The anti-inflammatory properties of conjugated linoleic acid and *trans*-11 vaccenic acid in immune mediated inflammatory disease

by

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Abstract

The prevalence and economic stain of immune mediated inflammatory diseases (IMID) is rising. To combat the population's reliance on costly and potentially toxic pharmaceutical treatment, natural dietary interventions have gained popularity. Conjugated linoleic acid (CLA) and *trans*-11 vaccenic acid (TVA) are two abundant fatty acids in dairy fat that have been suggested as effective dietary treatment options for IMID. There are potential health and cost savings associated with increased population health through the exploration of CLA and TVA as dietary therapies to prevent IMID altogether. The ability of these fatty acids to prevent IMID has barely started to be explored.

IMID prevention studies with CLA have only used preparations containing a mixture of the two common CLA isomers, CLA *trans*-10, *cis*-12 and CLA *cis*-9, *trans*-11. This combination does not mimic typical dietary CLA consumption, and some of CLA's effects are known to be isomer specific. Therefore, studies in this thesis were conducted with pure isomer CLAt10c12 or CLAc9t11 preparations. To model IMID, this thesis utilizes the murine collagen-induced arthritis (CA) model as a model of rheumatoid arthritis. Mice in this model are immunized twice, three weeks apart, with chick collagen type II. For nine weeks after the secondary immunization mice are monitored for arthritis symptoms, disease severity, anti-collagen antibody production, plasma and paw cytokine levels, and hepatic fatty acid accumulation.

An initial experiment was performed in which 0.25% CLAt10c12 and 0.5% CLAc9t11 were fed to mice in the CA model starting either before the primary (CLAc9t11) or at the secondary (CLAt10c12) immunization. Results from this study suggest that the CLA isomers may differentially regulate arthritis incidence and severity through the regulation of T helper cell (Th)1 or Th2 pathways. CLAt10c12 increased disease severity when fed starting at the secondary

immunization while CLAc9t11 prevented CA onset and increased the Th2 cytokines interleukin (IL)-4 and IL-10.

In a follow up study, CLAt10c12 and CLAc9t11 were directly compared by feeding mice dietary treatments containing 0.5% CLAt10c12 or CLAc9t11 starting three weeks before any collagen immunization. Interestingly, both isomers decreased the incidence of CA by at least 39%, and CLAt10c12 further reduced disease severity in mice that did develop arthritis. It was hypothesized that isomer specific polarization of T cell mediated immunity and attenuation of the arachidonic acid cascade may have been responsible for these effects.

Humans typically consume CLA through dairy fat, which heavily favors the CLAc9t11 isomer. Diary fat also contains abundant levels of TVA, the metabolic precursor to CLAc9t11. TVA deceases CA severity when fed after disease onset, but it is unknown if these effects are due to TVA itself or because of its conversion to CLAc9t11. A study was performed in which arthritic mice were fed 0, 0.25, 0.5, or 1% dietary TVA with and without the presence of a delta-9 desaturase inhibitor, cottonseed oil. This study demonstrated that TVA may have a lower than previously identified anti-inflammatory dose, and its effects on CA are linked to its conversion to CLAc9t11. A final study was conducted to assess the ability of a 0.5% dietary TVA to prevent CA. TVA reduced arthritis severity 29%, and had similar effects on CA as CLAc9t11.

CLAt10c12, CLAc9t11, and TVA decreased CA incidence by a minimum of 29% when fed prior to disease onset. CLAt10c12 was the only dietary treatment that decreased disease severity in mice with active CA. A lower minimum effective dose of 0.25% TVA was identified in mice treated after CA onset, but further exploration into minimally effective doses of these fatty acids in both treatment and prevention of IMID is require. These findings further support

the role dairy fat, enriched for maximum CLA and TVA content, can play as both a treatment and preventative for IMID.

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Abbreviations List

AA: arachidonic acid

AD: days post arthritis development

CLA: conjugated linoleic acid

CLAc9t11: conjugated linoleic acid *cis*-9, *trans*-11

CLAt10c12: conjugated linoleic acid *trans*-10, *cis*-12

CA: collagen-induced arthritis

CAS: clinical arthritic score

CO: corn oil

COX: cyclooxygenase

CSO: cottonseed oil

CII: collagen type II

c9t11: *cis*-9, *trans*-11

DGLA: di-homo gamma linoleic acid

DHA: docosahexaenoic acid

EDTA: ethylenediaminetetraacetic acid

EPA: eicosapentaenoic acid

FAME: fatty acid methyl esters

FITC: fluorescein isothyocyanate-dextran

IFN: interferon

Ig: immunoglobulin

IL: interleukin

IMID: immune mediated inflammatory diseases

HRP: horseradish peroxidase

LA: linoleic acid

NF: nuclear factor

ND: non-developed

NSAID: non-steroidal anti-inflammatory drug

NZB/W F1: F1 hybrid New Zealand Black and White cross

n/d: not detected

PAS/AB: Periodic acid-Schiff and Alcian Blue staining

PG: prostaglandin

PPAR: peroxisome proliferator-activated receptor

RA: rheumatoid arthritis

SCD-1: stearoyl CoA desaturase

SEM: standard error of the mean

TFF: trefoil factor

Th: T helper cell

TNF: tumor necrosis factor

TVA: *trans*-11 vaccenic acid

t10c12: *trans*-10, *cis*-12

2°: secondary

Chapter 1:

A review of the literature:

History of conjugated linoleic acid and *trans*-11 vaccenic acid as treatments for immune mediated inflammatory diseases and their futures as preventative therapies.

1.1 Introduction

Immune mediated inflammatory diseases (IMID) are a type of chronic inflammatory conditions that can be detrimental to human health, and often go undiagnosed or untreated. Approximately 32% of the United States population suffers from chronic inflammation, characterized by non-resolving activation of the immune system [1]. IMID are propagated by both the innate and adaptive immune systems, which can inhibit the body's ability to maintain homeostasis [2]. This review will focus on the autoimmune condition rheumatoid arthritis (RA), as a model for IMID. RA is characterized by persistent swelling of the synovial joints and is associated with weight loss, disability, and mortality [3]. RA affects between 0.5 and 1% of the population in developed nations [4], and is a major health and economic burden. There is a 60 to 70% increase in mortality risk in RA patients compared to the general population [5], and, in 2005, the annual societal cost of RA was estimated at $39.2 billon [6]. The extensive morbidity and costs associated with RA and other IMID make finding effective treatments essential, especially considering these diseases require chronic care management.

Murine collagen-induced autoimmune arthritis (CA) provides a mode for RA, as immune activation is brought on by a loss of self-tolerance at a targeted tissue and, if untreated, inflammation will continue unresolved. In the CA model, mice are immunized against chick collagen type II resulting in the development of arthritis [7]. The CA model was instrumental in the development of pharmaceuticals including anti-tumor necrosis factor (TNF) α [8], anti-interleukin (IL)-1 [9], and some non-steroidal anti-inflammatory (NSAID) drugs [10]. These drugs can have significant side effects and are not recommended for prolonged use [11]. In addition to the negative side effects of biological or pharmaceutical agents, the high prevalence

and economic strain of RA increases the need for interventions, like dietary treatment, that are more cost effective and have less adverse health effects.

Initially omega-3 polyunsaturated fatty acids, including eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), were explored as dietary treatments for IMID [12]. These fatty acids incorporate into membranes at the expense of arachidonic acid (AA), and compete with the enzymes responsible for the metabolism of AA to inflammatory mediators [13, 14]. EPA and DHA also decrease leukocyte chemotaxis, expression of major histocompatibility complex II, and production of pro-inflammatory cytokines [15–17]. RA clinical trials with dietary fish oils, which have high EPA and DHA content, reported an average decrease of 26% in arthritic severity [18–20], and a reduction in NSAID usage [21]. An anti-inflammatory threshold of 2g/day of EPA and DHA through fish oil supplementation has been suggested [13]. The high quantity of fish oil required for an anti-inflammatory benefit along with the associated cost, amount of pills, and fishy taste results in a low compliance rate for EPA and DHA supplementation [22, 23], and has increased interest in exploring other anti-inflammatory dietary treatment options.

The use of conjugated linoleic acid (CLA) and dairy fat as dietary anti-inflammatory agents has increased over recent years. CLA typical refers to a 50:50 mix of the two most prevalent isomers, CLA *trans*-10, *cis*-12 (t10c12) and CLA *cis*-9, *trans*-11 (c9t11) [24]. This mixed isomer CLA is the form commonly sold as a health supplement marketed for weight loss [25]. CLA is also consumed through the intake of ruminant lipids, which contain CLAt10c12 at 1.4 - 3.5% and CLAc9t11 at 96.5 - 98.6% of total CLA [26]. CLA was first identified in dairy fat in 1933 by Booth *et al*. [27] because of its interference with determination of vitamin A content. In 1987, Mike Pariza, at the University of Wisconsin-Madison, identified anti-carcinogenic

properties of CLA [28]. The effects of CLA on immune modulation were first identified by Mark Cook and Mike Pariza in 1994, when day old chicks fed 0.5% CLA gained weight and had elevated immune responses to sheep red blood cell immunization and endotoxin challenge, compared to chicks fed a corn oil (CO) or fish oil based diet [29, 30]. Today CLA has many known health benefits, including being anti-carcinogenic [28], anti-atherosclerotic [31], and anti-inflammatory [30]. This review will explore research that examines CLA and dairy fat's value in treating IMID.

1.2 Mixed Isomer Conjugated Linoleic Acid

The first dietary treatment studies with CLA used mixed isomer preparations that typically contained a 20/80 to 50/50 mix of CLAt10c12 and CLAc9t11. These mixed isomer preparations demonstrated anti-inflammatory effects in a variety of diseases including obesity [32], inflammatory bowel disease [33], and rheumatoid arthritis [34]. CLA modulated both the innate and adaptive immune response through cytotoxic T cells, cytokines, macrophages, immunoglobulins, prostaglandins, and other immune mediators [35, 36]. Specifically, CLA decreases the pro-inflammatory cytokines TNFα, interferon (IFN)γ, IL-1β, and IL-6 [37]. One suggested mechanism through which cytokine modulation may occur is through nuclear receptor peroxisome proliferator-activated receptor (PPAR)γ, which is known to regulate transcription factors including nuclear factor (NF)-κB [33, 38]. The PPARγ pathway is linked to severity of mouse CA and agonists of this pathway, such as CLA, reduced arthritis severity through the downregulation of NF-κB and associated pro-inflammatory cytokines [34, 38–40].

The modulation of PPARγ, NF-κB, and cytokines may play a role in the 70% reduction in CA severity Hubner *et al*. [34] observed after feeding mice 1% mixed isomer CLA ($P = 0.04$).

Analysis of paw thickness and joint histology supported the reduction of arthritis severity in CLA fed mice ($P = 0.03$ and $P = <0.0001$, respectively) [34]. No differences in anti-collagen immunoglobulin (Ig)G levels were observed; however, the pro-inflammatory cytokine IL-1β was reduced in the plasma of CLA fed mice compared to those fed CO ($P = 0.01$). Few human clinical trials assessing CLA's anti-inflammatory effects in RA have been performed. Aryaeian *et al*. [41] fed a 2g/day mixed isomer CLA supplementation to RA patients and saw a reduction in RA severity as well as NSAID and glucocorticoid use over three months.

Some of CLA's effects appeared to be isomer specific. For example, CLAt10c12 is linked to lipolysis and fat oxidation [42], while CLAc9t11 modulates the immune system to a greater extent [43]. CLAt10c12 is additionally associated with impaired glucose and insulin tolerance [44]. Studies with mixed isomer CLA make it difficult to determine any isomer specific effects. The value of a purified isomer study is increased by the fact that natural CLA sources, ruminant lipids, contain CLA that is 96.5-98.6% CLAc9t11 [26]. Dairy fat also contains *trans*-11 vaccenic acid (TVA), which is enzymatically converted to CLAc9t11 by delta-9 desaturase [45]. The varied mechanisms of action between CLA isomers, potential negative effects associated with CLAt10c12, and the natural abundance of CLAc9t11 has pushed anti-inflammatory CLA research to focus on the study of the CLA isomers individually.

