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Title: Alternate Day Calorie Restriction Improves Clinical Findings and Reduces Markers of Oxidative Stress and Inflammation in Overweight Adults with Moderate Asthma

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Corresponding Author: Dr. James B Johnson,

Corresponding Author's Institution: Louisiana State University Health Sciences Center

First Author: James B Johnson

Order of Authors: James B Johnson; Warren Summer; Roy G Cutler; Bronwen Martin; Dong-Hoon Hyun; Vishwa D Dixit; Michelle Pearson; Matthew Nassar; Stuart Maudsley; Olga Carlson; Sujit John; Donald R Laub; Mark P Mattson

Manuscript Region of Origin:

Abstract: Background: Asthma is an increasingly common disorder responsible for considerable morbidity and mortality. Although obesity is a risk factor for asthma and weight loss can improve symptoms, many patients do not adhere to low calorie diets and the impact of dietary restriction on the disease process is unknown.
Objective: A study was designed to determine if overweight asthma patients would adhere to an alternate day calorie restriction (ADCR) dietary regimen, and to establish the effects of the diet on their symptoms, pulmonary function and markers of oxidative stress and inflammation.

Methods: Ten subjects with BMI>30 were maintained for 8 weeks on a dietary regimen in which they ate ad libitum every other day, while consuming less than 20% of their normal calorie intake on the intervening days. At baseline, and at designated time points during the 8 week study, asthma control, symptoms and Quality of Life questionnaires (ACQ,ASUI, mini-AQLQ) were assessed and blood was collected for analyses of markers of general health, oxidative stress and inflammation. Peak Expiratory Flow (PEF) was measured daily on awakening. Pre and post bronchodilator spirometry was obtained at baseline and 8 weeks.

Results: Nine of the subjects adhered to the diet and lost an average of 8% of their initial weight during the study. Their asthma related symptoms, control and QOL improved significantly, and PEF increased significantly, within 2 weeks of diet initiation; these changes persisted for the duration of the study. Spirometery was unaffected by ADCR. Levels of serum □-hydroxybutyrate were increased and levels of leptin were decreased on CR days indicating a shift in energy metabolism towards utilization of fatty acids and confirming compliance with the diet. The improved clinical findings were associated with decreased levels of serum cholesterol and triglycerides, striking reductions in markers of oxidative stress (8-isoprostane, nitrotyrosine, protein carbonyls, and 4-hydroxynonenal adducts) and increased levels of the antioxidant uric acid. Indicators of inflammation, including serum tumor necrosis factor-□ and brain-derived neurotrophic factor, were also significantly decreased by ADCR.

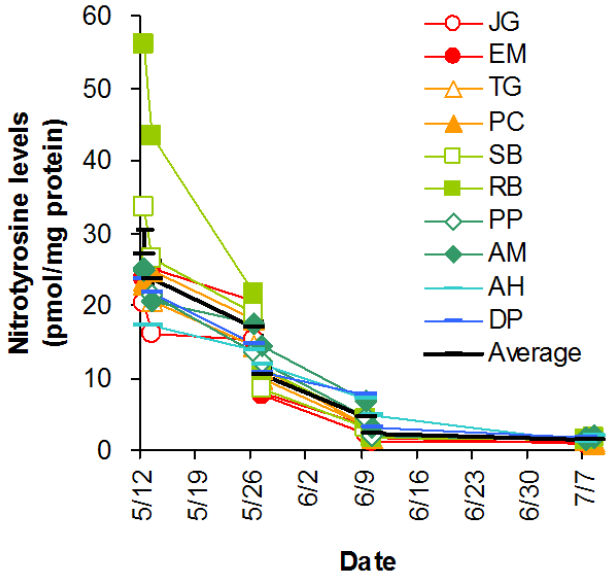
Conclusions: Compliance with the ADCR diet was high, symptoms and pulmonary function improved, and oxidative stress and inflammation declined in response to the dietary intervention. These findings demonstrate rapid and sustained beneficial effects of ADCR in subjects with asthma, suggesting a novel approach for therapeutic intervention in this disorder.

Dear Dr. Davies,

We are submitting our manuscript describing an IRB approved clinical trial in which ten moderate asthmatics followed a pattern of alternate day calorie restriction for eight weeks, one day eating ad lib and the next day consuming less than 20% of required daily calories. Parameters of pulmonary function and asthma symptoms showed highly significant improvement, and serum markers of oxidative stress and inflammation showed striking reductions. Nitrotyrosine levels declined by ten fold and protein carbonyl and isoprostane by five fold. In contrast, daily calorie restriction produces non significant changes in these markers.

The various assays were performed at the Gerontology Research Center, National Institute on Aging, NIH, under the supervision of Mark Mattson, PhD, Chief, Laboratory of Neurosciences and senior author on this paper.

The sample size is small and there was no control group, but the clinical results were dramatic. As shown below, the pattern of decline and end point levels of nitrotyrosine for all subjects was remarkably uniform and very low.



We believe that a stress response mechanism activated by the alternate day very low level of calories mediates the extraordinary reductions in oxidative stress likely due to upregulation of antioxidant enzyme activity, endogenous antioxidants and proteasome catabolic activity.

Thank for your attention.

Sincerely,
James B. Johnson, MD
Donald R. Laub, MD

Reviewer Suggestions

1. Kelvin J.A. Davies
   James E. Birren Chair of Gerontology
   Andrus Gerontology Center
   University of Southern California
   Los Angeles, CA

2. Stanley L. Hazen
   Cleveland Clinic Foundation
   Cleveland, OH

3. Robert A. Floyd
   Oklahoma Medical Research Foundation,
   Oklahoma City, OK

4. Daret St. Clair
   University of Kentucky,
   Lexington, KY

Dear Dr. Davies,

Thank you for the opportunity to respond to the referee's concerns and revise our manuscript. The major concerns of each reviewer were based on misunderstandings/oversights on their part. We were surprised at their comments because, in both cases, the information they overlooked was presented very clearly in the manuscript. Both reviewers seem not to have recognized the importance of our findings which demonstrate marked improvement in the asthma disease process and profound, and statistically highly significant reductions in markers of oxidative stress and inflammation, during the course of the alternate day calorie restriction (ADCR) regimen. These are novel and important findings of considerable interest from both basic science and clinical perspectives.

Reviewer #1

Comments: This is a very interesting pilot study regarding a very important biomedical problem. There seems to be a major problem with the experimental design or in the methods of analysis of some of the parameters. The experimental design was meant to be such that AL subjects remained on the same AL diet as before the start of the experiment. This obviously failed for data in several of the figures (3a,3b,4a,4b,4c,5a,5b,5c) all clearly show the AL subjects changed in a strikingly similar pattern as do the CR subjects. This most obvious problem was completely ignored in the discussion. Considerable discussion was devoted to time of sampling in the discussion section but not in the methods section. This remains a pilot study only.

Response: Reviewer #1 seems to think there were two groups being compared, an ad lib (AL) group and a calorie restriction (CR) group. The manuscript makes it clear, as does the cover letter, that there was only one group of subjects who were evaluated at baseline and at increasing time points after initiation of an alternating day pattern of AL and CR. The experimental design involved evaluation of clinical and biochemical variables in subjects at baseline and at designated time points during the course of a 2 month alternate day CR (ADCR) dietary regimen. In this longitudinal design, the baseline value for each subject served as the control value for that subject to which ADCR diet values were compared. After a 14 day pre-diet period during which baseline variables were recorded, all subjects initiated ADCR in which women consumed 320 calories and men 380 calories of a commercially available canned meal replacement shake every other day; they ate ad libitum (AL) on intervening days. For the data in Figures 3 – 6, blood samples taken on consecutive CR and AL days were analyzed in order to determine whether the variables being measured changed acutely. Most variables changed progressively with increasing time on the ADCR diet, but did not change acutely between consecutive AL and CR days. The referee misunderstood the experimental design and therefore his/her criticisms are irrelevant. Nevertheless, we have added several sentences to the "Experimental Design" section of the manuscript that clearly describe the longitudinal design of the study.

Reviewer #2

Comment. Very interesting paper. Found one typo on page 5 in experimental designs six lines from end of paragraph. "They" should read "The".

Response: The typo has been corrected.

Comment: Basic weakness of study was the small number of subjects being used (8 female and

two male). Thus with the positive results seen so far I would recommend to determine if the findings hold with larger numbers in a future study.
Response: We agree that a larger number of subjects is always desirable. However, given the longitudinal design of the study, and the high levels of statistical significance of the effects of the ADCR diet on many of the clinical and biochemical variables, the data provide clear and unambiguous results that are an important contribution to the field.

Comment: The tests for oxidative stress are reasonable, but I would recommend using additional tests being used in measuring changes in oxidative stress status in experimental animals on caloric restriction. For example tests measuring oxidative damage to nucleic acids, proteins and lipids. Isoprostane assays may be the best for these studies. In human studies it is well known that large differences in oxidative stress status are found between different individuals and sex. Thus it would also be interesting to known the reproduceability and reliability of the oxidative stress assays for human subjects.
Response: Surprisingly, this reviewer commented "I would recommend using additional tests being used in measuring changes in oxidative stress status in experimental animals on caloric restriction. For example tests measuring oxidative damage to nucleic acids, proteins and lipids. Isoprostane assays may be the best for these studies". We, in fact, DID measure levels of isoprostanes, protein carbonyls, HNE adducts, nitrotyrosine and ceramides. Clearly, we have thoroughly evaluated key markers of oxidative stress using state-of-the-art methods.
Regarding reproduceability, the error bars on the bar charts for the nitrotyrosine, protein carbonyls, and isoprostane indicate the narrow range in these values among the ten subjects. It is also important to note that the after eight weeks on the ADCR diet, the inter-subject variability decreases.

