that an increase in CLA dose is related to an increase in inflammation. This study design could be expanded to include other proinflammatory markers, such as IL-1ra and TNF-alpha. It is possible that this study was too short, and that CLA supplementation for longer than twelve weeks may produce more significant results. Additionally, the small sample size available limited the power of the analyses conducted. To obtain a larger and nationally dispersed sample, we would have to expand the study to other sites, which would require extra time, money, and resources.

Future Research

Overall, our results suggest that an increase in CLA dose is linked to an increase in serum levels of IL-6, but that this association could be attributed to age. Further research needs to be conducted to reveal the mechanism by which age can affect the serum levels of this proinflammatory marker.

This trial did not find a significant association between CLA supplementation and DNA damage, Nrf2 expression, and SOD1 expression. This could be attributed to the mixture of CLA isomers used and the different effects of each isomer. Limited information is available on the isomer-specific effects of CLA supplementation on these outcomes, which makes it a great subject of future research.

This study design could be expanded in several ways. Originally, only two proinflammatory markers were analyzed. Thus, this study design could be expanded to include other proinflammatory markers, such as IL-1ra and TNF-alpha. In addition, a CLA isomer mixture (equal amounts of t10c12 and c9t11) was used for this study. Previous research suggests that the two isomers have different effects on obesity and inflammation.[14,16,18] Future research could examine the isomer-specific effects of CLA supplementation on oxidative stress, gene expression, and inflammatory markers.

Furthermore, future studies could examine a broader and larger sample. Participation in this trial was limited to obese individuals. Because of this, these findings on oxidative stress and inflammatory markers cannot be applied to nonobese individuals. Studying the effects of CLA supplementation in nonobese individuals would provide insight into how CLA functions in these individuals.

These findings could then be combined with the findings from this trial to show how CLA supplementation could affect the general population. Additionally, the study criteria could be broadened to include participants over the age of fifty. Moreover, having a larger sample size would raise the power of these results.

It is also possible that the duration of the study was too short. Data from this study and similar studies have shown that CLA supplementation for fewer than twelve weeks does not significantly affect antioxidant metabolism and oxidative stress response genes.[33] Future studies could examine how CLA supplementation for longer than twelve weeks affects these outcomes.

Summary and Conclusions

In conclusion, we found that supplementation with CLA for twelve weeks increases the levels of CRP in obese, but otherwise healthy individuals. Since CRP is a marker of inflammation, this suggests that CLA supplementation may result in an increase in inflammation. Increases in IL-6 levels were also noticed, but these increases were no longer statistically significant after adjusting for age. Since obesity rates are rapidly rising and CLA is becoming more ubiquitous in commercial supplements, further research needs to be conducted to assess the mechanism by which CLA affects inflammation, and subsequently, obesity.

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Appendix

Table A1: Outcome Measurements Obtained at Each Visit

Dose Group	Time Point (Data Collected)		
	Baseline	Week 6	Week 12
Placebo (n = 16)	BMI, DEXA, DNA damage, gene expression	BMI, DEXA, DNA damage	BMI, DEXA, gene expression
CLA 3.2 g/day (n = 16)	BMI, DEXA, DNA damage, gene expression	BMI, DEXA, DNA damage	BMI, DEXA, gene expression
CLA 6.4 g/day (n = 16)	BMI, DEXA, DNA damage, gene expression	BMI, DEXA, DNA damage	BMI, DEXA, gene expression

Table A2: Changes in DNA Damage Levels from Participants' Lymphocytes (as measured by Comet assays)

(N = 18)

Dose Group	**Mean Change in Tail Moment (SD)**
Placebo	-15.16 (7.86)
3.2 g/day	-8.01 (4.17)
6.4 g/day	-2.65 (6.24)

Table A3: Mean Ratio of Changes in Nrf2 Expression from RNA

(N = 44)

Dose Group	**Mean Change in RNA Levels (SD)**
Placebo	1.21 (0.10)
3.2 g/day	1.15 (0.09)
6.4 g/day	1.71 (0.33)

Table A4: Mean Ratio of Changes in SOD1 Expression from RNA

(N = 43)

Dose Group	Mean Change in RNA Levels (SD)
Placebo	1.23 (0.09)
3.2 g/day	1.39 (0.16)
6.4 g/day	1.20 (0.90)

Table A5: Changes in CRP Expression from RNA

(N = 48)

Dose Group	Mean Change in RNA Levels (SD)
Placebo	-0.16 (0.10)
3.2 g/day	0.15 (0.13)
6.4 g/day	0.31 (0.06)

Table A6: Changes in IL-6 Expression from RNA

(N = 45)

Dose Group	Mean Change in RNA Levels (SD)
Placebo	-0.53 (0.31)
3.2 g/day	0.32 (0.31)
6.4 g/day	0.49 (0.21)

Table A7: DNA Damage Due to Oxidative Stress at Baseline, Week 6, and Changes from Baseline to Week 6

	Placebo	CLA 3.2 g/day	CLA 6.4 g/day
Baseline	3.62 (2.43)	-0.02 (7.97)	-0.35 (6.79)
Week 6	-12.73 (17.54)	-1.71 (11.02)	-1.92 (15.01)
Change	-15.17 (17.59)	-8.01 (9.33)	-2.65 (18.71)

Relative Oxidative Stress calculated with the formula FPG – N, where:

FPG measures both the oxidative and relative DNA damage. FPG can detect double-strand breaks by recognizing 8-OH-dG and clipping them, thus creating single-strand fragments.