1.3 Conjugated Linoleic Acid *trans*-10, *cis*-12

CLAt10c12 is primarily a synthetic product and only present in natural CLA sources at 0 to 6% of total CLA [26]. This isomer is often implicated for use in weight loss because of its anti-adipogenic effects; however, these effects are linked to impaired glucose and insulin tolerance [42, 44]. CLAt10c12 is also know to inhibit the delta-9 desaturase, stearoyl Co-A desaturase

(SCD)-1, and reduce hepatic apolipoprotein B secretion [46]. When it comes to inflammation, CLAt10c12 reduces cyclooxygenase (COX)-2 expression through the NF-κB pathway, which is known to regulate inflammatory cytokines [47, 48]. Cytokine modulation may vary between the two CLA isomers; for example, CLAt10c12 reduced TNFα to a lesser extent than CLAc9t11[49].

In the CA model of arthritis, diet containing 0.5% CLAt10c12 and 5.5% CO, fed starting at the onset of arthritis symptoms (arthritic day (AD) 0), decreased the change in arthritic severity by 93% compared to a 6% CO control diet (**Figure 1**) [34]. Hubner *et al*. [50] performed a dose response study, in the CA model, with 0.125, 0.25, 0.375, and 0.5% dietary CLAt10c12 supplementation, starting at AD 0. In this study, severity decreased between 93% and 99% compared to a 6% CO diet (**Figure 1**). Compared to CLAc9t11 or dairy fat supplementation, CLAt10c12 resulted in the highest reduction in arthritic severity.

Two studies have fed 0.5% CLAt10c12, from AD 0 to 84 days, and quantified treatment effects on plasma cytokine levels over time (**Figure 2**) [34, 50]. CLAt10c12 decreased pro-inflammatory IL-1β, IL-6, and TNFα, and increased levels of the anti-inflammatory cytokine IL-10 compared to CO [34, 50]. IL-1β and TNFα levels are decreased the most between AD 21 and 42 in CLAt10c12 fed mice compared to CO [34, 50]. IL-6 levels were the most reduced at AD 7 and 14, and then declined over time [34, 50]. IL-10 levels in CLAt10c12 fed mice were increased at each time point with the highest increase compared to CO at AD 84 [34, 50]. While onset of arthritis impacts cytokine profiles over time, this data supports the correlation between decreased pro-inflammatory cytokines, increased anti-inflammatory cytokines, and the reduction of arthritic severity due to CLAt10c12 treatment. A dose response study with CLAt10c12 demonstrated a dose dependent decrease in plasma IL-1β, and paw IL-1β, TNFα, and increased

plasma IL-10 levels [50]. The change in arthritis severity and cytokine levels at the lowest dose of CLAt10c12 fed, 0.125%, was still considered biologically relevant ($P = 0.002$) [51]. A dose response study for CLAt10c12 has never been carried out beyond the 0.125% dose, and is needed to identify a true minimum effective dose.

A 0.125% dose of CLAt10c12 is equivalent to a human intake of 3g CLAt10c12/day (using Kleiber's law for a 70kg person and 30g mouse) [52]. With dairy fat containing ≤ 1.1g CLAt10c12/kg fat, a person would need to consume about 2.73kg of dairy fat daily to receive a beneficial anti-inflammatory CLAt10c12 dose [53]. This amount of dairy fat consumption is not recommended; therefore, dietary supplements, which typically contain 400mg CLAt10c12/g, would be the easiest way achieve the anti-inflammatory CLAt10c12 daily dose. However, compliance with dietary supplements long term is often low, and, as mentioned, there are negative anti-adipogenic effects associated with this much CLAt10c12 intake. Possible mechanism differences, side effects, and the low natural abundance of CLAt10c12 have increased interest in exploration into CLAc9t11's anti-inflammatory benefits.

1.4 Conjugated Linoleic Acid *cis*-9, *trans*-11

Another common CLA isomer is CLAc9t11, and dietary intake of this isomer is much greater than that of CLAt10c12 [26]. The Cook laboratory has demonstrated a greater than 60% decrease in arthritic severity (normalized to AD 0) with as low as a 0.125% dose of CLAc9t11, compared to a CO control diet, in the CA model (**Figure 1**) [34, 51]. This 0.125% dose equates to a human intake of 3g CLAc9t11/day [51, 52]. At a 0.5% CLAc9t11 dose, a decrease in pro-inflammatory plasma cytokines IL-1β, TNFα, and IL-6 was observed, while the anti-inflammatory cytokine IL-10 was increased (**Figure 3**) [34, 51, 54]. While similar effects on inflammation markers were

observed with CLAc9t11 as CLAt10c12, CLAt10c12 may have a greater overall anti-inflammatory effect resulting in the further decrease in overall CA severity. It should be noted that in the dose response studies CLAt10c12 was fed for three weeks longer than CLAc9t11 when the percent change in severity compared to CO was calculated. More studies on the accumulation of these fatty acids in tissue over time, as it relates to the anti-inflammatory effect, are required to fully understand the impact of this timing difference. The potentially harmful CLAt10c12 side effects and the increased ease of access to CLAc9t11, increases interest in CLAc9t11 as it still has beneficial anti-inflammatory effects at a more easily achievable dietary level.

Olson *et al*. [54] compared the anti-inflammatory equivalency of a 0.5% CLAc9t11 dose to a commonly used anti-inflammatory RA drug, celecoxib. Results from this study indicate that 0.5% CLAc9t11 (570mg/kg bw) reduced arthritic severity 34%, which was representative of a 1.5mg/kg body weight dose of celecoxib [54]. As a COX-2 inhibitor drug, celecoxib is effective at reducing inflammation; however, it has many harmful side effects, including myocardial infarction [55]. To avoid side effects and high cost, RA patients may turn to NSAIDs over celecoxib or other COX-2 inhibitors [56]. While NSAIDs may come at a lower cost, they also have unwanted side effects including gastrointestinal irritation and ulcers [56]. Supplementing or even replacing RA pharmaceuticals with low cost, dairy fat products containing CLAc9t11 may be efficacious.

Dairy fat provides an abundant natural source of CLAc9t11, through its production, in the rumen, by bacterial biohydrogenation of linoleic and α-linolenic acid [58, 59]. CLAc9t11 levels can be enriched in dairy fat through adjusting the feeding strategies of cattle to increase their intake of linoleic and α-linolenic acid; for example, a low foliage diet supplemented with

sunflower oil [60]. CLAc9t11 can be enriched to make up 6% of the total fatty acids in dairy fat [53].

Epidemiological studies and clinical trials also supported the link between dairy fat and reduced inflammation [61–63]. One study fed butter, enriched with CLAc9t11, to adults and observed an increase in serum IL-10, and decreased NF-κB and IL-2 levels [64]. In the CA model, Huebner *et al*. [51] showed that both CLAc9t1-enriched butter and non-enriched butter fed at 6% of the diet decreased arthritis severity to a similar extent as mice fed 0.125% pure CLAc9t11 (**Figure 1**). The pro-inflammatory cytokines IL-1β and IL-6 were also decreased by these butter based diets. The similar decrease in arthritis severity between the non-enriched butter, which provided 0.02% CLAc9t11 in the diet, and the pure 0.125% CLAc9t11 treatment suggests that there may be additional anti-inflammatory components in dairy fat. It was hypothesized, and later demonstrated, that this extra anti-inflammatory component is TVA, a metabolic precursor to CLAc9t11 [51, 65].

1.5 *Trans*-11 Vaccenic Acid

TVA is abundant in dairy fat, at up to 14% of total fatty acids [53]. Approximately 12% (mice) [66] to 19% (humans) [67] of dietary TVA is converted to CLAc9t11 by SCD-1, a delta-9 desaturase. TVA may have anti-inflammatory potential both on its own and through the actions of CLAc9t11. Dietary treatment with TVA reduced cardiovascular disease [68–70], anti-inflammatory mediators [71], metabolic syndrome [72], and arthritis [54].

Olson [65] conducted a does response study with 0.375, 0.75, and 1.5% TVA in the CA model to assess TVA's anti-inflammatory effects. Arthritic severity decreased linearly from 21, to 42, to 65%, compared to 6% CO control diet, as the dose increased [65]. The longer TVA was

fed, the higher the levels of TVA and CLAc9t11 found in mouse tissue [65]. When plotted over time the amount of TVA continued to rise through nine weeks of feeding in adipose and paw tissue; however, levels plateaued after five weeks in the liver [65]. Conversion of TVA to CLAc9t11, calculated as hepatic TVA dived by the total of hepatic TVA and CLAc9t11, was 48% in the liver, 56% in adipose, and up to 100% in paws [65]. Based on this conversion and the known minimally effective CLAc9t11 dose, 0.125%, the predicted minimally effective dose of TVA was 0.31% of diet [51, 65]. This is equivalent to 10.4g TVA/day for a 70kg human [52].

Studies are still needed to determine if TVA's anti-inflammatory properties are linked to or independent of its conversion to CLAc9t11. It has been suggested that TVA may have beneficial health properties of its own [73, 74]. TVA treatment i*n vitro* caused the reduction of IL-2 and TNFα production through direct activation of PPARγ, independent of its conversion to CLAc9t11 [74]. The anti-carcinogenic effects of TVA on mammary tumors was attenuated when the conversion of TVA to CLAc9t11 was partially blocked by sterculic acid, a delt-9 desaturase inhibitor [75]. TVA and CLAc9t11 may also have synergistic anti-inflammatory effects. In a rodent allergic airway inflammation model, milk fat reduced inflammatory leukocyte infiltration in airways and plasma of mice only when TVA and CLAc9t11 were fed in combination [76].

Human clinical trials have shown an increase in 8-iso-prostaglandin-$F_2\alpha$, a sign of arachidonic acid degradation, after dietary supplementation with 3g TVA/day of TVA [77]; however, this was not associated with an increase in pro-inflammatory cytokines [78]. Just as humans can consume CLAc9t11 naturally through dairy fat, TVA can also be easily consumed in this manner. TVA can also be enriched in dairy fat in a similar manner to CLAc9t11 enrichment [53].

Based on the CLAc9t11 and TVA content in maximal enriched butter, and the predicted minimally effective anti-inflammatory doses of CLAc9t11 and TVA, one would need to consume 30g dairy fat/day to receive an anti-inflammatory benefit [51, 52, 65]. This level is within the recommended daily intake for dairy fat as set by the United States Department of Agriculture [79], but would decrease further if a lower minimally effective CLAc9t11 or TVA dose was identified, greater enrichment was possible, or if the TVA to CLAc9t11 conversion was increased. TVA may also be β-oxidized to palmitelaidic acid, which may be another potentially anti-inflammatory fatty acid of dairy fat [80]. Other short chain fatty acids in dairy fat may also have unidentified anti-inflammatory potential [81].

1.6 The Next Steps: Moving from Treatment to Prevention

Rula *et al.* [82] estimated that preventative treatments for chronic inflammatory conditions, health promotion, and chronic care management could save Medicare between $650 billion and $1.43 trillion in the next ten years. Most studies conducted to date, start dietary exposure to omega-3 polyunsaturated fatty acids, CLA, or TVA after disease onset [18, 34, 50, 51, 65]; little research has been done on the preventative effects of these dietary components on IMID, such as rheumatoid arthritis.

It has been suggested that CLA, or a component of it, may push T helper cell type (Th) 1 responses, actually accelerating the onset of a Th1 diseases like arthritis and lupus [83–85]. Increased IL-2 production and decreased IgE and IL-4 production after CLA treatment support a push toward a Th1 response [86–88]. Yang *et al.* [84] showed that feeding mixed isomer CLA prior to spontaneous lupus onset accelerated the appearance of antinuclear antibodies and proteinuria in F1 hybrid New Zealand Black and White cross (NZB/W F1) lupus-prone mice.

Although these examples have suggested CLA could accelerate inflammatory disease onset, some studies conflict with this idea. In the CLA fed NZB/W F1 mice with early onset lupus, weight loss was decreased [85] and post-proteinuria survival was extended 1.5 fold in mice with lupus [84]. Butz *et al*. [83] fed CLA to mice three weeks before induction of collagen antibody induced arthritis. CLA reduced clinical arthritic scores by 60% compared to CO control diet, and prevented ankle joint remodeling caused by the arthritis [83]. CLA has also been shown to reduce symptoms in other antibody dependent disease models including airway inflammation [89, 90]. The differences in effect may be a result of the individual CLA isomer's properties.

CLA has been extensively studied as treatment for CA and other IMID, but its preventative capabilities have yet to be fully examined. This thesis aims to investigate the hypothesis that the individual CLA isomers have different effects on IMID prevention: while CLAt10c12 could increase CA incidence and severity through modulation of Th1 immune responses, CLAc9t11 and its precursor, TVA, would decrease CA incidence and severity. In addition, this thesis will further explore the hypothesis that TVA's anti-inflammatory properties are due to its conversion to CLAc9t11. If true, this hypothesis would support the use of CLAc9t11, TVA, and dairy fat as dietary supplements to prevent the development of IMID.