Comment: It would also have been valuable to the reader if the authors included in the discussion section some ideas or hypothesis about possible mechanism of action of effect observed.
Response: We agree with the reviewer and have therefore added the following paragraph to the Discussion.
The mechanism(s) by which ADCR reduces oxidative stress and inflammation in asthmatic subjects remains to be established. However, based upon previous studies of the effects of alternate day fasting on cellular physiology in rodents, two general mechanisms are likely. First, because subjects on ADCR exhibit a reduction in overall energy intake and lose weight, there is likely a reduction in cellular oxygen free radical production[24, 41, 42]. The latter effect of ADCR would be associated with lower levels of oxidatively modified proteins and lipid peroxidation products in the blood. Second, ADCR may impose a mild beneficial stress, to which cells respond adaptively by up-regulating the expression of antioxidant systems. Such increased cellular stress resistance has been shown occur in rodents on an alternate day energy restriction regimen, resulting in increased disease resistance[24]. It will be of considerable interest to determine the effects of ADCR on gene expression in tissue involved in the pathogenesis of asthma.

We believe we have fully addressed the concerns of the referees, and that the revised manuscript will provide a valuable addition to the field.

Best Regards,

James B. Johnson, MD
Mark P. Mattson, PhD

Alternate Day Calorie Restriction Improves Clinical Findings and Reduces Markers of Oxidative Stress and Inflammation in Overweight Adults with Moderate Asthma

James B. Johnson[1]*, Warren Summer[2], Roy G. Cutler[3], Bronwen Martin[3], Dong-Hoon Hyun[3], Vishwa D. Dixit[4], Michelle Pearson[3], Matthew Nassar[3], Stuart Maudsley[3], Olga Carlson[5], Sujit John[6], Donald R. Laub[7], and Mark P. Mattson[3]

[1]Department of Surgery and [2]Department of Pulmonary Medicine, Louisiana State University Medical Center, New Orleans, LA. [3]Laboratory of Neurosciences, [4]Laboratory of Immunology, [5]Diabetes Section, National Institute on Aging Intramural Research Program, Baltimore, MD. [6]Deparment of Mathematics, University of New Orleans, New Orleans, LA [7]Department of Surgery, Stanford University, Palo Alto, CA.

*Correspondence: James B. Johnson, jim@jbjmd.com

Key Words: AQLQ; isoprostanes; peak expiratory flow; protein carbonyls; nitrotyrosine; BDNF; spirometry; tumor necrosis factor, oxidative stress

Acknowledgements: This research was supported, in part, by the National Institute on Aging Intramural Research Program, NIH.

ABSTRACT

Background: Asthma is an increasingly common disorder responsible for considerable morbidity and mortality. Although obesity is a risk factor for asthma and weight loss can improve symptoms, many patients do not adhere to low calorie diets and the impact of dietary restriction on the disease process is unknown.

Objective: A study was designed to determine if overweight asthma patients would adhere to an alternate day calorie restriction (ADCR) dietary regimen, and to establish the effects of the diet on their symptoms, pulmonary function and markers of oxidative stress and inflammation.

Methods: Ten subjects with BMI>30 were maintained for 8 weeks on a dietary regimen in which they ate ad libitum every other day, while consuming less than 20% of their normal calorie intake on the intervening days. At baseline, and at designated time points during the 8 week study, asthma control, symptoms and Quality of Life questionnaires (ACQ,ASUI, mini-AQLQ) were assessed and blood was collected for analyses of markers of general health, oxidative stress and inflammation. Peak Expiratory Flow (PEF) was measured daily on awakening. Pre and post bronchodilator spirometry was obtained at baseline and 8 weeks.

Results: Nine of the subjects adhered to the diet and lost an average of 8% of their initial weight during the study. Their asthma related symptoms, control and QOL improved significantly, and PEF increased significantly, within 2 weeks of diet initiation; these changes persisted for the duration of the study. Spirometery was unaffected by ADCR. Levels of serum β-hydroxybutyrate were increased and levels of leptin were decreased on CR days indicating a shift in energy metabolism towards utilization of fatty acids and confirming compliance with the diet. The improved clinical findings were associated with decreased levels of serum cholesterol and triglycerides, striking reductions in markers of oxidative stress (8-isoprostane, nitrotyrosine, protein carbonyls, and 4-hydroxynonenal adducts) and increased levels of the antioxidant uric acid. Indicators of inflammation, including serum tumor necrosis factor-α and brain-derived neurotrophic factor, were also significantly decreased by ADCR.

Conclusions: Compliance with the ADCR diet was high, symptoms and pulmonary function improved, and oxidative stress and inflammation declined in response to the dietary intervention. These findings demonstrate rapid and sustained beneficial effects of ADCR on the underlying disease process in subjects with asthma, suggesting a novel approach for therapeutic intervention in this disorder.

INTRODUCTION

The cause(s) and pathogenic mechanisms of asthma are poorly understood, and available treatments can alleviate symptoms but do not reverse the disease process[1]. The prevalence of asthma in industrialized countries throughout the world has increased significantly during the past 30 years, particularly in children where rates have nearly doubled[2]. This recent surge of asthma prevalence does not appear to be the result of increases in specific allergens. Instead, increasing evidence points to a link between overeating/obesity and asthma. Weight loss often improves asthma symptoms in obese subjects[3], and low calorie diets and exercise programs result in weight loss and can reduce asthma symptoms in overweight children and adults[4, 5]. However, while obesity is a risk factor for asthma-related symptoms such as wheezing, it may not be a cause of airway hyperresponsiveness[5, 6]. It is therefore unclear whether weight loss modifies the asthma disease process.

The molecular and cellular mechanisms underlying airway hyper-responsiveness and asthma symptoms are complex and poorly understood. Two general alterations in the lungs are increased oxidative stress and inflammation[7-11]. The local changes in the lungs are associated with increases in markers of inflammation and oxidative stress in the blood including TNF[12], interleukin-6[13] and lipid peroxidation products[14]. In addition, circulating levels of brain-derived neurotrophic factor (BDNF) are increased in patients with asthma and other allergic disorders[15, 16]. Although capable of transiently relieving asthma symptoms, agents such as corticosteroids and β-adrenoreceptor agonists do not block or reverse the underlying disease process and their long-term use poses a considerable risk of morbidity and mortality[17, 18].

Caloric restriction (CR) improves numerous health indicators in rodents, monkeys and humans, including those associated with risk of cardiovascular disease, type 2 diabetes and cancers[19-21]. Similarly to daily CR (on a long-term basis), intermittent CR can extend lifespan and protect multiple organ systems against disease in rodents[22-24]. However, despite considerable evidence that intermittent CR is beneficial in rodent disease models, the potential application of intermittent CR to human diseases is largely untested[25]. In light of the poor adherence of subjects to continuous CR diets and adverse consequences associated with gastric bypass surgery and pharmacological interventions[26], we designed a pilot study aimed at

determining the feasibility and efficacy of an intermittent CR diet in treating overweight patients with moderate asthma.

METHODS

Subjects. This study was approved by an independent Review Board (Cresent City IRB) and analyses of serum samples was approved by the IRB of the National Institute on Aging Intramural Research Program. Participants were recruited through newspaper advertisements in the New Orleans metropolitan area. Inclusion and exclusion criteria were assessed by telephone, an in person interview, and a physician-conducted examination. Participants meeting the following criteria were included in the study: stable body weight with BMI>30 and less than 300 pounds; prior diagnosis of stable moderate persistent asthma as defined by the "Expert Panel Report 2 (NHLBI)[27]; FEV_1 or peak expiratory flow (PEF) >50%; daily symptoms with use of inhaled short-acting $beta_2$-agonist and controller, medication regimen stable for at least 30 days prior to the screening visit; medical history provided by the subject or the subject's physician did not indicate any potential risk to the subject as the result of the study. The subjects were in general good health based on assessment by the investigators, willing to follow instructions and complete study procedures as required by the protocol. All subjects had demonstrated a >12% post-bronchodilator increase in FEV1 documented in the past two years. Subjects were excluded if they had a history of smoking, were taking systemic corticosteroids within the prior six weeks, were using hypoglycemic agents or insulin at screening or if it was felt such medication might be needed during the study. The dosage of all medications, including over the counter, herbals, and dietary supplements were recorded.