N measures the relative DNA damage (by only recognizing single-strand breaks).

Tables A8a-c: Clinical Laboratory Values of Serum Proinflammatory Markers at Baseline and After 12 Weeks of Supplementation

Table A8a: Baseline

Marker	Function	Mean (SD)		
		Placebo	CLA 3.2 g/day	CLA 6.4 g/day
CRP	Proinflammatory Marker	1.12 (1.07)	0.60 (0.21)	0.72 (0.35)
IL-6	Proinflammatory Marker	2.08 (1.29)	1.75 (1.46)	1.63 (0.78)

Table A8b: Week 12

Marker	Function	Mean (SD)		
		Placebo	CLA 3.2 g/day	CLA 6.4 g/day
CRP	Proinflammatory Marker	0.96 (0.77)	0.75 (0.51)	1.03 (0.67)
IL-6	Proinflammatory Marker	1.71 (0.91)	2.07 (1.71)	2.07 (1.13)

Table A8c: Change from Baseline to Week 12

Marker	Function	Mean (SD)		
		Placebo	CLA 3.2 g/day	CLA 6.4 g/day
CRP	Proinflammatory Marker	-0.16 (0.39)	0.15 (0.53)	0.31 (0.51)
IL-6	Proinflammatory Marker	-0.53 (1.15)	0.32 (1.25)	0.49 (0.83)

Table A9: Association of CLA and DNA Damage Due to Oxidative Stress

Variable(s) Adjusted For	Direction of Association between DNA Damage and Dose Group	P-value
Unadjusted	6.19	0.10
Age	4.91	0.10
Sex	5.10	0.11
Age and Sex	3.91	0.06

Table A10: Association of CLA and Nrf2 Expression

Variable(s) Adjusted For	Direction of Association between Nrf2 Expression and Dose Group	P-value
Unadjusted	0.25	0.11
Age	0.25	0.28
Sex	0.25	0.11
Age and Sex	0.25	0.22

Table A11: Association of CLA and SOD1 Expression

Variable(s) Adjusted For	Direction of Association between SOD1 Expression and Dose Group	P-value
Unadjusted	-0.01	0.88
Age	-0.01	0.99
Sex	-0.01	0.97
Age and Sex	-0.01	0.99

Table A12: Association of CLA and Serum CRP Levels

Variable(s) Adjusted For	Coefficient (to indicate direction)	P-value
Unadjusted	0.23	0.007
Age	0.23	0.02
Sex	0.23	0.01
Age and Sex	0.23	0.03

Table A13: Association of CLA and Serum IL-6 Levels

Variable(s) Adjusted For	Coefficient (to indicate direction)	P-value
Unadjusted	0.50	0.02
Age	0.50	0.06
Sex	0.50	0.04
Age and Sex	0.50	0.08

Figure A1: Selection and Randomization Process

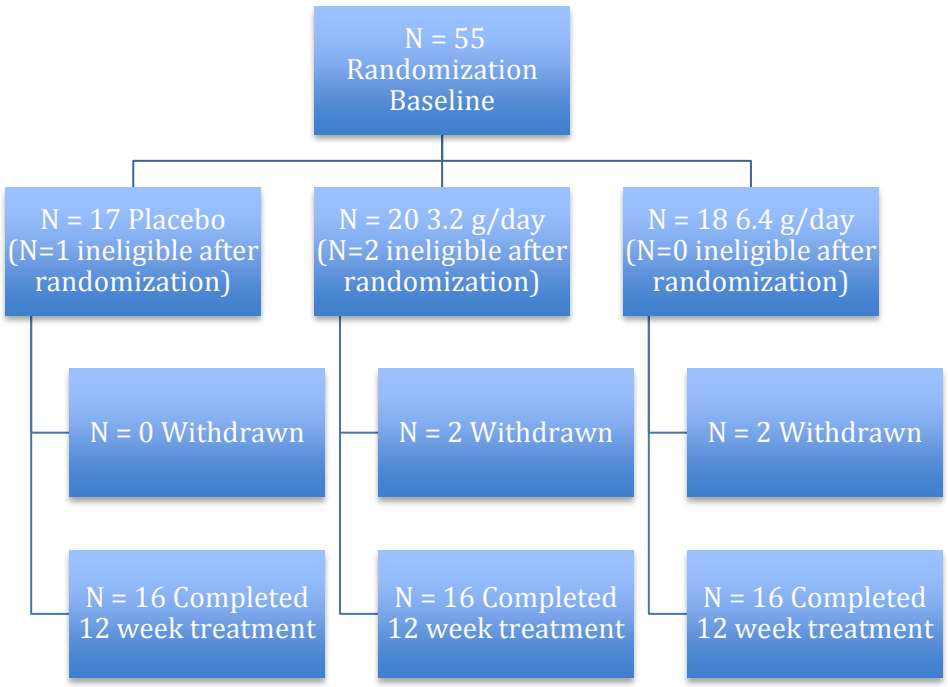

Table A14: Study Compliance Data

Dose Group	Percentage of Participants Who Complied
Placebo	88%
CLA 3.2 g/day	92%
CLA 6.4 g/day	92%