1.7 Figures

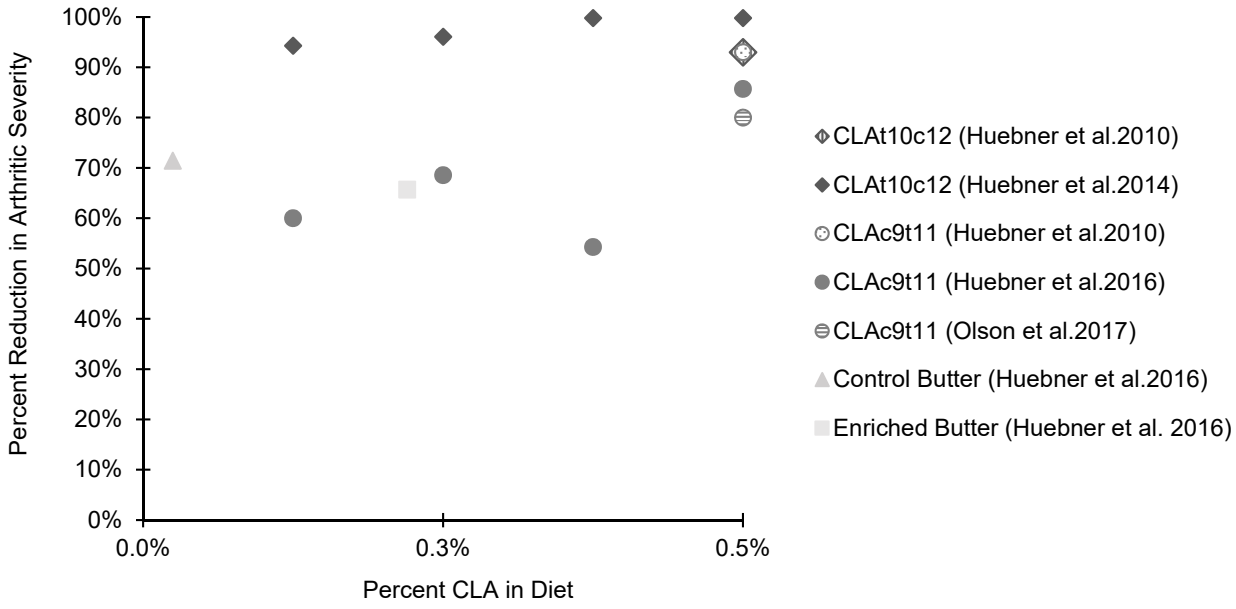

Figure 1: Percent reduction in normalized collagen-induced arthritis severity of mice fed diets containing CLAt10c12 or CLAc9t11, supplemented at the expense of corn oil (CO), compared to a 6% CO control diet. Normalized arthritic severity is the difference between the overall mean clinical arthritic score at arthritic day 0, day on arthritis onset, and at the end of the study period. Huebner *et al.* 2010 fed 0.5% CLAt10c12 (◈) or CLAc9t11 (◌) for 84 days after arthritis onset [34]. Huebner *et al.* 2014 fed 0.125, 0.25. 0.375, and 0.5% CLAt10c12 for 84 days after arthritis onset (◆) [50]. Huebner *et al.* 2016 fed 0.125, 0.25. 0.375, and 0.5% CLAc9t11 (●), 6% control butter (▲), or 6% enriched butter (■) for 63 days after arthritis onset [51]. Olson *et al.* 2017 (⊜) fed 0.5% CLAc9t11 for 42 days after arthritis onset [54].

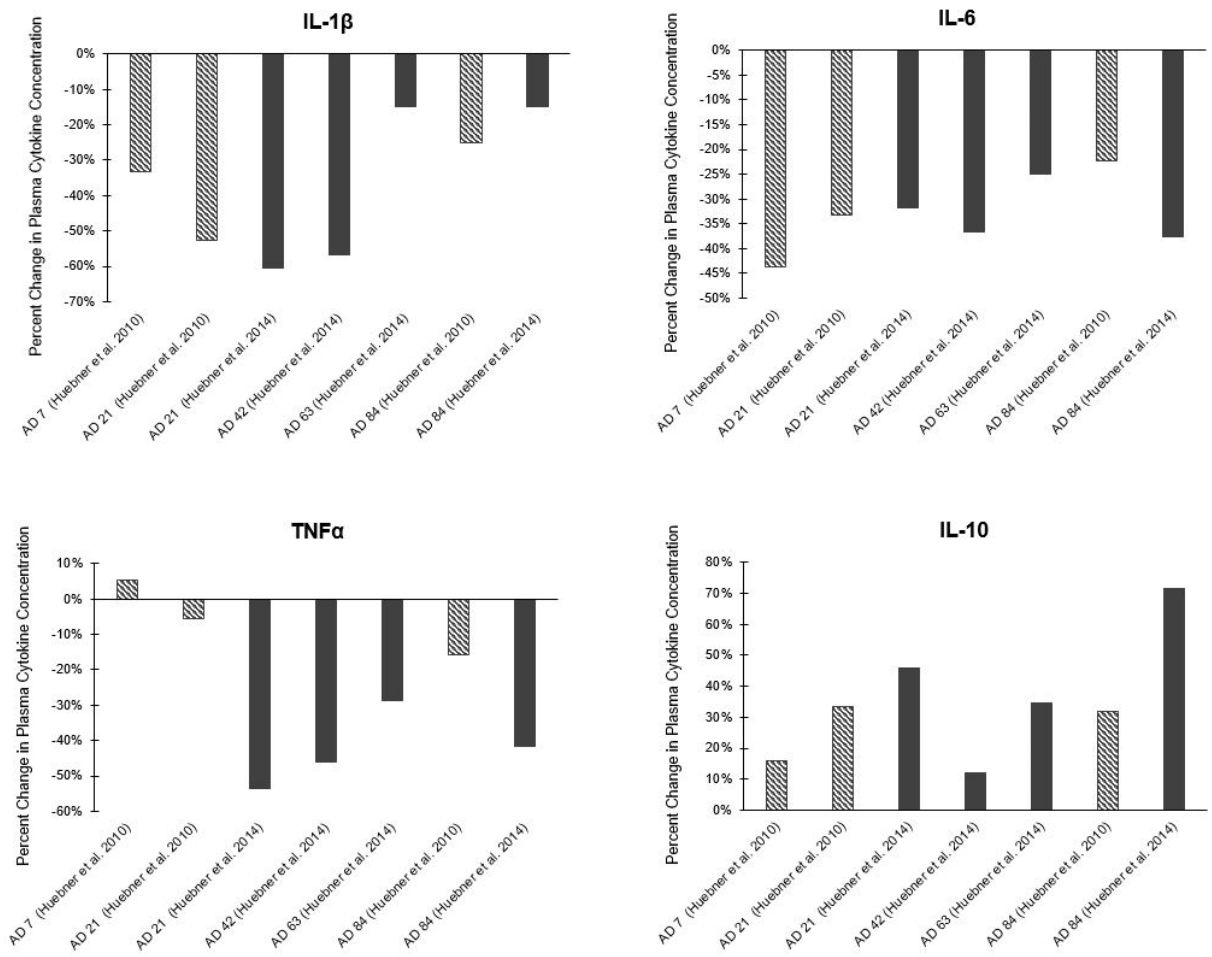

Figure 2: Percent change in plasma cytokine concentration after dietary supplementation with 0.5% CLAt10c12 and 5.5% corn oil compared to a 6% corn oil diet. Arthritic day (AD) represents the day post onset of arthritis, with AD 0 being the day of onset. In both studies reported, Huebner *et al.* 2010 (▨) and 2014 (■) fed 0.5% CLAt10c12 for 84 days after arthritis onset [34, 50]. Bar shading distinguishes the two studies.

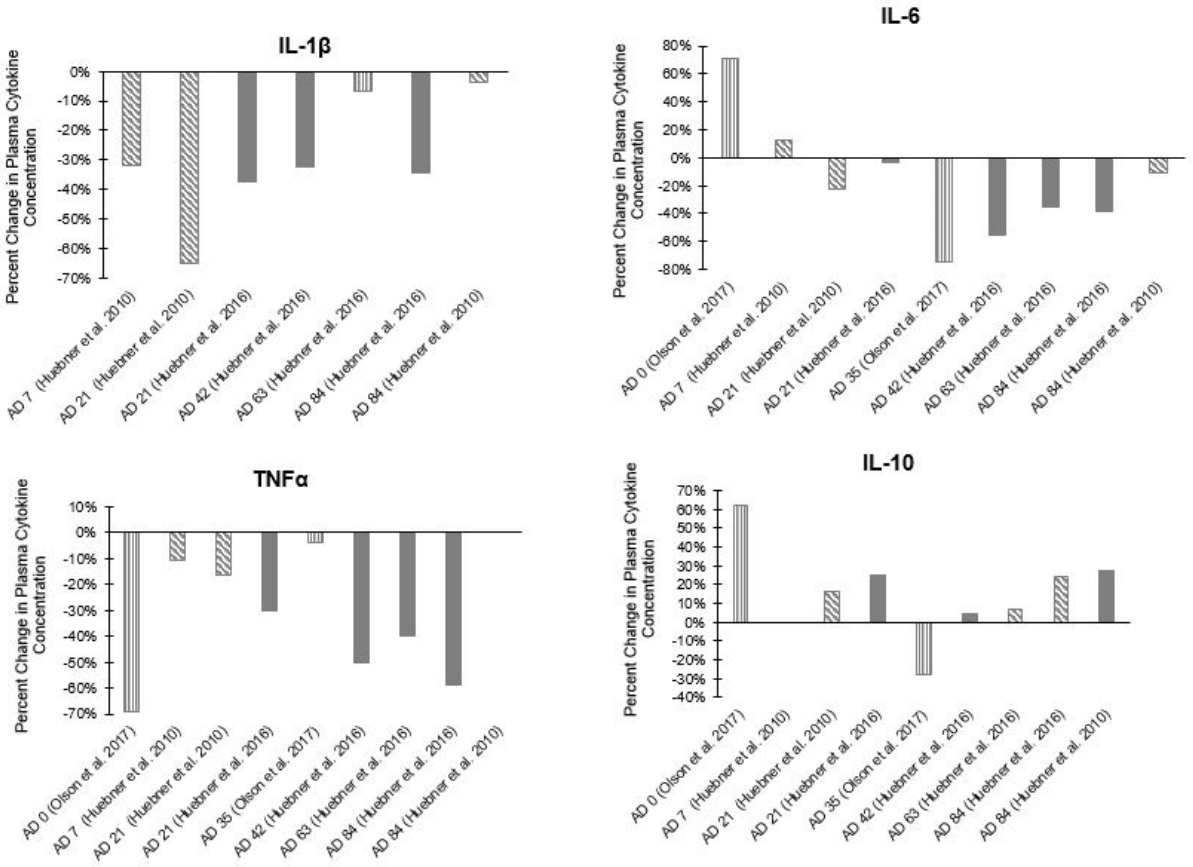

Figure 3: Percent change in plasma cytokine concentration after dietary supplementation with 0.5% CLAc9t11 and 5.5% corn oil compared to a 6% corn oil diet. Arthritic day (AD) represents the day post onset of arthritis, with AD 0 being the day of onset. Huebner *et al.* 2010 fed 0.5% CLAc9t11 for 84 days after arthritis onset (▨) [34]. Huebner *et al.* 2016 fed 0.5% CLAc9t11 for 63 days after arthritis onset (■) [51]. Olson *et al.* 2017 fed 0.5 % CLAc9t11 for 42 days after arthritis onset (▥) [54]. Bar shading distinguishes the reported studies.

1.8 References

1. Ward BW, Schiller JS, Goodman RA (2014) Multiple Chronic Conditions Among US Adults: A 2012 Update. Prev Chronic Dis 11:130389. doi: 10.5888/pcd11.130389

2. Straub RH, Schradin C (2016) Chronic inflammatory systemic diseases: An evolutionary trade-off between acutely beneficial but chronically harmful programs. Evol Med public Heal 2016:37–51. doi: 10.1093/emph/eow001

3. Vandenbroucke, Jan P; Hazevoet, Hermina M; Cats A (1984) Survival and Cause of Death in Rheumatoid Arthritis: A 25-year prospective followup. 158–161.