Experimental Design. Ten subjects (8 females and 2 males) with inactive lifestyles and stable moderate persistent asthma with daily symptoms were enrolled in the study as a single cohort. The experimental design involved evaluation of clinical and biochemical variables in subjects at baseline and at designated time points during the course of a 2 month alternate day CR (ADCR) dietary regimen. In this longitudinal design, the baseline value for each subject served as the control value for that subject to which ADCR diet values were compared. After a 14 day pre-diet period during which baseline variables were recorded, all subjects initiated ADCR in which women were instructed to consume 320 calories and men 380 calories of a commercially

available canned meal replacement shake (Atkins Advantage or Carb Solutions) provided to the subjects. On the other day subjects ate ad libitum (AL). Diary cards and instructions were given to the subjects during the 14 day baseline period. On the last day of the baseline period subjects returned their diary cards and were given new cards and instructions in how to follow the diet, including the number of calories to be consumed on each CR day. They were told to eat on the AL day whatever they normally ate and to the point of satisfaction but not to intentionally overeat. The subjects were told to continue taking the vitamins and herbal supplements they were taking prior to the study. The principal investigator and ancillary personnel met each week with all the participants for 1 hour in the evening to provide group support. Topics of discussion were limited to subjects' reaction to the dietary pattern. Subjects were weighed on days 1, 15, 29, 57 using a calibrated balance scale. Blood draws were taken at baseline and on consecutive AL and CR days at the 2, 4 and 8 week time points

Evaluation of Asthma Symptoms and Pulmonary Function. Three different questionnaires were used. The Juniper mini-Asthma Quality of Life Questionnaire (mini-AQLQ) and the Juniper Asthma Control Questionnaire (ACQ) were completed baseline and end of study. The Asthma Symptom Utility Index (ASUI) was completed at baseline and every two weeks. The mini-AQLQ has 4 domains: symptoms, activity limitations, emotional function, and environmental stimuli. The ASUI has five domains, all of which are symptoms: cough, wheeze, dyspnea, sleeplessness, and medication side effects. The ACQ has six domains and spirometry. It measures degree of control of the disease, mainly with questions related to symptoms. Thus, the mini-AQLQ measures perceived QOL improvement and emotional response, whereas the ASUI and ACQ measure primarily symptoms. Scores for the mini-AQLQ and ACQ were analyzed using the package provided by Dr. Juniper. The ASUI was scored according to published methods[28]. Participants were trained in the use of the peak flow meter (mini-Wright by Ferraris). The best of three Peak Flow (PEF) measurements were recorded on awakening, during a 14 day baseline period and daily during the 58 day study period. Spirometry before and after albuterol was performed during baseline and at 8 weeks by a certified respiratory therapist using the Schilling spirometer (Model: Type SP-1) under the supervision of the pulmonologist. The best of three attempts was recorded before and after albuterol during baseline and at the end of the study.

Assessments of Hunger and Mood. A hunger/ mood/energy scale was created for this study because of anecdotal reporting by previous patients of improved mood and energy levels when on a similar diet, and the lack of a mood/energy level measure in existing asthma or psychological questionnaires. Subjects recorded the level of hunger and mood/energy for each two hour segment daily during baseline and throughout the study. The hunger scale ranged from 1 to 10 with 1 being "not at all hungry, the thought of food is distasteful" and 10 being "extremely hungry, never been hungrier". The mood/energy scale ranged from 1 to 10 with 1 being "lowest energy level ever" and 10 being "highest energy level ever"

Analyses of Serum Samples. Fasting blood samples were drawn after an overnight fast on consecutive AL and CR days (days 1, 2, 15, 16, 29, 30, 57 and 58). Samples taken on consecutive CR and AL days were analyzed in order to determine whether the variables being measured changed daily in response to the ADCR regimen. Most variables changed progressively with increasing time on the ADCR diet, but did not change acutely between consecutive AL and CR days. Serum lipids, insulin, glucose and C-reactive protein were measured in the clinical laboratory (Qwest Diagnostics, New Orleans, LA) using standard methods in samples drawn on days 1, 15, 29 and 57 (only days 1 and 57 were used for statistical analysis). Serum TNFα, BDNF, protein carbonyls, nitrotyrosine, 8-isoprostane, 4-hydroxynonenal adducts and ceramides were measured in samples drawn on days 1, 2, 15, 16, 29, 30, 57 and 58. TNFα levels were measured using a commercially available ultra-sensitive ELISA kit (Biosource Int., Camarillo, CA). Serum BDNF concentrations were measured using a commercially available ELISA kit (Promega, San Luis Obispo, CA). Levels of protein carbonyls, nitrotyrosine and 8-isoprostane were quantified using methods described previously[29, 30]. Levels of lysine and histidine adducts of 4-hydroxynonenal, and long-chain ceramides were measured by tandem mass spectrometry methods described previously[31]. Serum leptin and ghrelin concentrations were quantified using ELISA kits from Linco Research Inc. (St. Charles, MO) and Phoenix Pharmaceuticals (Belmont, CA), respectively. Concentrations of total ketone bodies (acetoacetate and 3-hydroxybutyrate) were measured using a Total Ketone Bodies kit (catalog no. 415-73301 & 411-73401) from Wako Diagnostics USA, Richmond VA, on a Roche Cobas Fara II robotic chemical analyzer according to the manufacturers specifications. The

Total Ketone Body calibrator set (catalog no. 412-73791) was used to produce the standard curve and the Total Ketone Body control (catalog no. 418-73891) was used to insure accuracy between assay runs. Uric acid was measured using a Uric Acid kit (catalog no. 237-60) from Diagnostic Chemicals Limited, Oxford, CT, on a Roche Cobas Fara II robotic chemical analyzer according to the manufacturer's specifications.

Statistical Analyses. For those measurements that were normally distributed, paired t-tests and Pearson's correlation coefficients were used for the analyses. Statistical comparisons of variables in serum samples during the course of the study with the baseline values were made using ANOVA and either the Student-Newman-Keuls or Bonferroni post-hoc tests. For non-normal measurements, Wilcoxon signed rank sum test and Spearman's correlation coefficients were used. Two-sided tests were used for all the comparisons and a p value of 0.05 or less was considered statistically significant and a p value of 0.01 or less was considered highly statistically significant. All the analyses were done using SAS version 9.1.

RESULTS

Alternate Day Calorie Restriction Improves Asthma Symptoms and Pulmonary Function

Of 40 responders to the newspaper advertisement, 23 met inclusion and exclusion criteria and fourteen agreed to enroll in the study. Of these, one died of unknown causes during the baseline, one dropped out due to a change in vacation plans during baseline, one decided not to continue during the first study week, and one dropped out the second study week due to work-related travel. Of the remaining 10, nine completed the study; one subject did not complete the study because she volunteered that she was non-compliant with the CR regimen. Subjects lost an average of 8%(8.5 kg.) of their body weight during the course of the study, confirming their adherence to the ADCR regimen (Fig. 1a). The perceived mood and energy of the subjects increased progressively during the first 3 weeks of the ADCR diet and remained significantly elevated for the duration of the study (Fig. 1b). Analysis of the hunger rating scale indicated that the subject's perceived hunger did not increase significantly over baseline values during the course of the study (Fig. 1c). There was a significantly higher level of hunger on CR days compared to the ad libitum days throughout the study. PEF increased by a highly significantly amount from a baseline level of 335 L/min to a level of 382 L/min during the first three weeks of

the ADCR period, and remained elevated throughout the 8 week study period (Fig. 1d) ($p< 0.009$ at 8 weeks). There were no significant differences between FEV1 (forced expiratory flow in 1 sec) values at baseline and at 8 weeks (Table 1). However, the FEV1 after albuterol administration was significantly greater at 8 weeks compared to baseline (Table 1), suggesting that the ADCR diet resulted in improved bronchial responsiveness.

There was also a highly significant improvement in the ASUI scores (0.25+- 0.17 ($p<0,002$) Table1;Fig 2a) which occured within 2 weeks and was maintained throughout the 8 week ADCR diet. The mini-AQLQ scores of the subjects were significantly higher in all four domains (asthma symptoms, activity limitations, emotional function and environmental stimuli) at the end of the study compared to baseline, demonstrating a beneficial effect of the ADCR diet on weight related or on asthma quality of life (Fig. 2b). The overall change in the mini-AQLQ was 2.1 ± 1.4 units ($p<0.004$) or 61%. Similarly, there were significant positive effects of the ADCR diet on the ACQ score which changed -1.3 ± 0.7 ($p<0.0015$) or 54%

Effects of ADCR on Markers of Lipid and Energy Metabolism in Asthma Patients

Body weight reduction in obese subjects is often associated with decreases in risk factors for cardiovascular disease and diabetes. We therefore measured concentrations of lipids (total cholesterol, LDL cholesterol, HDL cholesterol and triglycerides), C-reactive protein (CRP), glucose and insulin in serum samples taken at baseline and after 8 weeks on the ADCR diet. Levels of total cholesterol and triglycerides were significantly lower at 8 weeks compared to baseline, while levels of HDL cholesterol were significantly increased at 8 weeks (Fig. 3a; Table 2). The ADCR diet had no significant effect on serum levels of LDL, glucose, insulin or CRP (Table 2).

The body weight of subjects on the ADCR diet decreased progressively suggesting that they were compliant with the diet throughout the study. To confirm compliance and to provide insight into the effects of the ADCR diet on energy metabolism we measured concentrations of ketone bodies (acetoacetate and 3-hydroxybutyrate) in serum samples taken on consecutive ad libitum and CR days at baseline and at 2, 4 and 8 weeks. Levels of ketone bodies reliably increase during extended periods of fasting or caloric restriction[32]. We found that levels of ketone bodies were elevated 4-6 fold on CR days compared to ad libitum days, consistent with adherence of the subjects to the diet (Fig. 3b). There was a significant increase in levels of 3-

hydroxybutyrate on ad libitum days at 4 and 8 weeks of the ADCR regimen compared to baseline levels (Fig. 3b). Levels of circulating leptin increase in the fed state and suppress appetite, whereas ghrelin levels increase during fasting and increase appetite[33]. We found that leptin levels were lower on CR days compared to AL days throughout the study, and there was a progressive decrease in leptin levels on AL days during the 8 week diet period (Fig. 3c). In the case of ghrelin there was a transient increase in levels on the AL day at the 2 week diet point, but ghrelin levels were not significantly affected by diet on either AL or CR days at the 4 and 8 week time points (Fig. 3d). There were no significant differences in ghrelin levels on AL compared to CR days at baseline, 4 and 8 weeks.