4. Gabriel SE, Michaud K, Kroenke K, et al (2009) Epidemiological studies in incidence, prevalence, mortality, and comorbidity of the rheumatic diseases. Arthritis Res Ther 11:229. doi: 10.1186/ar2669

5. Mikuls TR (2010) Rheumatoid arthritis incidence: What goes down must go up? Arthritis Rheum 62:1565–1567. doi: 10.1002/art.27432

6. Birnbaum H, Pike C, Kaufman R, et al (2010) Societal cost of rheumatoid arthritis patients in the US. Curr Med Res Opin 26:77–90. doi: 10.1185/03007990903422307

7. Brand DD, Latham KA, Rosloniec EF (2007) Collagen-induced arthritis. Nat Protoc 2:1269–1275. doi: 10.1038/nprot.2007.173

8. Williams RO, Feldmann M, Maini RN (1992) Anti-tumor necrosis factor ameliorates joint disease in murine collagen-induced arthritis. Proc Natl Acad Sci U S A 89:9784–8.

9. Joosten LA, Helsen MM, Saxne T, et al (1999) IL-1 alpha beta blockade prevents cartilage and bone destruction in murine type II collagen-induced arthritis, whereas TNF-

alpha blockade only ameliorates joint inflammation. J Immunol 163:5049–55.

10. Bendele A, Mccomb J, Gould TY, et al Animal Models of Arthritis: Relevance to Human Disease.

11. Singh JA, Furst DE, Bharat A, et al (2012) 2012 Update of the 2008 American College of Rheumatology recommendations for the use of disease-modifying antirheumatic drugs and biologic agents in the treatment of rheumatoid arthritis. Arthritis Care Res (Hoboken) 64:625–639. doi: 10.1002/acr.21641

12. Simopoulos AP (2002) Omega-3 fatty acids in inflammation and autoimmune diseases. J Am Coll Nutr 21:495–505.

13. Calder PC (2015) Marine omega-3 fatty acids and inflammatory processes: Effects, mechanisms and clinical relevance. Biochim Biophys Acta - Mol Cell Biol Lipids 1851:469–484. doi: 10.1016/j.bbalip.2014.08.010

14. Healy DA, Wallace FA, Miles EA, et al (2000) Effect of low-to-moderate amounts of dietary fish oil on neutrophil lipid composition and function. Lipids 35:763–8.

15. Schmidt EB, Varming K, Pedersen JO, et al (1992) Long-term supplementation with n-3 fatty acids, II: Effect on neutrophil and monocyte chemotaxis. Scand J Clin Lab Invest 52:229–36.

16. Hughes DA, Pinder AC, Piper Z, et al (1996) Fish oil supplementation inhibits the expression of major histocompatibility complex class II molecules and adhesion molecules on human monocytes. Am J Clin Nutr 63:267–72. doi: 10.1093/ajcn/63.2.267

17. Kelley DS, Taylor PC, Nelson GJ, et al (1999) Docosahexaenoic acid ingestion inhibits

natural killer cell activity and production of inflammatory mediators in young healthy men. Lipids 34:317–24.

18. Stamp LK, James MJ, Cleland LG (2005) Diet and Rheumatoid Arthritis: A Review of the Literature. Semin Arthritis Rheum 35:77–94. doi: 10.1016/j.semarthrit.2005.05.001

19. Kremer JM, Bigauoette J, Michalek A V, et al (1985) Effects of manipulation of dietary fatty acids on clinical manifestations of rheumatoid arthritis. Lancet (London, England) 1:184–7.

20. Nielsen GL, Faarvang KL, Thomsen BS, et al (1992) The effects of dietary supplementation with n-3 polyunsaturated fatty acids in patients with rheumatoid arthritis: a randomized, double blind trial. Eur J Clin Invest 22:687–91.

21. Belch JJ, Ansell D, Madhok R, et al (1988) Effects of altering dietary essential fatty acids on requirements for non-steroidal anti-inflammatory drugs in patients with rheumatoid arthritis: a double blind placebo controlled study. Ann Rheum Dis 47:96–104.

22. Bradberry JC, Hilleman DE (2013) Overview of omega-3 Fatty Acid therapies. P T 38:681–91.

23. Zargar A, Ito MK (2011) Long Chain Omega-3 Dietary Supplements: A Review of the National Library of Medicine Herbal Supplement Database. Metab Syndr Relat Disord 9:255–271. doi: 10.1089/met.2011.0004

24. Reaney MJT, Lui Y-D, Westcott ND (1999) Commercial Production of Conjugated Linoleic Acid. In: Yurawecz MP, Mossoba MM, Kramer JK., et al (eds) Adv. Conjug. Linoleic Acid Res. Vol. 1. AOCS Press, Champaign, Illinois, pp 39–54

25. Larsen TM, Toubro S, Astrup A (2003) Efficacy and safety of dietary supplements containing CLA for the treatment of obesity: evidence from animal and human studies. J Lipid Res 44:2234–41. doi: 10.1194/jlr.R300011-JLR200

26. Parodi PW (2003) Conjugated Linoleic Acid in Food. In: Sébédio J-L, Christie WW, Adolf R (eds) Adv. Conjug. Linoleic Acid Res. Vol. 2. AOCS Press, Champaign, Illinois, pp 101–122

27. Booth RG, Kon SA, Dann WJ, Moore T (1933) A study of seasonal variation in butter-fat: Seasonal variations in carotene, vitamin A and the antimony trichloride reaction. Biochem J 27:1189–96.

28. Ha YL, Grimm NK, Pariza MW (1987) Anticarcinogens from fried ground beef: heat-altered derivatives of linoleic acid. Carcinogenesis 8:1881–1887.

29. Miller CC, Park Y, Pariza MW, Cook ME (1994) Feeding Conjugated Linoleic Acid to Animals Partially Overcomes Catabolic Responses Due to Endotoxin Injection. Biochem Biophys Res Commun 198:1107–1112. doi: 10.1006/bbrc.1994.1157

30. Cook ME, Miller CC, Park Y, Pariza M (1993) Immune modulation by altered nutrient metabolism: nutritional control of immune-induced growth depression. Poult Sci 72:1301–1305.

31. Nicolosi RJ, Rogers EJ, Kritchevsky D, et al (1997) Dietary conjugated linoleic acid reduces plasma lipoproteins and early aortic atherosclerosis in hypercholesterolemic hamsters. Artery 22:266–277.

32. Takahashi Y, Kushiro M, Shinohara K, Ide T (2002) Dietary conjugated linoleic acid

reduces body fat mass and affects gene expression of proteins regulating energy metabolism in mice. Comp Biochem Physiol B Biochem Mol Biol 133:395–404.

33. Bassaganya-Riera J, Reynolds K, Martino-Catt S, et al (2004) Activation of PPAR γ and δ by conjugated linoleic acid mediates protection from experimental inflammatory bowel disease. Gastroenterology 127:777–791. doi: 10.1053/j.gastro.2004.06.049

34. Huebner SM, Campbell JP, Butz DE, et al (2010) Individual Isomers of Conjugated Linoleic Acid Reduce Inflammation Associated with Established Collagen-Induced Arthritis in DBA/1 Mice. J Nutr 140:1454–1461. doi: 10.3945/jn.109.120527

35. Bassaganya-Riera J, Pogranichniy RM, Jobgen SC, et al (2003) Conjugated Linoleic Acid Ameliorates Viral Infectivity in a Pig Model of Virally Induced Immunosuppression. J Nutr 133:3204–3214. doi: 10.1093/jn/133.10.3204

36. Viladomiu M, Hontecillas R, Bassaganya-Riera J (2016) Modulation of inflammation and immunity by dietary conjugated linoleic acid. Eur J Pharmacol 785:87–95. doi: 10.1016/j.ejphar.2015.03.095

37. O'Shea M, Bassaganya-Riera J, Mohede IC (2004) Immunomodulatory properties of conjugated linoleic acid. Am J Clin Nutr 79:1199S–1206S. doi: 10.1093/ajcn/79.6.1199S

38. Yu Y, Correll PH, Vanden Heuvel JP (2002) Conjugated linoleic acid decreases production of pro-inflammatory products in macrophages: evidence for a PPAR gamma-dependent mechanism. Biochim Biophys Acta 1581:89–99.

39. Tomita T, Kakiuchi Y, Tsao PS (2006) THR0921, a novel peroxisome proliferator-activated receptor gamma agonist, reduces the severity of collagen-induced arthritis.

Arthritis Res Ther 8:R7. doi: 10.1186/ar1856

40. Setoguchi K, Misaki Y, Terauchi Y, et al (2001) Peroxisome proliferator-activated receptor-γ haploinsufficiency enhances B cell proliferative responses and exacerbates experimentally induced arthritis. J Clin Invest 108:1667–1675. doi: 10.1172/JCI13202

41. Aryaeian N, Shahram F, Djalali M, et al (2009) Effect of conjugated linoleic acids, vitamin E and their combination on the clinical outcome of Iranian adults with active rheumatoid arthritis. Int J Rheum Dis 12:20–28. doi: 10.1111/j.1756-185X.2009.01374.x

42. Chung S, Brown JM, Sandberg MB, McIntosh M (2005) Trans-10,cis-12 CLA increases adipocyte lipolysis and alters lipid droplet-associated proteins: role of mTOR and ERK signaling. J Lipid Res 46:885–95. doi: 10.1194/jlr.M400476-JLR200

43. Wang T, Lee HG (2015) Advances in Research on *cis*- 9 *, trans*- 11 Conjugated Linoleic Acid: A Major Functional Conjugated Linoleic Acid Isomer. Crit Rev Food Sci Nutr 55:720–731. doi: 10.1080/10408398.2012.674071

44. Hamilton M, Hopkins LE, AlZahal O, et al (2015) Feeding butter with elevated content of trans-10, cis-12 conjugated linoleic acid to obese-prone rats impairs glucose and insulin tolerance. Lipids Health Dis 14:119. doi: 10.1186/s12944-015-0122-2

45. Pollard MR, Gunstone FD, James AT, Morris LJ (1980) Desaturation of positional and geometric isomers of monoenoic fatty acids by microsomal preparations from rat liver. Lipids 15:306–14.

46. Park Y, Storkson JM, Ntambi JM, et al (2000) Inhibition of hepatic stearoyl-CoA desaturase activity by trans-10,cis-12 conjugated linoleic acid and its derivatives. Biochim

Biophys Acta - Mol Cell Biol Lipids 1486:285–292. doi: 10.1016/S1388-1981(00)00074-3

47. Li G, Barnes D, Butz D, et al (2005) 10t,12c-Conjugated Linoleic Acid Inhibits Lipopolysaccharide-Induced Cyclooxygenase Expression in Vitro and in Vivo. J Lipid Res 46:2134–2142. doi: 10.1194/jlr.M500064-JLR200

48. Zhang YH, Lin JX, Vilcek J (1990) Interleukin-6 induction by tumor necrosis factor and interleukin-1 in human fibroblasts involves activation of a nuclear factor binding to a kappa B-like sequence. Mol Cell Biol 10:3818–23.

49. Pariza MW, Park Y, Cook ME (2000) Mechanisms of action of conjugated linoleic acid: evidence and speculation. Proc Soc Exp Biol Med 223:8–13.

50. Huebner SM, Olson JM, Campbell JP, et al (2014) Dietary trans-10,cis-12 CLA reduces murine collagen-induced arthritis in a dose-dependent manner. J Nutr 144:177–184. doi: 10.3945/jn.113.182550

51. Huebner SM, Olson JM, Campbell JP, et al (2016) Low Dietary c9t11-Conjugated Linoleic Acid Intake from Dairy Fat or Supplements Reduces Inflammation in Collagen-Induced Arthritis. Lipids 51:807–819. doi: 10.1007/s11745-016-4163-8

52. Kleiber M (1947) Body Sizes and Metabolic Rate. Physiol. Rev. 27:

53. Shingfield KJ, Reynolds CK, Hervás G, et al (2006) Examination of the Persistency of Milk Fatty Acid Composition Responses to Fish Oil and Sunflower Oil in the Diet of Dairy Cows. J Dairy Sci 89:714–732. doi: 10.3168/jds.S0022-0302(06)72134-8

54. Olson JM, Haas AW, Lor J, et al (2017) A Comparison of the Anti-Inflammatory Effects

of Cis-9, Trans-11 Conjugated Linoleic Acid to Celecoxib in the Collagen-Induced Arthritis Model. Lipids 52:151–159. doi: 10.1007/s11745-016-4228-8

55. Brownstein JS, Sordo M, Kohane IS, Mandl KD (2007) The Tell-Tale Heart: Population-Based Surveillance Reveals an Association of Rofecoxib and Celecoxib with Myocardial Infarction. PLoS One 2:e840. doi: 10.1371/journal.pone.0000840

56. Sundy JS (2001) COX-2 inhibitors in rheumatoid arthritis. Curr Rheumatol Rep 3:86–91.

57. Aryaeian N, Djalali M, Shahram F, et al (2014) Effect of conjugated linoleic Acid, vitamin e, alone or combined on immunity and inflammatory parameters in adults with active rheumatoid arthritis: a randomized controlled trial. Int J Prev Med 5:1567–77.