ADCR Reduces Markers of Inflammation and Oxidative Stress in Asthma Patients

The concentration of TNFα in serum was unchanged after 2 weeks on the ADCR diet. However, there was a highly significant reduction in serum TNFα levels in the CR day sample at 4 weeks, and in both the ad libitum and CR samples at 8 weeks (Fig. 4a). There was a significant decrease in circulating BDNF levels that occurred within the first 2 weeks of the dietary intervention, decreased further at 4 weeks and remained low at 8 weeks (Fig. 4b). Ceramides are liberated from membrane sphingomyelin in response to inflammatory cytokine receptor activation and oxidative stress and levels of ceramides are elevated in affected tissues and body fluids in several inflammatory and infectious diseases[34-36]. Levels of ceramides C16:0, C18:0, C22:0 and C24:1 were significantly decreased on both ad libitum and CR days within 2 weeks of ADCR diet initiation and remained at the lower levels for the duration of the 8 week period (Fig. 4c). These reductions in levels of circulating TNFα, BDNF and ceramides in response to the ADCR diet, suggests that this dietary intervention reduces inflammation in asthma patients

Levels of protein carbonyls, a measure of protein oxidation, decreased significantly on both ad libitum and CR days within 2 weeks of diet initiation, continued to decrease through 4 weeks and remained low through 8 weeks (Fig. 5a). Progressive and highly significant decreases in serum levels of nitrotyrosine and 8-isoprostane also occurred during the course of the 8 week ADCR diet period (Fig. 5b, c). Levels of histidine and lysine 4-hydroxynonenal adducts were progressively and significantly decreased during the course of the 8 week ADCR diet period; levels of these adducts were decreased on both ad libitum and CR days (Fig. 5d). The

magnitude of the decreases in each marker of oxidative stress were large; at the end of the 8 week study levels of protein carbonyls and 8-isoprostane were less than 20% of baseline levels, levels of nitrotyrosine were less than 10% of baseline values, and levels of 4-hydroxynonenal adducts decreased by approximately 50% (Fig. 5). Finally, we measured levels of uric acid, a major antioxidant scavenger of hydroxyl radical and peroxynitrite[37], in serum samples from the subjects. Uric acid levels increased significantly (by approximately 20%) within 2 weeks of ADCR diet initiation and remained elevated through 8 weeks (Fig. 6), consistent with less oxidative stress.

DISCUSSION

Nine of the 10 asthma subjects who began the ADCR regimen complied with the diet, as indicated by progressive weight loss, and completed the study. All 9 subjects exhibited improved asthma symptoms, control and quality of life, demonstrating a clinical benefit of the ADCR diet. An improvement of ACQ or mini AQLQ score of 0.5 is considered clinically important and has been repeatedly shown to be useful in research and management of individual asthma patients. In a recent clinical study of 1414 asthma patients newly started on either fluticosone proprionate or montelucast an improvement in ACQ and mini-AQLQ scores of >1 unit or 47% and 25% respectively was observed[38]. Our ADCR study recorded a 54% improvement in ACQ and 61% improvement in mini- AQLQ in patients already on baseline controller therapy. Although medical and surgical induced weight loss is also associated with similar degrees of quality of life improvement (SF-36) our patients also demonstrated improvement in asthma specific control (ACQ) and symptoms (ACUI) score. Our study demonstrated a 0.25 improvement in ASUI within 3-4 weeks, when weight loss was only 4%. In studies using the ASUI a change of >0.25-0.3 is associated with a clinically detectable difference in asthma severity classification Although all these scoring systems could be linked simply to weight loss the rapid change associated with change in inflammatory markers are consistent with improvement in asthma burden. The improvement in PEF of 44.6 L/min+-3.8 in our study is consistent with the improvement usually observed after "optimizing" controller medications in mild/moderate asthmatics. Although major weight loss (13%) is known to result in some improvement in pulmonary function, our PEF improvement occurred within 3-4 weeks when weight loss was 4%; PEF thereafter remained constant, whereas

continued through 8 weeks, suggesting that the change in pulmonary function in response to the ADCR diet was not due solely to weight loss.

The significant increase in the FEV1 after albuterol in the subjects during the ADCR diet compared to baseline suggests an effect of the ADCR diet on airway smooth muscle responsiveness, consistent with an anti-inflammatory effect. In a study of 58 obese women losing >13% of body weight over six months, there was no change in response to metacholine challenge[5], suggesting that the changes were independent of airway reactivity. Although we did not evaluate methacholine responsiveness, the improved airway response to bronchodilators should not be caused by weight loss per se and is consistent with an anti-inflammatory response from the ADCR diet.

Particularly striking were the reductions in levels of TNFα, BDNF and markers of oxidative stress (protein carbonyls, nitrotyrosine and 8-isoprostane) in the serum of the asthma patients during the course of the ADCR diet period. Levels of these markers of inflammation and oxidative stress were decreased on both ad libitum and CR days, indicating a sustained effect of the ADCR diet that did not fluctuate in response to the level of energy intake on the day prior to blood sampling. The decreased levels of TNFα and BDNF suggest that ADCR suppresses inflammation, which may contribute to the beneficial effects of ADCR on asthma symptoms and hyperresponsiveness. Indeed, studies of asthma patients and animal models of asthma have provided evidence that TNFα[10, 12] and BDNF[16, 39] are important mediators of airway inflammation and associated symptoms. It was previously reported that levels of protein carbonyls, nitrites and nitrates, and lipid peroxidation products were increased in plasma from patients with bronchial asthma compared to control subjects[40]. The consistent and progressive decrease in levels of oxidative stress in our subjects may therefore be a marker of, or to have contributed to, the improvement in symptoms on the ADCR diet. The striking reduction in markers of oxidative damage which we observed have not been described in daily calorie restriction studies. Other authors have reported modest or non-significant changes in levels of protein carbonyls with various CR regimes[41, 42, 43]. Similarly, in a previous weight loss study nitrotyrosine levels declined 23% in the Caucasian women and remained unchanged in African American women[44], suggesting that different groups of subjects exhibit differential reductions in oxidative stress in response to weight loss.

The mechanism(s) by which ADCR reduces oxidative stress and inflammation in asthmatic subjects remains to be established. However, based upon previous studies of the effects of alternate day fasting on cellular physiology in rodents, two general mechanisms are likely. First, because subjects on ADCR exhibit a reduction in overall energy intake and lose weight, there is likely a reduction in cellular oxygen free radical production[24, 41, 42]. The latter effect of ADCR would be associated with lower levels of oxidatively modified proteins and lipid peroxidation products in the blood. Second, ADCR may impose a mild beneficial stress, to which cells respond adaptively by up-regulating the expression of antioxidant systems. Such increased cellular stress resistance has been shown occur in rodents on an alternate day energy restriction regimen, resulting in increased disease resistance[24]. It will be of considerable interest to determine the effects of ADCR on gene expression in tissue involved in the pathogenesis of asthma.

We found that serum leptin levels were lower in subjects on CR compared to AL days throughout the 8 week study period, and that leptin levels on AL days decreased progressively during the 8-week study period. Leptin has been shown to exert pro-inflammatory actions[45], and it is therefore possible that the reduction in leptin levels contribute to the anti-inflammatory effects of the ADCR diet. On the other hand, the ADCR diet did not significantly affect circulating levels of this hormone, a result consistent with our evidence that the ADCR does not result in a sustained overactivation of the hunger response.

Humans are unable to consistently comply with a long-term daily caloric reduction of 40% (consuming 60% of maintenance), as has been used in most animals studies to date. The authors of a recent three week trial in which 16 volunteers alternated eating ad lib for 24 hours and nothing the next 24 hours concluded that, due to persistent hunger and irritability, it was unlikely subjects would stay on the regime for extended periods of time[46]. We designed the ADCR pattern of eating intended as an accommodation to human needs and adaptation to human meal pattern of the alternate day total fasting pattern used in rodent studies. When rats or mice are maintained on an alternate day fasting regimen they maintain body weights 10-25% lower than ad libitum fed control animals, live up to 30% longer and exhibit improvement in a range of health indicators[47]. A regimen which allows ad libitum feeding on one day and reduced food/caloric intake on the next day (for longer periods of time), whereby a stable weight is maintained, may prolong lifespan and healthspan in humans[48]. Low levels of oxidative stress

may be necessary to reach very old age; at least two studies have shown lower oxidative stress in centenarians than in 70 year olds[49, 50].