58. Parodi PW (1977) Conjugated Octadecadienoic Acids of Milk Fat. J Dairy Sci 60:1550–1553. doi: 10.3168/jds.S0022-0302(77)84068-X

59. Viviani R (1970) Metabolism of long-chain fatty acids in the rumen. Adv Lipid Res 8:267–346.

60. Bauman DE, Barbano DM, Dwyer DA, et al Technical Note: Production of Butter with Enhanced Conjugated Linoleic Acid for Use in Biomedical Studies with Animal Models 1,2. doi: 10.3168/jds.S0022-0302(00)75131-9

61. Wijga AH, Smit HA, Kerkhof M, et al (2003) Association of consumption of products containing milk fat with reduced asthma risk in pre-school children: the PIAMA birth cohort study. Thorax 58:567–72. doi: 10.1136/THORAX.58.7.567

62. Dawczynski C, Schubert R, Hein G, et al (2009) Long-term moderate intervention with n-3 long-chain PUFA-supplemented dairy products: effects on pathophysiological

biomarkers in patients with rheumatoid arthritis. Br J Nutr 101:1517. doi: 10.1017/S0007114508076216

63. Sofi F, Buccioni A, Cesari F, et al (2010) Effects of a dairy product (pecorino cheese) naturally rich in cis-9, trans-11 conjugated linoleic acid on lipid, inflammatory and haemorheological variables: A dietary intervention study. Nutr Metab Cardiovasc Dis 20:117–124. doi: 10.1016/j.numecd.2009.03.004

64. Penedo LA, Nunes JC, Gama MAS, et al (2013) Intake of butter naturally enriched with cis9,trans11 conjugated linoleic acid reduces systemic inflammatory mediators in healthy young adults. J Nutr Biochem 24:2144–2151. doi: 10.1016/j.jnutbio.2013.08.006

65. Olson J (2016) The ruminant-derived fatty acids cis-9, trans-11 conjugated linoleic acid and trans-11 vaccenic acid are anti-inflammatory in the collagen-induced arthritis model: Evidence of intestinal barrier dysfunction associated with arthritis-induced inflammation.

66. Turpeinen AM, Mutanen M, Aro A, et al (2002) Bioconversion of vaccenic acid to conjugated linoleic acid in humans. Am J Clin Nutr 76:504–10.

67. Santora JE, Palmquist DL, Roehrig KL (2000) Trans-vaccenic acid is desaturated to conjugated linoleic acid in mice. J Nutr 130:208–15.

68. Tyburczy C, Major C, Lock AL, et al (2008) Individual Trans Octadecenoic Acids and Partially Hydrogenated Vegetable Oil Differentially Affect Hepatic Lipid and Lipoprotein Metabolism in Golden Syrian Hamsters. J Nutr 139:257–263. doi: 10.3945/jn.108.098004

69. Rice BH, Kraft J, Destaillats F, et al (2012) Ruminant-produced trans-fatty acids raise plasma HDL particle concentrations in intact and ovariectomized female Hartley guinea

pigs. J Nutr 142:1679–83. doi: 10.3945/jn.112.160077

70. Wang Y, Jacome-Sosa MM, Ruth MR, et al (2009) Trans-11 Vaccenic Acid Reduces Hepatic Lipogenesis and Chylomicron Secretion in JCR:LA-cp Rats. J Nutr 139:2049–2054. doi: 10.3945/jn.109.109488

71. Sun X, Zhang J, MacGibbon AKH, et al (2011) Bovine milk fat enriched in conjugated linoleic and vaccenic acids attenuates allergic dermatitis in mice. Clin Exp Allergy 41:729–738. doi: 10.1111/j.1365-2222.2011.03723.x

72. Blewett HJ, Gerdung CA, Ruth MR, et al (2009) Vaccenic acid favourably alters immune function in obese JCR:LA-cp rats. Br J Nutr 102:526–36. doi: 10.1017/S0007114509231722

73. Lim J-N, Oh J-J, Wang T, et al (2014) trans-11 18:1 vaccenic acid (TVA) has a direct anti-carcinogenic effect on MCF-7 human mammary adenocarcinoma cells. Nutrients 6:627–36. doi: 10.3390/nu6020627

74. Jaudszus A, Jahreis G, Schlörmann W, et al (2012) Vaccenic acid-mediated reduction in cytokine production is independent of c9,t11-CLA in human peripheral blood mononuclear cells. BBA - Mol Cell Biol Lipids 1821:1316–1322. doi: 10.1016/j.bbalip.2012.06.010

75. Lock AL, Corl BA, Barbano DM, et al (2004) The anticarcinogenic effect of trans-11 18:1 is dependent on its conversion to cis-9, trans-11 CLA by delta9-desaturase in rats. J Nutr 134:2698–704.

76. Kanwar RK, MacGibbon AK, Black PN, et al (2007) Bovine milk fat enriched in

conjugated linoleic and vaccenic acids attenuates allergic airway disease in mice. Clin Exp Allergy 0:071115150625003–??? doi: 10.1111/j.1365-2222.2007.02868.x

77. Kuhnt K, Wagner A, Kraft J, et al (2006) Dietary supplementation with 11trans- and 12trans-18:1 and oxidative stress in humans. Am J Clin Nutr 84:981–8.

78. Kuhnt K, Kraft J, Vogelsang H, et al (2007) Dietary supplementation with trans-11- and trans-12-18 : 1 increases cis-9, trans-11-conjugated linoleic acid in human immune cells, but without effects on biomarkers of immune function and inflammation. Br J Nutr 97:1196. doi: 10.1017/S0007114507685183

79. How Much Food from the Dairy Group is Needed Daily? In: US Dep. Agric. https://www.choosemyplate.gov/dairy. Accessed 6 Mar 2017

80. Jaudszus A, Kramer R, Pfeuffer M, et al (2014) trans Palmitoleic acid arises endogenously from dietary vaccenic acid. Am J Clin Nutr 99:431–435. doi: 10.3945/ajcn.113.076117

81. Jenkins B, West J, Koulman A (2015) A Review of Odd-Chain Fatty Acid Metabolism and the Role of Pentadecanoic Acid (C15:0) and Heptadecanoic Acid (C17:0) in Health and Disease. Molecules 20:2425–2444. doi: 10.3390/molecules20022425

82. Rula EY, Pope JE, Hoffman JC (2011) Potential Medicare savings through prevention and risk reduction. Popul Health Manag 14 Suppl 1:S35-44. doi: 10.1089/pop.2010.0063

83. Butz DE, Li G, Huebner SM, Cook ME (2007) A mechanistic approach to understanding conjugated linoleic acid's role in inflammation using murine models of rheumatoid arthritis. Am J Physiol Regul Integr Comp Physiol 293:R669–R676. doi: 10.1152/ajpregu.00005.2007

84. Yang M, Cook ME (2003) Dietary CLA decreased weight loss and extended survival following the onset of kidney failure in NZB/W F1 mice. Lipids 38:21–24. doi: 10.1007/s11745-003-1026-8

85. Yang M, Pariza MW, Cook ME (2000) Dietary Conjugated Linoleic Acid Protects Against End Stage Disease of Systemic Lupus Erythematosus in the NZB/W F1 Mouse. Immunopharmacol Immunotoxicol 22:433–449.

86. Yang M, Cook ME (2003) Dietary Conjugated Linoleic Acid Decreased Cachexia, Macrophage Tumor Necrosis Factor-α Production, and Modifies Splenocyte Cytokines Production. Exp Biol Med Diet 228:51–58.

87. Cook ME, Butz D, Li G, et al (2003) Conjugated Linoleic Acid Enhances Immune Responses but Protects Against the Collateral Damage of Immune Events. In: Sébédio J-L, Christie WW, Adolf R (eds) Adv. Conjug. Linoleic Acid Res. Vol. 2. AOCS Press, Champaign, Illinois, pp 283–291

88. Sugano M, Tsujita A, Yamasaki M, et al (1998) Conjugated linoleic acid modulates tissue levels of chemical mediators and immunoglobulins in rats. Lipids 33:521–527.

89. Whigham LD, Cook EB, Stahl JL, et al (2001) CLA reduces antigen-induced histamine and PGE2 release from sensitized guinea pig tracheae. Am J Physiol Regul Integr Comp Physiol 49:R908–R912.

90. Whigham LD, Higbee A, Bjorling DE, et al (2002) Decreased antigen-induced eicosanoid release in conjugated linoleic acid-fed guinea pigs. Am J Physiol Regul Integr Comp Physiol 282:R1104–R1112. doi: 10.1152/ajpregu.00075.2001

Chapter 2:

Dietary Conjugated Linoleic Acid-c9t11 Prevents Collagen-Induced Arthritis, whereas Conjugated Linoleic Acid-t10c12 Increases Arthritic Severity.

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2.1 Abstract

Two conjugated linoleic acid (CLA) isomers, *cis*-9, *trans*-11 (c9t11) and *trans*-10, *cis*-12 (t10c12), reduce inflammation in a number of animal models, including collagen-induced arthritis (CA). However, little is known about the ability of individual CLA isomers to prevent autoimmune disease onset. Evidence that mixed isomer CLA drives T helper cell (Th) 1 responses suggests that CLA, or a specific isomer, exacerbates onset of Th1 autoimmune diseases. In two experiments, we examined if prior dietary exposure to CLAt10c12 (experiment 1) or CLAc9t11 (experiment 2) affected the incidence or severity of CA. DBA/1 mice were fed a semi purified diet with either 6 % corn oil (CO, w/w), 5.75 % CO plus 0.25 % CLAt10c12, or 5.5 % CO plus 0.5 % CLAc9t11 prior to arthritis development. Arthritis incidence and severity, anti-collagen antibodies, paw cytokines, and hepatic fatty acids were measured. CLAt10c12 had no effect on arthritis incidence but increased arthritic severity (42 %, $P = 0.02$); however, CLAc9t11 decreased arthritis incidence 39 % compared to CO fed mice ($P = 0.01$), but had no effect on disease severity. CLAt10c12-induced increase in anti-collagen type II IgG antibodies may be a mechanism by which this isomer increased arthritic severity, and CLAc9t11-induced increase in Th2 paw cytokines (IL-4 and IL-10, $P \leq 0.04$) may explain how CLAc9t11 reduced the arthritis incidence. While both isomers are well known to reduce inflammation in arthritic mice, this new data suggests isomer differences when fed prior to autoimmune disease.

2.2 Introduction

The benefits of dietary conjugated linoleic acid (CLA) are well-recognized [1–3]. Many of these studies are based on commercial preparations of CLA, which typically contain two CLA isomers, CLA *cis*-9, *trans*-11 (CLAc9t11) and CLA *trans*-10, *cis*-12 (CLAt10c12), in an approximate ratio of 1:1 [4]. The relative abundance of these two isomers, as a percent of total CLA, in ruminant lipids such as milk and beef is 96.5 - 98.6% CLAc9t11 and 1.4 - 3.5% CLAt10c12 [5]. Hence, exposure to CLA from natural sources as opposed to commercially prepared supplements favor the CLAc9t11 isomer.

Most CLA research focuses on examining the effects of mixed isomer CLA, which is well established to be anti-carcinogenic [6], anti-atherosclerotic [7], and anti-inflammatory [8]. Mixed isomer CLA alleviated inflammation in diseases including atherosclerosis [9], lupus [10, 11], type I airway hypersensitivity [12], inflammatory bowel disease [13], and arthritis [14]. Individually, CLAc9t11 and CLAt10c12 reduced collagen-induced arthritis (CA) severity when fed after arthritis induction [14]. Both isomers had similar effects on reducing clinical arthritic score, paw thickness, histological abnormalities in the joint, and pro-inflammatory cytokine levels compared to a corn oil (CO) based diet [14]. In studies conducted to date CLA was fed post disease onset; little research has been done on preventative effects of CLA individual isomers on autoimmune inflammatory diseases such as arthritis.

CLA studies often vary in timing of treatment relative to disease, as well as isomer ratios. Butz *et al.* [15] showed feeding mixed isomer CLA prior to collagen type II (CII) immunization resulted in slightly earlier appearance of CA. Yang *et al.* [11] showed feeding mixed isomer CLA prior to spontaneous lupus onset accelerated the appearance of antinuclear antibodies and proteinuria in F1 hybrid New Zealand Black and White cross (NZB/W F1), lupus-prone, mice.

Although early onset of disease was observed with NZB/W F1 mice, survival post proteinuria was extended 1.5 fold [16]. Both authors suggested that feeding mixed isomer CLA prior to disease onset drives a T helper cell type (Th) 1 response leading to an earlier onset of Th1 type diseases [11, 15, 16]. CLA's ability to drive Th1 responses is supported by its ability to decrease immunoglobulin (Ig)E antibody and IL-4 production, and increase IL-2 production [16–18]. CLA's ability to select for a Th1 cytokine response explains the earlier appearance of antibodies and disease symptoms in models where mixed isomer CLA was fed prior to disease onset.

CLAc9t11 and CLAt10c12 affect different inflammatory pathways, and in some cases the same pathway differently [19, 20]. The CLAt10c12 isomer reduced cyclooxygenase 2 expression and prostaglandin E2 through inhibition of nuclear factor (NF)-κB pathway [21]. CLAc9t11's anti-inflammatory effects have been reported to occur independent of eicosanoid synthesis, based on no change in arachidonic acid production after treatment [22]. CLAt10c12 also was reported to decrease the Th1 cytokine IL-12 by a mechanism not dependent on the Th2 cytokine IL-10, while the decrease in IL-12 caused by CLAc9t11 was linked to IL-10 [23]. The specific mechanisms by which CLAc9t11 influences the immune system have yet to be defined, but it was reported to inhibit tumor necrosis factor (TNF)-α production to a greater extent than CLAt10c12 [14, 16, 20]. CLAc9t11 has been reported to be antiatherogenic [24] and anti-inflammatory [25], and decrease plasma lipids [26]. CLAt10c12 alters atherogenic biomarkers (e.g. decreased high density lipoprotein and increased plasma lipids) [24, 26], is anti-adipogenic [27], and has been linked to increased liver lipids [24]. The isomer's varying effects, specifically involving the immune system and lipid metabolism, may help explain the confounding results seen in individual isomer compared to mixed isomer CLA studies.

Using the CA model of arthritis, this study attempts to determine if the CLAc9t11 and CLAt10c12 isomers have different effects on arthritis induction when fed prior to disease onset. Based on earlier work [15], two experiments were conducted to determine if individual CLA isomers behave differently than reported mixed isomer CLA results. The first experiment utilizes CLAt10c12 in the CA model, with dietary treatments starting 21 days after the primary collagen immunization, at the secondary collagen immunization. In the second experiment, CLAc9t11 was fed 21 days prior to collagen immunization. Results from these experiments provide insight into impact of specific CLA isomers on CA incidence and severity, relative to CO. However, due to differences in study design the results of the two studies cannot be directly compared.

2.3 Materials and Methods

Experiments were conducted in accordance with an animal protocol, A005438, approved by the College of Agricultural and Life Sciences Animal Care and Use Committee.

Collagen-induced arthritis model.

Experiment one was based on the design of previous experiments with mixed isomer CLA [15]. Briefly, four-week-old DBA/1 mice were purchased from Harlan (Indianapolis, IN), housed in shoebox units (3 mice per box), and maintained on a 12:12 h light-dark cycle. After the acclimation period of one week on chow diet, all mice were fed a 99% complete diet (containing 5% CO, TD94060; Harlan-Teklad, Madison, WI, **Table 1**) supplemented with 1% CO (n = 27). Mice (grouped by shoebox units) were randomly assigned to receive a 100µl intradermal injection, at the base of the tail, of 100 µg chick CII in 0.05 M acetic acid (Chondrex, Redmound, WA, n = 18 mice) or a sham injection of 0.05 M acetic acid (n = 9 mice) emulsified in a 1:1 ratio

with complete Freund's adjuvant (2mg/ml heat-killed *Mycobacterium tuberculosis*; Sigma, St. Louis, MO) [15, 28]. The date of primary injection was designated as day 0. On day 21, mice received a 100µl intraperitoneal booster injection of either 100µg chick CII in 0.05 M acetic acid or a sham injection of 0.05 M acetic acid emulsified in a 1:1 ratio with Freund's incomplete adjuvant (Sigma). This day was designated secondary (2°) immunization day 0. At the 2° immunization mice immunized with chick CII were randomly assigned to either remain on the 6% CO diet or start a 0.25% CLAt10c12 (in place of CO) treatment diet (n = 9 mice per diet, **Figure 1a**). Following 2° immunization mice were monitored daily for clinical signs of arthritis (see Clinical Arthritic Score).

In experiment two, the housing and immunization protocol remained the same as described above. Freund's complete (4mg/ml heat-killed *Mycobacterium tuberculosis*) and incomplete adjuvant was supplied by Chondrex. After one week on chow diet, three weeks before primary immunization, mice were switched to a 99% complete diet (5% CO) supplemented with 1% CO or 0.5% CLAc9t11 and 0.5% CO (TD94060; Harlan-Teklad, Madison, WI). Mice were randomized in a 2 x 2 factorial arrangement of diet and arthritis (n = 38 per diet, n = 20 in chick CII immunized groups, n = 9 in sham groups, **Figure 1b**).

CO and CLA isomer fatty acid content was assessed by gas chromatography 100m CP-Sil 88 column (0.25mm inside diameter, 0.3µm film thickness) as previously described [29] (**Table 2**).

Clinical arthritic score.

As previously described, mice were examined by a trained observer blinded to treatments [14, 15, 28, 30]. After 2° immunization mice were monitored daily for clinical signs of arthritis, and

after the first day post arthritis development (AD 0) mice were scored 3 times per week. Briefly, individual paws were assigned an inflammation severity score between 0 and 4 based on the following criteria: 0 = normal, no inflammation; 1 = mild, definite redness and swelling of the ankle or wrist or apparent redness and swelling of the digits; 2 = moderate, redness and swelling of the ankle and wrist; 3 = sever redness and swelling of the entire paw including digits; 4 = maximally inflamed limb involving multiple joints. Clinical arthritic score (CAS) was the sum of the 4 individual paw scores [28]. Normalized arthritic score was calculated by subtracting AD 0 from a given AD score post AD 0. Mice receiving a primary and secondary injection of CII that never developed arthritis were designated as non-developed (ND).

Plasma collection and preparation.

Blood was collected from the retroorbital venous plexus using heparinized capillary tubes, while mice were under light isoflurane anesthesia. Blood was collected at 35 days after the primary immunization (experiment 1), AD 7 (experiment 2), and 70 days after primary immunization (end of experiment 2). Plasma was separated by centrifugation at 3000xg for 10 minutes and stored at -80°C until analyzed.

Plasma anti-CII antibody ELISA.

Plasma samples were analyzed as previously described for total concentration of anti-CII IgG_1 and IgG_{2a} [15]. In experiment two anti-CII IgG_{2a} antibody levels were quantified with respect to anti-CII IgG_{2a} monoclonal antibody standard (Chondrex).

Paw tissue collection and cytokine extraction.

In experiment two, after mice were euthanized by exsanguination (AD 28 for arthritic mice and the respective sham mice), only arthritic paws were harvested in arthritic mice and all paws in sham mice. All paws were stored at -80°C until cytokine analysis. Prior to preforming the paw cytokine assay, paws were weighed and crushed under liquid nitrogen and total protein was extracted through tissue homogenization in cell lysing solution (Bio-Rad Laboratories, Hercules, CA) containing phenylmethyl sulfonyl fluoride (MP Biomedicals, Santa Ana, CA) [30]. Samples were incubated at -80 °C for one hour then centrifuged at 3000xg for 10 minutes at 4°C. Supernatant was collected and stored at -80°C. Samples were diluted to 650μg protein/ml following bicinchoninic acid protein assay results (Thermo Scientific, Waltham, MA) before final cytokine quantification [31].

Cytokine analysis.

Paw cytokine concentrations of interleukin (IL)-2, IL-12 (p70), Interferon (IFN)-γ IL-4, IL-5, and IL-10 were quantified using Bio-Plex Pro Th1/Th2 mouse cytokine assays according to manufacturer's instructions (Bio-Rad Laboratories). Fluorescence was measured using Luminex 100 system (Bio-Rad Laboratories), and results were analyzed using Bio-Plex Manager software (Bio-Rad Laboratories).

Hepatic fatty acid determination.

Total lipids of 0.2g liver samples were extracted using chloroform:methanol (2:1, v/v) according to the methods of Folch *et al*. [32]. Fatty acid methyl esters (FAME) were prepared by acid-catalyzed methylation [33, 34]. Relative percentages of liver FAME were determined using an Agilent 6890N GC (Agilent Technologies Santa Clara, CA) equipped with a 100m CP-Sil 88

column (Varian Palo Alto, CA) [29]. FAME were identified against a custom qualitative FAME standard (Matreya, LLC, Pleasant Gap, PA, #SPL4833) by comparing retention times.

Statistical analysis.

Incidence data were analyzed using the SAS procedure LIFETEST (SAS Institute) to determine difference due to dietary treatment. Development of arthritis was considered a fail, non-survival, in this analysis. The dependent variables arthritic score, antibodies, cytokines, and liver fatty acid were analyzed using the SAS procedure MIXED (SAS Institute) to determine difference due to the fixed effects of dietary treatment and time. In order to account for repeated measures of arthritic score across time, a REPEATED statement was employed with the SAS procedure MIXED with a first order autoregressive covariance structure. Each dependent variable was tested utilizing unequal variance among groups and a Levene's test was performed determine the appropriate model ($P \leq 0.05$). When dependent variables were identified to have a significant diet, arthritis, time, or interaction ($P \leq 0.05$) ANOVA analysis was followed by post hoc least squared difference test to identify individual differences among groups. Differences were considered significant at $P \leq 0.05$ and a trend at $P \leq 0.10$. Values are displayed as mean ± standard error of the mean (SEM).

2.4 Results

Experiment 1. CLAt10c12 and arthritis incidence/score.

In experiment 1, half of the CII immunized mice were switched from CO diet to CLAt10c12 diet at 2° immunization day 0. At their maximums, 78% of CLAt10c12 and CO mice developed arthritis, while sham immunized mice did not show arthritic symptoms at any time (**Figure 2a**).

CLAt10c12 and CO fed arthritic mice had significantly different overall incidence compared to sham mice (*P = 0.001*), but CO and CLAt10c12 fed arthritic mice were not significantly different from each other (*P = 0.59*, **Table 3**).

CAS of only mice that developed arthritis had an average score of 7.4 ± 0.5 for CLAt10c12 mice and 5.2 ± 0.3 for CO mice. Normalized score was 5.3 ± 0.5 in CLAt10c12 mice and 1.5 ± 0.2 in CO mice. A significant diet by time interaction for absolute and normalized CAS (*P = 0.02* and *P = 0.008*, respectively, **Table 3**) showed that during peak arthritis score, CLAt10c12 fed mice had more severe CAS than CO fed mice (**Figure 2b** and **2c**).

Experiment 1. CLAt10c12 and anti-CII antibodies.

In plasma samples collected 35 days after primary immunization there was a trend toward increased plasma anti-CII IgG_1 antibodies levels (*P = 0.06*) in arthritic mice fed CLAt10c12, compared to CO fed mice (**Table 4**). The increase in anti-CII IgG_{2a} antibodies in arthritic CLAt10c12 fed mice compared to arthritic CO fed mice was not significant (*P = 0.33*). No anti-CII antibodies were detected in sham mice**.**

Experiment 2. CLAc9t11 and incidence/score.

In experiment two, half of the mice were switched onto CLAc9t11 diets three weeks prior to immunization. At their maximums, 69% of CO mice and 30% of CLAc9t11 fed mice developed arthritis (**Figure 3a**), which was statistically different over the course of this study (*P=0.01*, **Table 5**). When analysis of arthritis incidence was conducted by day, a significant difference became apparent between sham and arthritic CO fed mice at 18 days post 2° immunization (*P <*

0.05). A significant difference became apparent at 34 days post 2° immunization between sham and arthritic CLAc9t11 fed mice ($P < 0.05$).