In our study, the ADCR pattern of eating consisted of repeating cycles of a (approximately) 36 hour period of very low calorie intake and a 12 hour period of AL eating was tolerable and efficacious in treating asthma symptoms, at least in obese subjects. Larger studies that include a control group or a cross-over design with measures of airway reactivity and inflammation will be required to further elucidate the full impact of ADCR diets on obese asthma patients. Further studies to improve asthma outcome are desirable since current therapies do not seem to modify the underlying process or factors that determine disease progression. It will also be important to determine if such diets benefit patients with other disorders that involve inflammation and oxidative stress such as atherosclerotic heart disease[51].

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Figure Legends

Figure 1. Asthma subjects lose weight and exhibit improved mood and peak airflow when maintained on and alternate day calorie restriction diet. Body weights (a), mood/energy scores (b), hunger scores (c) and peak expiratory flow (d) were measured at baseline and at the indicated time points during the 2 month ADCR period.

Figure 2. Alternate day calorie restriction results in improved symptoms in subjects with asthma. a. MiniAQLQ scores for four domains (symptoms, activity limitations, emotional function and environmental stimuli) in subjects at baseline and after 8 weeks of ADCR. The differences between the 8 week and baseline values were significantly different for each of the four domains ($p<0.004$). b. ASUI scores increased rapidly and significantly ($p<0.003$) within 2 weeks of diet initiation.

Figure 3. Alternate day calorie restriction results in changes in lipid and energy metabolism indicative of improved health in asthma subjects. Levels of total cholesterol (a), 3-hydroxybutyrate (b), leptin (c) and ghrelin (d) were measured in serum samples from asthma subjects on successive ad libitum (AL) and CR days at baseline and at 2, 4 and 8 weeks of ADCR. *$p<0.05$, **$p<0.01$ compared to the baseline value; ##$p<0.01$ compared the corresponding CR value.

Figure 4. Markers of inflammation are reduced in asthma subjects in response to the ADCR diet. Levels of TNF-α (a), BDNF (b) and ceramides (c) were measured in serum samples from

asthma subjects on successive ad libitum (AL) and CR days at baseline and at 2, 4 and 8 weeks of ADCR. *$p<0.05$, **$p<0.01$, ***$p<0.001$ compared to the baseline value.

Figure 5. Markers of oxidative stress are reduced in asthma subjects in response to the ADCR diet. Levels of total protein carbonyls (a), nitrotyrosine (b), 8-isoprostanes (c) and lysine and histidine adducts of 4-hydroxynonenal (d) were measured in serum samples from asthma subjects on successive ad libitum (AL) and CR days at baseline and at 2, 4 and 8 weeks of ADCR. *$p<0.05$, **$p<0.01$, ***$p<0.001$ compared to the baseline value.

Figure 6. Levels of the antioxidant uric acid are increased in asthma subjects in response to the ADCR diet. Levels of uric acid were measured in serum samples from asthma subjects on successive ad libitum (AL) and CR days at baseline and at 2, 4 and 8 weeks of ADCR. *$p<0.05$ compared to the baseline value.

Table 1: Results of analyses of pulmonary variables

Variable	Baseline	After 8 weeks	Change	P value
Peak Flow (L/min)	334.7 ± 26.0	379.3 ± 27.9	14.4 ± 4.1 (%)	0.0081
FEV1 (predicted)	67.4 ± 5.7	69.8 ± 5.3	5.3 ± 3.7 (%)	0.2152
FEV1 (predicted after Albuterol)	71.9 ± 4.8	77.5 ± 4.0	10.5 ± 5.1 (%)	0.0156
FEV1 (Albuterol) – FEV1	4.4 ± 1.6	7.8 ± 1.7	3.5 ± 1.3	0.0425
Mini-AQLQ	3.4 ± 0.3	5.6 ± 0.3	2.1 ± 0.5	0.0039
ACQ	2.4 ± 0.3	1.0 ± 0.1	-1.3 ± 0.7	0.0015
ASUI	0.66 ± 0.20	0.91 ± 0.10	0.25 ± 0.17	0.0022

Table 2: Results of analyses of non-pulmonary variables

Variable	Baseline	After 8 weeks	Change	P value
Weight (kg)	104.9 ± 6.2	96.4 ± 5.5	-8.5 ± 1.7	0.0011
Weight (%)			-8.0 ± 1.4 (%)	0.0009
Total Cholesterol	204.1 ± 7.9	183.6 ± 7.1	-9.3 ± 4.0 (%)	0.0480
Triglyceride	279.3 ± 105.4	161.0 ± 40.5	-118.3 ± 66.8	0.0391
HDL	44.0 ± 5.6	48.1 ± 5.9	4.1 ± 1.3	0.0111
LDL	116.8 ± 9.5	103.4 ± 11.4	-10.5 ± 8.9	0.4295
Trig/HDL	9.3 ± 4.3	4.6 ± 2.0	-4.6 ± 2.4	0.0273
HDLC	4.9 ± 0.6	4.3 ± 0.5	-0.9 ± 0.3	0.0202
Glucose	75.3 ± 6.9	80.4 ± 3.6	5.1 ± 4.3	0.2679
CRP	4.6 ± 0.8	5.6 ± 1.1	1.0 ± 0.9	0.2777
Insulin	23.7 ± 12.4	14.9 ± 3.3	-8.8 ± 9.9	0.6797

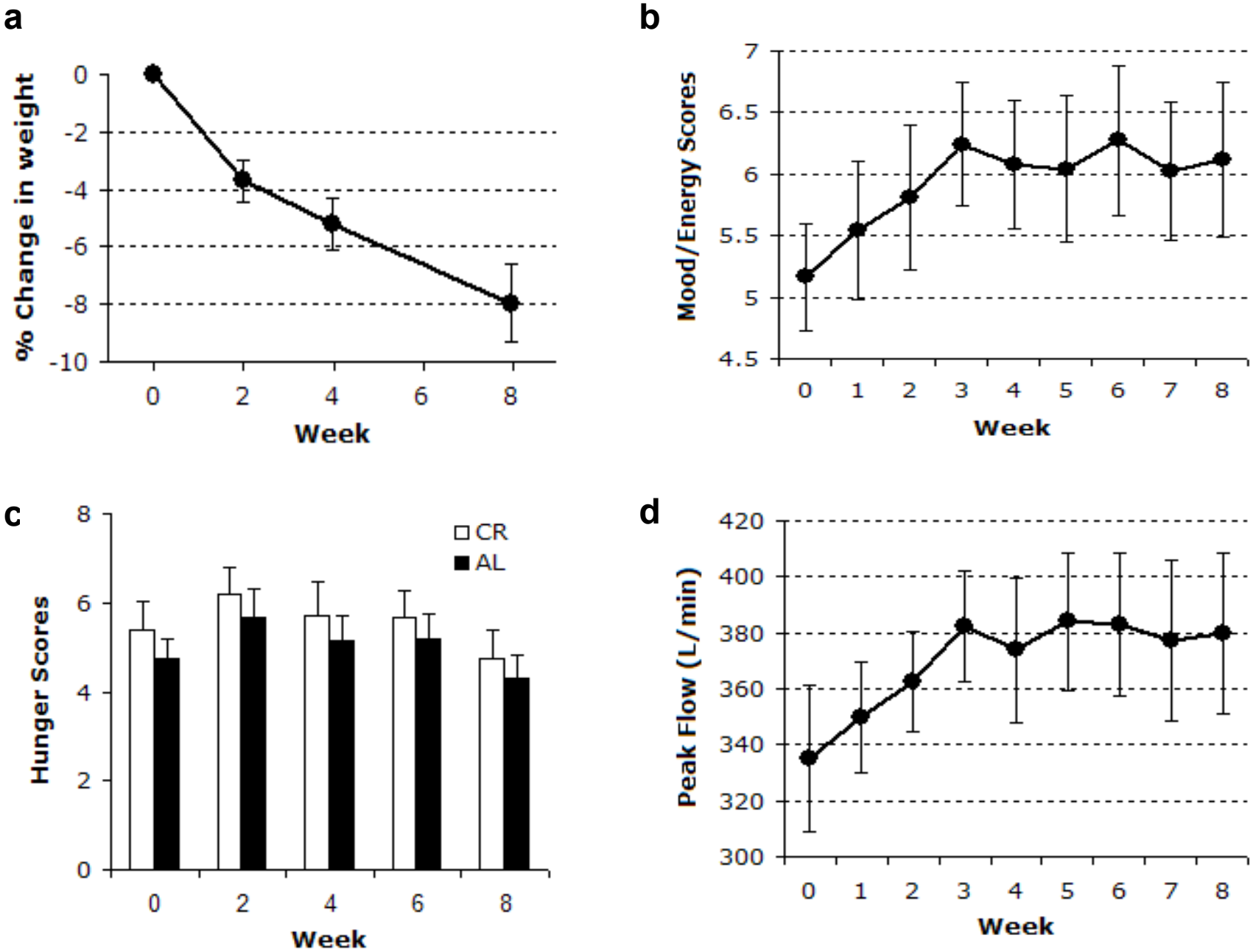
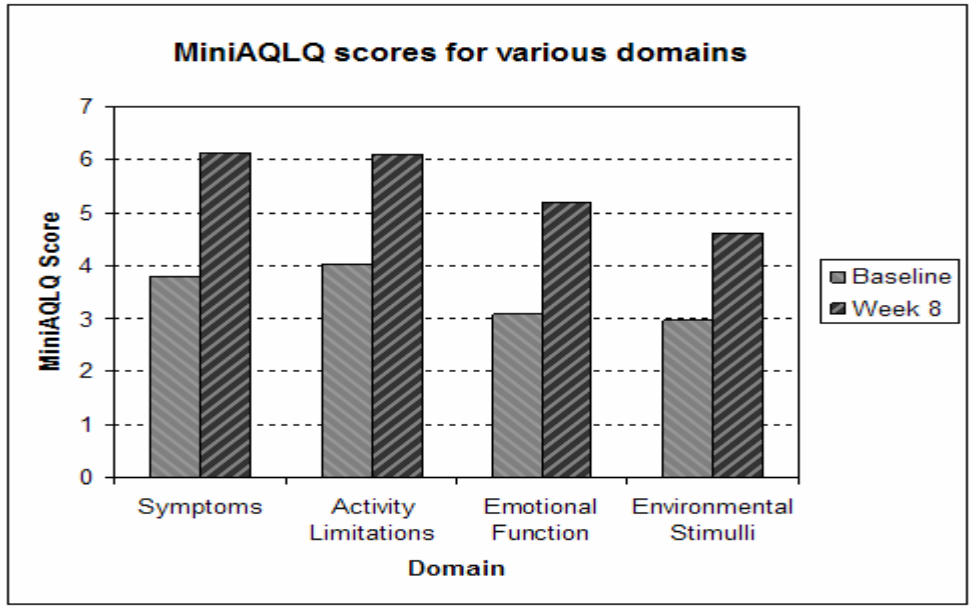

a
% Change in weight
0
-2
-4
-6
-8
-10
0
2
4
6
8
Week
b
Mood/Energy Scores
7
6.5
6
5.5
5
4.5
0
1
2
3
4
5
6
7
8
Week
c
Hunger Scores
8
6
4
2
0
CR
AL
0
2
4
6
8
Week
d
Peak Flow (L/min)
420
400
380
360
340
320
300
0
1
2
3
4
5
6
7
8
Week

Figure 2

a


MiniAQLQ scores for various domains
MiniAQLQ Score
0
1
2
3
4
5
6
7
Symptoms
Activity Limitations
Emotional Function
Environmental Stimulli
Domain
Baseline
Week 8

b

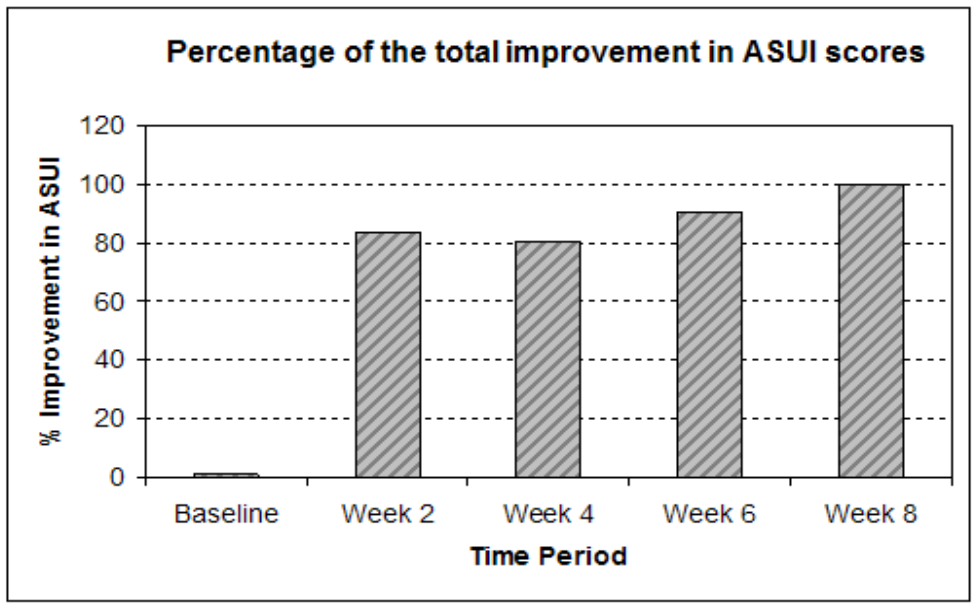
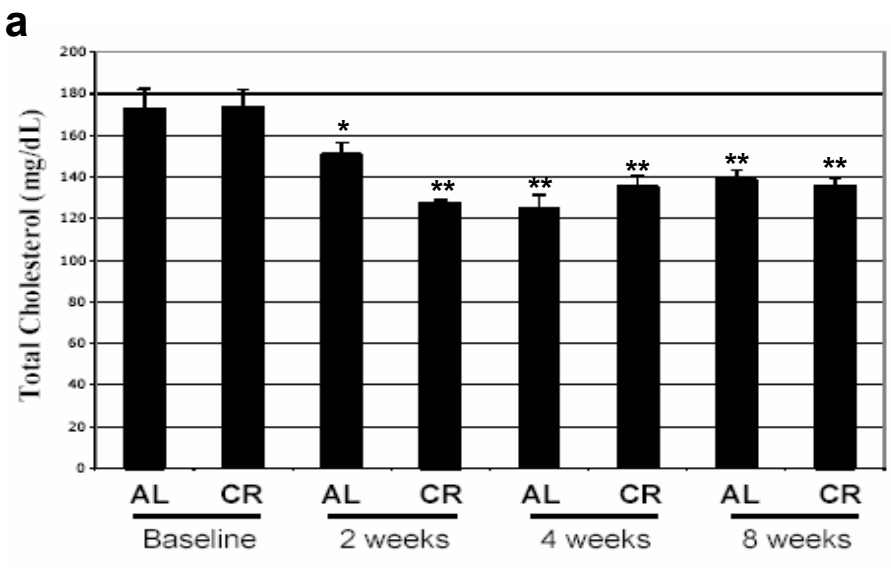
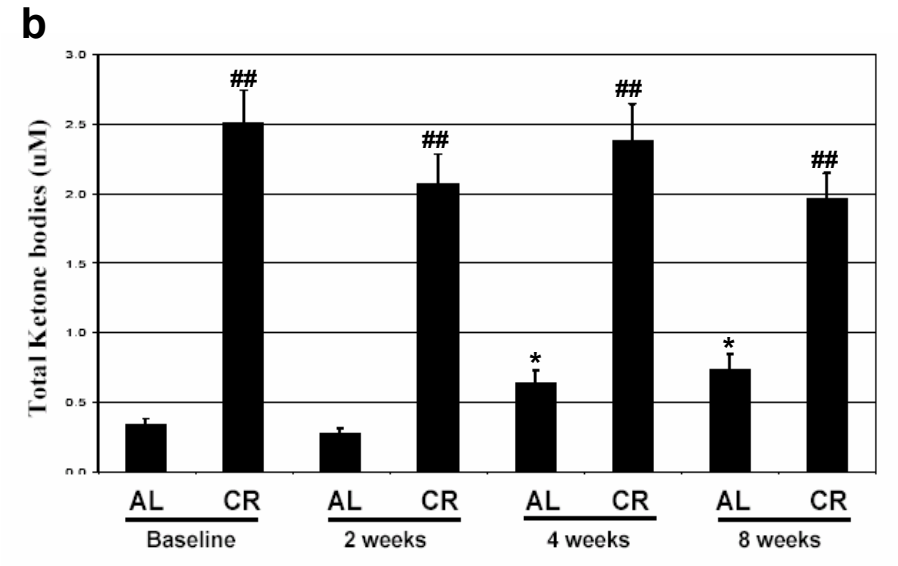
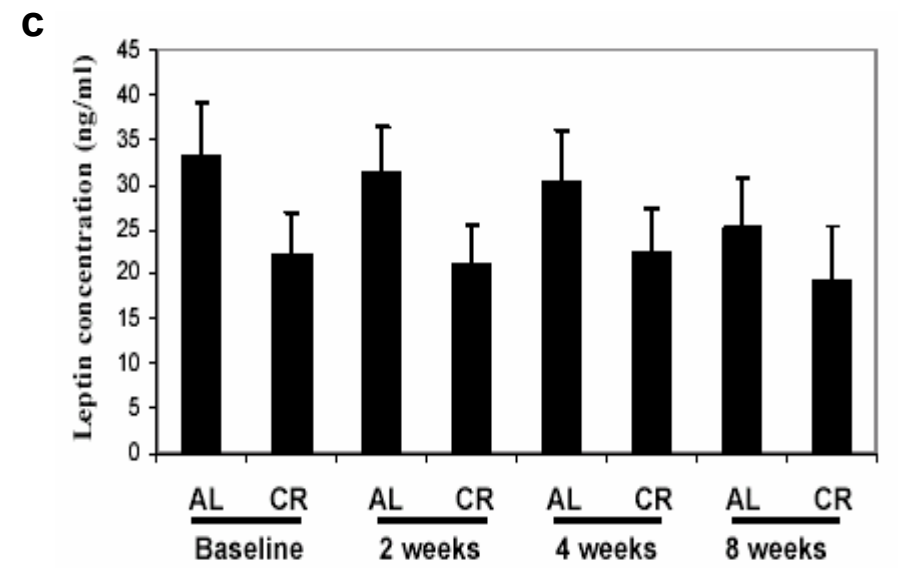

Percentage of the total improvement in ASUI scores
% Improvement in ASUI
0
20
40
60
80
100
120
Baseline
Week 2
Week 4
Week 6
Week 8
Time Period