Mean CAS of arthritic mice (CAS = 4.1 ± 0.1) was similar between CO fed mice (n = 11) and CLAc9t11 (n = 6) mice ($P = 0.47$, **Figure 3b**, **Table 5**). Normalized score of CLAc9t11 and CO arthritic mice also followed a similar pattern over the course of this study (**Figure 3c**) and no differences in absolute or normalized CAS over the entire feeding period was observed in mice that developed arthritis (**Table 5**).

Experiment 2. CLAc9t11 and anti-CII antibodies.

Plasma samples for arthritic and ND mice were from different time points; therefore, antibody levels were not compared across arthritic classification. No anti-CII antibodies were detected in sham mice; however, both mice immunized mice against chick CII that developed arthritis and those that did not develop arthritis (ND) had detectable anti-CII IgG_1 and IgG_{2a} levels (**Table 6a** and **6b**). Feeding CO versus CLAc9t11 had no impact on IgG_1 or IgG_{2a} antibody levels in arthritic mice; however, there was a significant a 49% reduction in anti-CII IgG_{2a} antibody levels in ND CLAc9t11 fed mice compared to ND mice fed CO ($P = 0.05$).

Experiment 2. CLAc9t11 and paw cytokines.

Paw cytokine levels showed that IL-2 levels, but no other cytokines, were significantly elevated in the paws of arthritic mice compared to sham mice independent of diet ($P = 0.04$, **Figure 4**). CLAc9t11 fed mice had significantly increased levels of IL-4 and IL-10 compared to mice fed CO independent of arthritis induction ($P = 0.02$ and 0.04, respectively, **Figure 4d** and **4f**). Arthritis and diet had no significant effect on IL-12, IFN-γ, and IL-5 (**Figure 4b, 4c,** and **4e**).

Experiment 2. CLAc9t11 and hepatic fatty acids.

Hepatic CLAc9t11 was only detectable in mice fed CLAc9t11 and the levels were unaffected by arthritic condition (**Table 7**). The CLAt10c12 isomer was not detected in any samples from this study. In CLAc9t11 fed mice, independent of arthritis, hepatic 16:1c9 was significantly increased (*P = 0.02*), and 18:0 and 22:6n-3 were significantly decreased when compared to mice fed CO (*P = 0.03* and *0.02*, respectively). The presence of arthritis did not affect hepatic lipid levels and no interactions between diet and arthritis were observed.

2.5 Discussion

An objective of this study was to assess the effects of individual CLA isomers on CA incidence when compared to a CO based diet. While in retrospect it would have been more appropriate to conduct a direct comparison of the two isomers in a single study, the studies were conduct by two researchers on separate projects (Butz for experiment 1 [35] and Muhlenbeck for experiment 2). Consequently, the timing of feeding the CLA isomers prior to arthritis onset was not identical and largely based on prior literature at the time of the experiment. Regardless of the design differences and the comparison made from separate experiments, for the first time we were able to show that CLAt10c12 and CLAc9t11, when fed pre-arthritic development, differentially impact arthritic development and early severity of arthritic score.

Many experiments studying the effects of CLA have used mixed isomer preparations of CLAt10c12 and CLAc9t11 in near equal ratios. The results of our two experiments clearly show that the feeding mixed isomer CLA may have confounding effects; the data presented here helps to differentiate these opposing effects. When the effects of specific isomers on arthritis were

studied using the murine CA model, these studies focused on treating arthritic mice after arthritis development [14, 30, 36]. In these studies [14, 30, 36], both CLAt10c12 and CLAc9t11 had anti-arthritic effects when arthritic mice were treated with the individual isomers. These studies should not be confused with what is being reported here, as in these previous works the individual CLA isomers were fed after development of the adaptive immune response to CII. The work reported here asks if effects of these CLA isomers on onset and early severity of arthritis differ when mice are treated during the development of the adaptive immune response to CII. Since each isomer was studied in separate experiments, results will be discussed within each experiment. Until an experiment is conducted where the isomers are compared directly, it is difficult to blend the discussion across the isomers.

CLAt10c12.

The murine CA model is well established to represent a dominant Th1 disease [37, 38]. The increase in Th1 associated responses including IgG_{2a} [39], IL-2 [40], and IFN-γ [37] support CA as a Th1 disease model. Fed prior to the onset of Th1 autoimmune disease, mixed isomer CLA has been suggested to exacerbate disease onset. NZB/W F1 mice spontaneously develop systemic lupus erythematosus, which is considered a Th1 dominant autoimmune disease early on [41, 42] marked by increased IgG_{2a} [43] and IFN-γ [44]. Yang *et al*. [10] showed that NZB/W F1 mice fed a mixed isomer preparation of CLAt10c12 and CLAc9t11 had auto-antibodies to single and double stranded deoxyribonucleic acid and onset of proteinuria earlier in life. The isomer responsible for this earlier onset of autoimmunity was never determined due to the lack of purified CLA isomers. Similarly, a paper by Butz *et al*. [15] showed evidence that feeding mixed isomer CLA 21 days before the primary immunization with CII, to induce arthritis, was driving a

Th1 type response in the CA model by showing an earlier onset of disease. This earlier onset was not observed in mice fed mixed isomers of CLA and directly injected with antibodies to collagen, suggesting that mixed isomer CLA altered the adaptive immune Th1 response thereby promoting arthritic disease. In the spleen, CLAt10c12 is suggested as the CLA isomer playing a major role in Ig production; CLAc9t11 was found to reduce splenic B cells [45]. The Th1 response of increased IgG_{2a} has been associated with increased severity of CA [15, 46, 47]. Taking these findings together, we postulated that the CLAt10c12 isomer was the isomer promoting the Th1 shift and was responsible for the early occurrence of Th1 disease. Interestingly, in our study, we did not see an increase in the incidence of arthritis, but did see a more severe initial inflammatory response when mice were fed CLAt10c12 at the 2° immunization. While Huebner *et al*. [30] observed a dose effect when feeding CLAt10c12 at arthritis onset, future studies are necessary to understand any dose effects that may result from CLA isomer exposure prior to arthritis onset.

Unfortunately, plasma and paw cytokines were not measured in this study; however, anti-CII antibody levels trended toward significantly increased IgG_1 in the CLAt10c12 fed mice ($P = .06$) compared to arthritic CO fed mice. Variance for arthritic CO fed mice was similar to what was reported by Butz *et al*. [15]. However, small sample size available for measuring anti-CII antibodies likely influenced the high variation in these studies. Anti-CII IgG antibodies may have pathogenic effects on collagen, independent of the arthritic inflammatory response, and are required for CA development [48, 49]. The 2° immunization is given to increase IgG levels and induce the onset of CA [50]. The elevation in anti-CII antibodies caused by feeding CLAt10c12 at the 2° immunization may explain why the severity of the arthritic score was increased.

CLAc9t11.

CLAc9t11 has been shown to exhibit anti-inflammatory effects when fed post induction of autoimmune arthritis [14, 36]. These anti-inflammatory effects may be driven by CLAc9t11's ability to reduce pro-inflammatory cytokines such as IL-1β, IL-6 [14, 36], and TNF-α [16, 20], through a process linked to NF-κB signal transduction [23, 51]. Changes in these cytokine profiles may explain CLAc9t11's ability to reduce inflammation post arthritis induction, but fail to explain how CLAc9t11 reduces the incidence of disease when fed prior to onset. In Huebner *et al*. [14] feeding CLAc9t11 at onset of arthritis decreased total anti-CII IgG; a finding not supported in this study where IgG_1 and IgG_{2a} were similar between arthritic mice fed CO or CLAc9t11. Interestingly, CLAc9t11 fed mice injected with collagen that did not develop arthritis had a significant reduction in IgG_{2a} versus CO fed mice ($P = 0.05$). The decreased anti-CII antibody production, specifically IgG_{2a}, by CLAc9t11 feeding may be a mechanism by which CLAc9t11 helps protect against arthritis incidence by decreasing Th1 responses [47], possibly by preventing a threshold level of antibodies required for disease onset. A future study tracking anti-CII IgG production over the course of CA development may further support this conclusion.

A more plausible explanation of how pre-arthritic development feeding of CLAc9t11 may impact the incidence of arthritis is that CLAc9t11 shifts the cytokine profile during the adaptive immune response to CII to Th2 cytokines. Previous work in the CA model showed that if Th2 cytokine profiles are increased, during the adaptive immune response, the incidence of arthritic disease is reduced [52]. Loscher *et al.* [23] has shown that the treatment of dendritic cells with CLAc9t11 decreased the Th1 cytokines IL-12, and increased the Th2 cytokine IL-10. Our finding that feeding CLAc9t11 prior and during the adaptive immune response to CII increased Th2 cytokines, IL-4 and IL-10, in paws by the end of the study suggests that CLAc9t11 drives a

Th2 dominant cytokine profile during adaptive immunity. An assumption made in our analysis is that cytokine levels observed in paws at the end of the study reflect cytokine levels at the time of arthritis. Unfortunately, we cannot say how cytokines were different between arthritic and ND mice in this study. Interestingly, the drive toward Th2 cytokine profile was not associated with a change in the severity of arthritis in mice. The increase in IL-4 and IL-10 seen in CLAc9t11 fed mice may provide some insight into changes in cytokine profile created by CLAc9t11 that may protect against arthritis development. An important future direction will be to observe cytokine profiles over time starting before immunization and continuing through arthritis induction, preferably in paws.

Fatty acids play a significant role in autoimmune and inflammatory responses [53, 54]. Immune and inflammatory modulating effects are often attributed to the cyclooxygenase or lipoxygenase products of 20:4 (arachidonate) or 20:5n-3 (eicosapentaenoate) [53]. The liver is a primary site for the processing lipids and can be useful in discerning the effects of CLA on fatty acid composition [55, 56]. As expected, feeding CLAc9t11 did increase hepatic relative abundance of CLAc9t11. Previously, extended feeding of CLAt10c12 or CLAc9t11 post arthritis development was found to have no effect on hepatic 20:4 or 20:5 n-3 [30, 36]. In a similar manner feeding CLAc9t11 before arthritis development had no impact on these fatty acids. CLAt10c12 is a known stearoyl-CoA desaturase (SCD)-1 inhibitor, and CLAc9t11 has not been reported to affect this enzyme but did decrease levels of fatty acid synthase [57–59]. Unlike CLAt10c12, which increases 18:0 and decreases 18:1 and its 16 carbon precursors due to its inhibition of SCD-1, CLAc9t11 had the opposite effect in this study as 18:0 was decreased 12% and 16:1 was increased 40% due to CLAc9t11 feeding. Effects of CLAc9t11 on liver 18:0 and 16:1 composition have not been previously reported. While not significant, Huebner *et al.* [36]

showed up to a 12% decrease in 18:0 and a 14% increase in 16:1 due to CLAc9t11 consumption. Feeding the precursor to CLAc9t11, trans vaccenic acid, also caused a 20% decrease in 18:0 and a 24% increase in 16:1 (again not statistically significant) [60]. An explanation for these changes in hepatic 16:1 and 18:0 is not readily apparent based on the scientific literature, but we hypothesize that they may be linked to SCD-1 activity during immune activation since SCD-1 expression is suggested to attenuate pro-inflammatory signaling [61].

In conclusion, this is the first report that shows pure isomers of CLAt10c12 and CLAc9t11 have unique effects on arthritis development and early severity when fed to mice prior to the onset of arthritis. The finding that CLAt10c12 may drive Th1 type responses during the adaptive immune response, whereas CLAc9t11 may drive a Th2 dominant adaptive immune response provides a unique opportunity in the development of pure CLA compositions, specifically for the prevention of Th1 type diseases in susceptible animals and humans. These data seem to also support the consumption of foods rich or enriched in CLAc9t11, such as those of ruminant origin or through fermentation, as a potential health benefit to humans and animals at risk of Th1 mediated diseases. However, the ability to direct Th1 versus Th2 pathways through CLA isomer feeding or supplementation may have consequences to health beyond autoimmune disease.