Figure 3


a
Total Cholesterol (mg/dL)
200
180
160
140
120
100
80
60
40
20
0
*
**
**
**
**
**
AL CR AL CR AL CR AL CR
Baseline
2 weeks
4 weeks
8 weeks


b
Total Ketone bodies (uM)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
##
##
##
##
*
*
AL CR AL CR AL CR AL CR
Baseline
2 weeks
4 weeks
8 weeks


c
Leptin concentration (ng/ml)
45
40
35
30
25
20
15
10
5
0
AL CR AL CR AL CR AL CR
Baseline
2 weeks
4 weeks
8 weeks

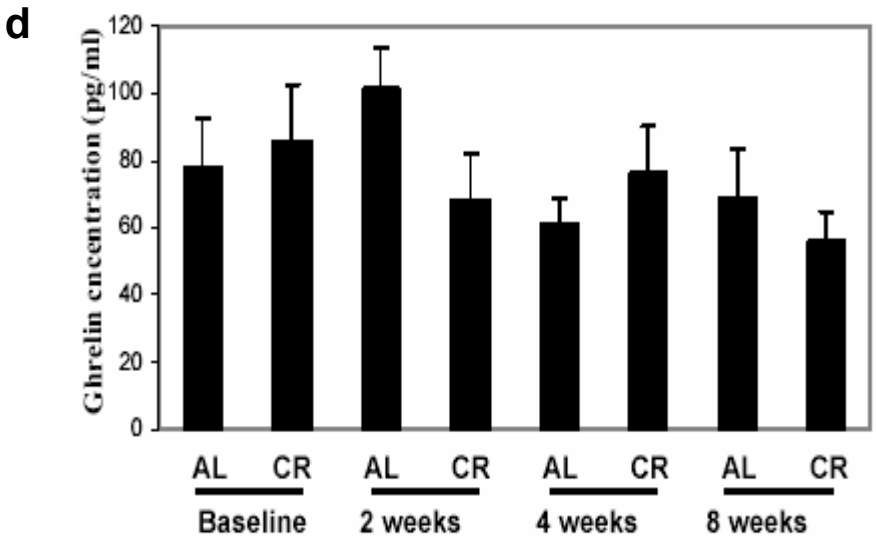

d
Ghrelin encentration (pg/ml)
120
100
80
60
40
20
0
AL CR AL CR AL CR AL CR
Baseline
2 weeks
4 weeks
8 weeks

Figure 4a, b

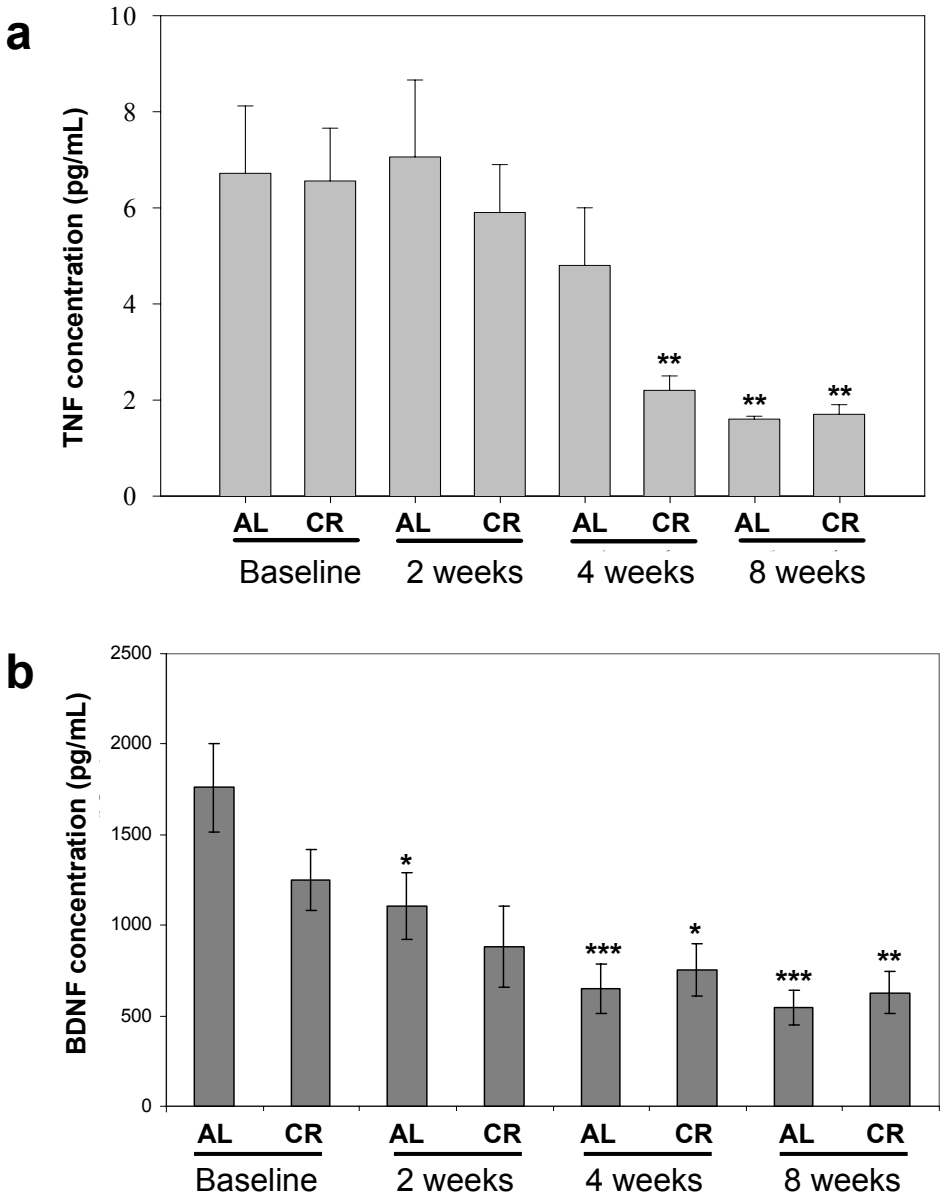

a
TNF concentration (pg/mL)
10
8
6
4
2
0
**
**
**
AL CR AL CR AL CR AL CR
Baseline
2 weeks
4 weeks
8 weeks
b
BDNF concentration (pg/mL)
2500
2000
1500
1000
500
0
*
***
*
***
**
AL CR AL CR AL CR AL CR
Baseline
2 weeks
4 weeks
8 weeks

Figure 4c

c

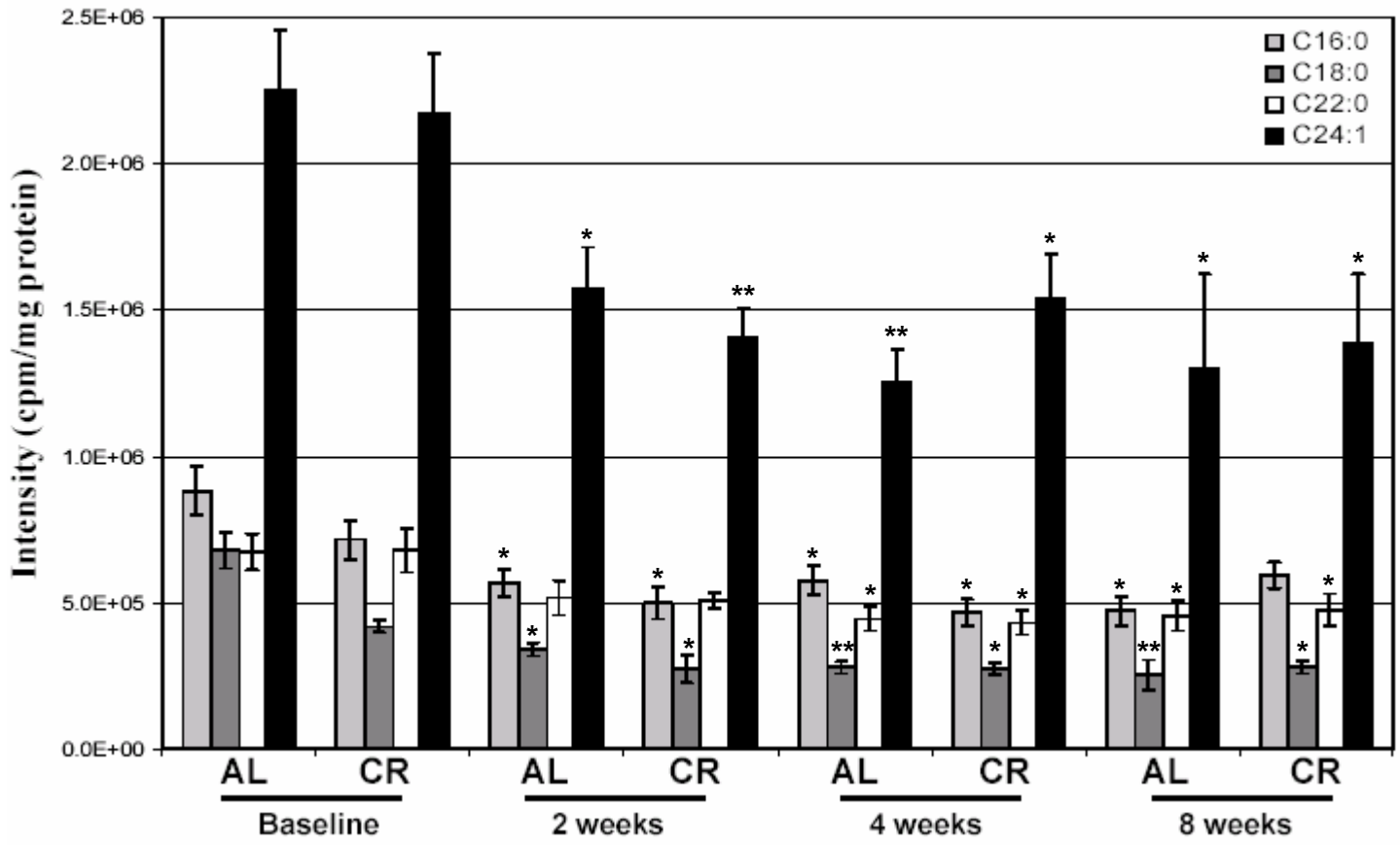

C16:0
C18:0
C22:0
C24:1
Intensity (cpm/mg protein)
2.5E+06
2.0E+06
1.5E+06
1.0E+06
5.0E+05
0.0E+00
AL
CR
Baseline
2 weeks
4 weeks
8 weeks

Figure 5a, b, c

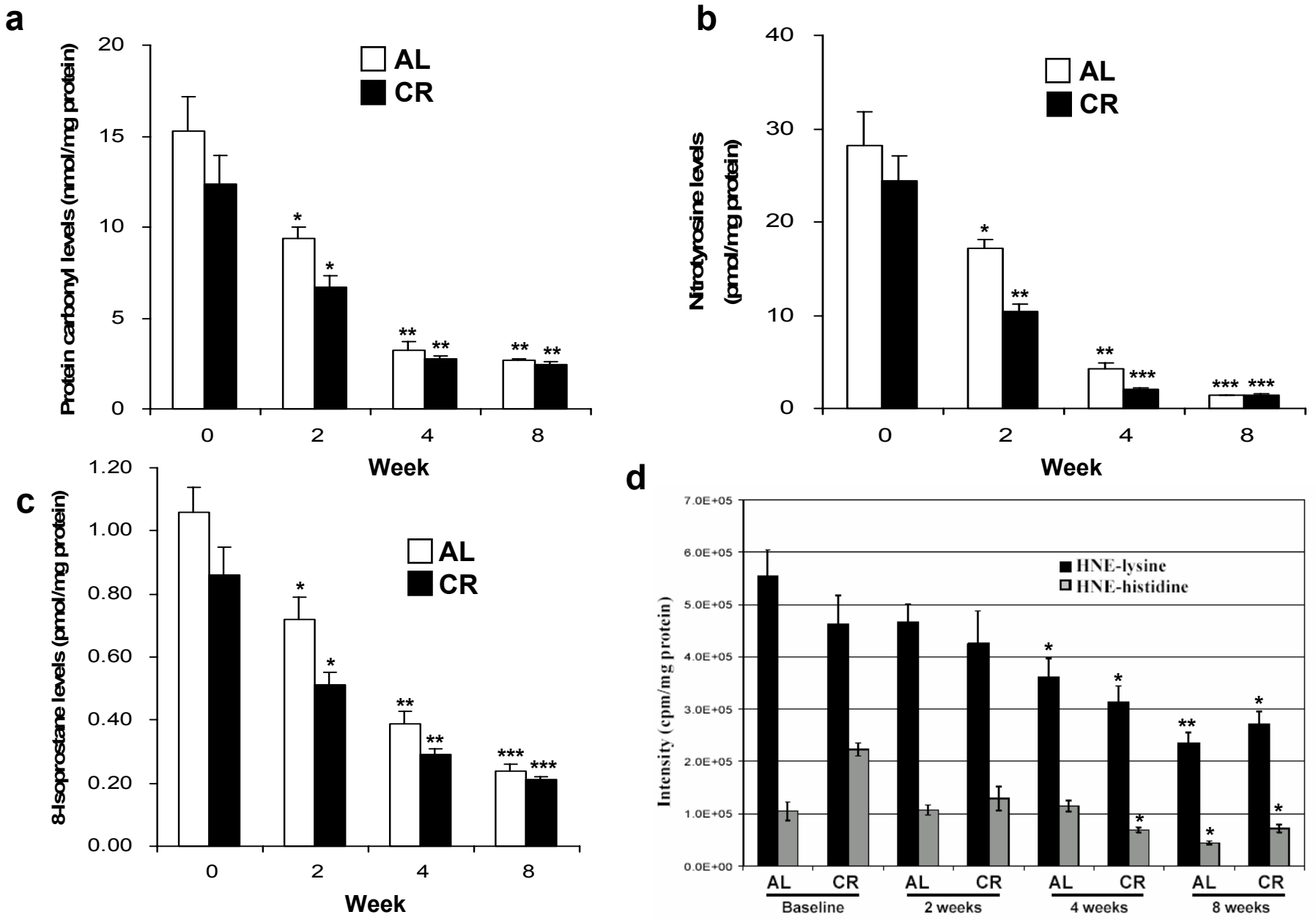
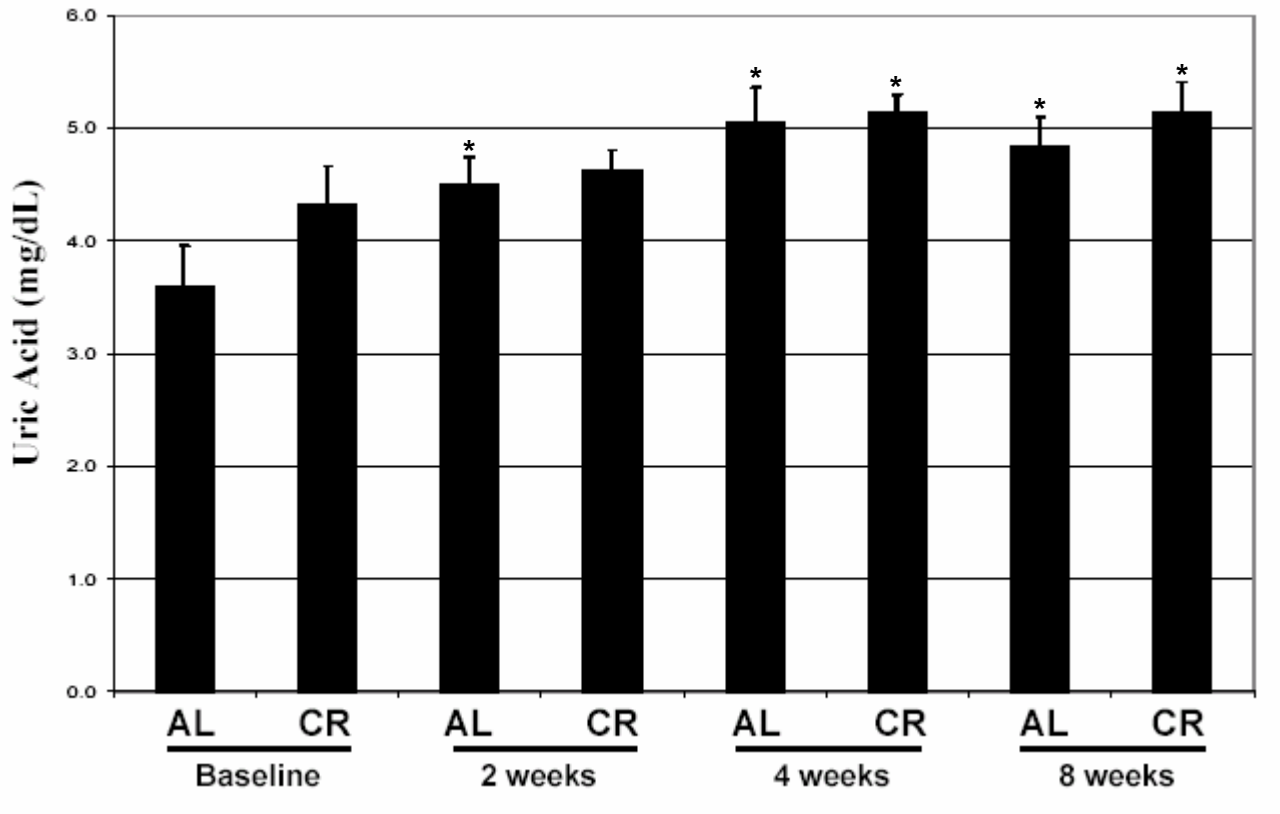

a
Protein carbonyl levels (nmol/mg protein)
AL
CR
Week
b
Nitrotyrosine levels (pmol/mg protein)
AL
CR
Week
c
8-Isoprostane levels (pmol/mg protein)
AL
CR
Week
d
Intensity (cpm/mg protein)
HNE-lysine
HNE-histidine
AL
CR
Baseline
2 weeks
4 weeks
8 weeks

Figure 6


Uric Acid (mg/dL)
6.0
5.0
4.0
3.0
2.0
1.0
0.0
AL
CR
AL
CR
AL
CR
AL
CR
Baseline
2 weeks
4 weeks
8 weeks
*
*
*
*
*

Charity Houston
Free Radical Biology and Medicine

Dear Charity,

I am writing this to serve as a cover letter for the submission of our revised article FRBM-D-06-00742 with Response to Reviewers attached.

Sincerely,

James B. Johnson, M.D.