2.6 Acknowledgments

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2.7 Tables

Table 1 Composition of experimental diet contents used in experiment 1 and 2

Ingredient	g/100g
Sucrose	47.6
Casein[1]	21
Corn starch	15
Corn oil[2]	6.0
Cellulose	5
AIN-76 Mineral mix	3.5
AIN-76 Vitamin mix	1.0
Calcium carbonate	0.4
DL-Methionine	0.3
Choline bitartrate	0.2
Ethoxyquin	0.001

[1]Casein was vitamin-free tested and contained no detectable CLA

[2]Conjugated linoleic acid (CLA) t10c12 or CLAc9t11 was added at 0.25 % or 0.5 %, respectively, at the expense of corn oil

Table 2 Fatty acid composition of dietary lipid sources

	g/100g		
Fatty Acid	CO	CLAt10c12	CLAc9t11
16:0	12.8	n/d	n/d
18:0	2.4	n/d	n/d
18:1c9	29.1	n/d	0.5
18:1c11	0.8	n/d	n/d
18:2n-6	53.2	3	n/d
18:2c9t11	n/d	n/d	92.2
18:2t10c12	n/d	82.5	n/d
18:2cc	n/d	1.8	-
18:2tt	n/d	8.6	-
18:3c9c12c15	1.2	n/d	-
20:0	0.5	n/d	n/d
Unknown	0	4.1	7.3

CO = corn oil, CLA = conjugated linoleic acid, n/d = not detected, - = standard not available during experiment 2

Table 3 Experiment 1. Overall mean arthritis incidence and score of mice fed CO or CLAt10c12. Incidence is based on n = 9 mice per group. Arthritic score values include only mice that developed arthritis (n = 7 mice per group). Values shown are means ± SEM

	Arthritic				
	CO	CLAt10c12	*P*-value		
Maximum Incidence	78%	78%	0.59		
			Diet	Day	Interaction
Clinical Arthritic Score	5.2 ± 0.3	7.4 ± 0.5	0.20	<0.0001	0.02
Normalized Score	1.5 ± 0.2	5.3 ± 0.5	0.06	<0.0001	0.008

CO = corn oil, CLA = conjugated linoleic acid, and SEM = standard error of the mean

Table 4 Anti-collagen type II IgG concentration (µg/ml) 35 days post primary collagen immunization. CO n = 4 and CLAt10c12 n = 3. Values shown are means ± SEM

	Sham	Arthritic		
µg/ml	CO	CO	CLA t10c12	*P*-value[1]
IgG_1	n/d	816 ± 284	4062 ± 1593	0.06
IgG_{2a}	n/d	1886 ± 621	6435 ± 4913	0.33

CO = corn oil, CLA = conjugated linoleic acid, AD = day post arthritis development, SEM = standard error of the mean, and n/d = not detected

[1]Statistical analysis includes only arthritic mice

Table 5 Experiment 2. Overall mean arthritis incidence and score of mice fed CO, or CLAc9t11. Incidence is based on n = 16 for CO fed mice and n = 20 for CLAc9t11 fed mice. Arthritic score averages include only mice that developed arthritis (n = 11 CO and n = 6 CLAc9t11). Values shown are means ± SEM

	Arthritic				
	CO	CLAc9t11	*P*-value		
Maximum Incidence	69%	30%	0.01		
			Diet	Day	Interaction
Clinical Arthritic Score	4.1 ± 0.1	4.1 ± 0.2	0.86	0.18	0.47
Normalized Score	0.77 ± 0.1	1.1 ± 0.2	0.57	0.12	0.29

CO = corn oil, CLA = conjugated linoleic acid, and SEM = standard error of the mean

Table 6 Anti-collagen type II IgG concentration (µg/ml) at study end, day 70 for mice that never developed arthritis (CO fed mice n = 5 and CLAc9t11 fed mice n = 14, a) or AD 7 for arthritic mice (CO fed mice n = 11 and CLAc9t11 fed mice n = 6, b). Values shown are means ± SEM

a		Sham	Non-developed		
	µg/ml	CO & CLAc9t11	CO	CLAc9t11	*P*-value[1]
	IgG_1	n/d	426 ± 128	219 ± 34	0.14
	IgG_{2a}	n/d	1379 ± 373[a]	823 ± 96[b]	0.05
b		Sham	Arthritic		
	µg/ml	CO & CLAc9t11	CO	CLAc9t11	*P*-value[1]
	IgG_1	n/d	571 ± 114	415 ± 94	0.37
	IgG_{2a}	n/d	1702 ± 259	1966 ± 394	0.57

CO = corn oil, CLA = conjugated linoleic acid, AD = day post arthritis development, SEM = standard error of the mean, and n/d = not detected

Means within a row without a common letter differ by $P \leq 0.05$

[1]Statistical analysis was performed across dietary treatment within arthritic classification, but not across arthritic classification. Antibody levels in shams were undetectable; therefore, not included in analysis

Table 7 Relative hepatic fatty acid compositions of arthritic, non-developed, and sham mice fed corn oil or CLAc9t11 dietary treatments. Sham CO n = 8, sham CLAc9t11 n = 6, non-developed CO n = 5, non-developed CLAc9t11 n = 14, arthritic CO n = 11, and arthritic CLAc9t11 n = 6. Values are mean relative fatty acid content in g/100g.

	Sham		Non-developed		Arthritic		SEM[1]	*P*-value		
Fatty Acid (g/100g)	CO	CLAc9t11	CO	CLAc9t11	CO	CLAc9t11		Diet	Arthritis	Interaction
14:0	9.86	11.56	9.94	9.68	11.15	11.93	0.40	0.41	0.20	0.49
16:0	30.28	30.1	30.31	29.63	29.29	29.11	0.20	0.42	0.14	0.86
16:1c9	1.28	1.26	0.95	1.53	0.84	1.51	0.09	0.02	0.89	0.22
18:0	8.61	8.81	9.76	8.03	8.93	7.23	0.23	0.03	0.33	0.18
18:1c9	16.66	14.95	15.38	16.45	15.68	17.93	0.40	0.54	0.57	0.17
18:1c11	1.87	1.68	1.85	2.12	1.84	2.28	0.07	0.21	0.22	0.16
18:2n-6	21.23	20.05	20.27	20.62	20.94	20.17	0.25	0.33	0.96	0.50
20:0	0.16	0.15	0.17	0.2	0.09	0.15	0.03	0.62	0.65	0.85
18:3n-3	0.02	0.02	n/d	n/d	0.01	0.01	0.00	0.80	0.28	0.89
20:1c11	0.16	0.16	0.06	0.22	0.13	0.12	0.03	0.48	0.89	0.45
18:2c9t11	n/d	0.85	n/d	0.95	n/d	0.79	0.07	<0.0001	0.10	0.10
18:2t10c12	n/d	n/d	n/d	n/d	n/d	n/d	-	-	-	-
20:2n-6	0.03	0.06	n/d	0.04	0.07	0.03	0.02	0.77	0.68	0.58
20:3n-6	0.19	0.26	0.21	0.37	0.21	0.21	0.04	0.31	0.66	0.72
20:4n-6	6.89	7.21	7.81	6.93	7.78	6.07	0.19	0.06	0.65	0.13
22:6n-3	0.98	0.94	1.04	0.92	1.10	0.75	0.04	0.02	0.53	0.19
Unknown	1.78	1.95	2.25	2.32	1.95	1.70	0.14	0.99	0.41	0.85

CO = corn oil, CLA = conjugated linoleic acid, SEM = standard error of the mean, and n/d = not detected

[1]Pooled SEM

2.8 Figures

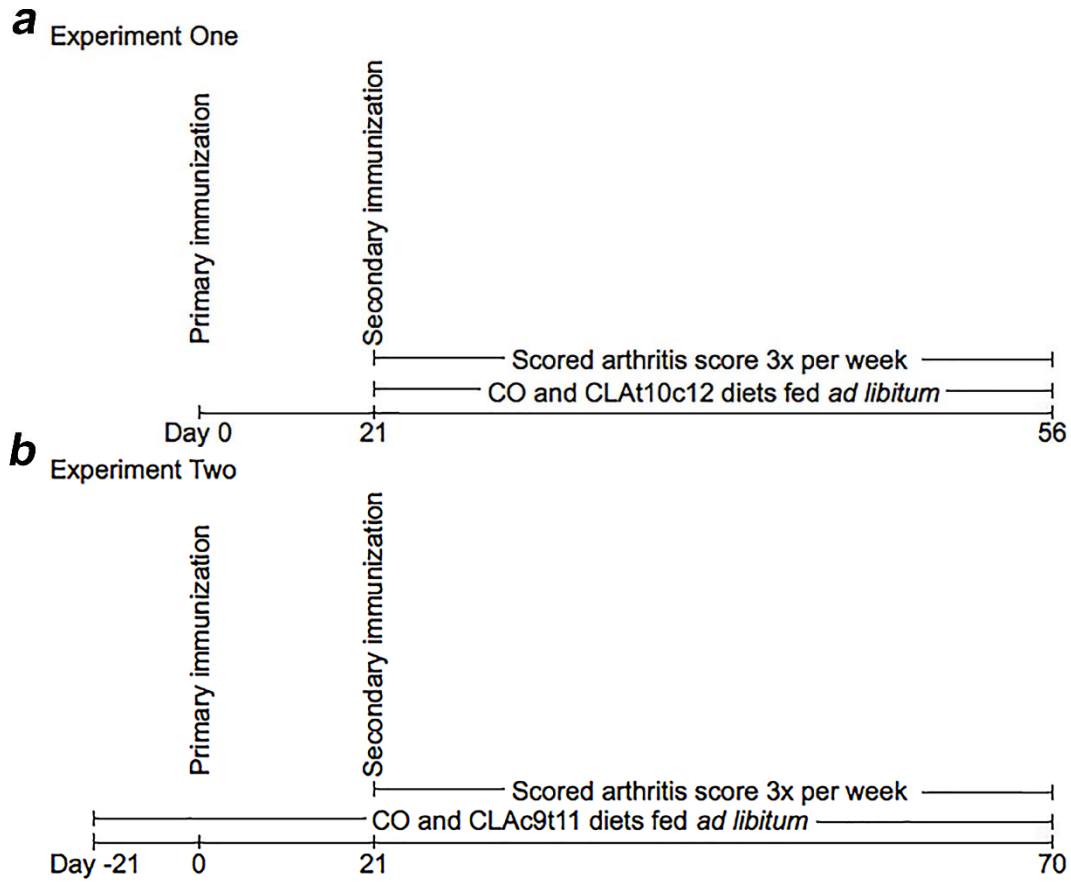

Figure 1: Illustration of experimental designs. Both studies utilized the collagen induced arthritis (CA) model. In experiment one (a), mice were fed diet supplemented with 1% corn oil (CO) or 0.25% conjugated linoleic acid (CLA) *trans*-10, *cis*-12 (CLAt10c12) at the expense of CO (n = 9 per group) starting at the secondary inoculation. Mice continued on diet until the end of the study (56 days after primary immunization). In experiment two (b), mice were fed diets supplemented with 1% CO or 0.5% CLA *cis*-9, *trans*-11 (CLAc9t11) at the expense of CO (n = 20 per group) starting three weeks before primary immunization. Mice continued on diet for four weeks after arthritis development or until the end of the study (70 days post primary immunization).

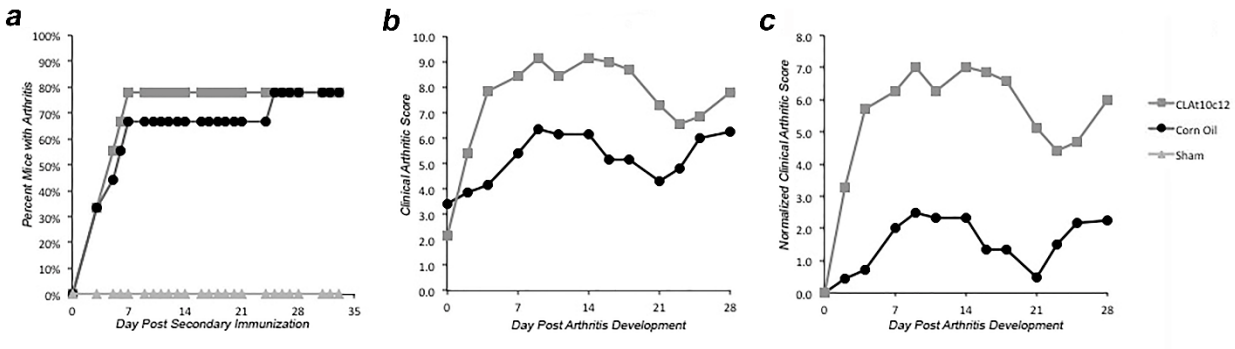

Figure 2: Experiment 1. Arthritis incidence and absolute or normalized arthritic score of arthritic mice fed diet supplemented with 1% CO or 0.25% CLAt10c12 beginning at secondary inoculation with chick collagen type II. The incidence of arthritis (a) in CO and CLAt10c12 fed mice reached a maximum of 78%. The absolute clinical arthritic score (b) and normalized clinical arthritic score (c) were increased in CLAt10c12 fed mice. See table 3 for summary of statistical data and error around the mean.

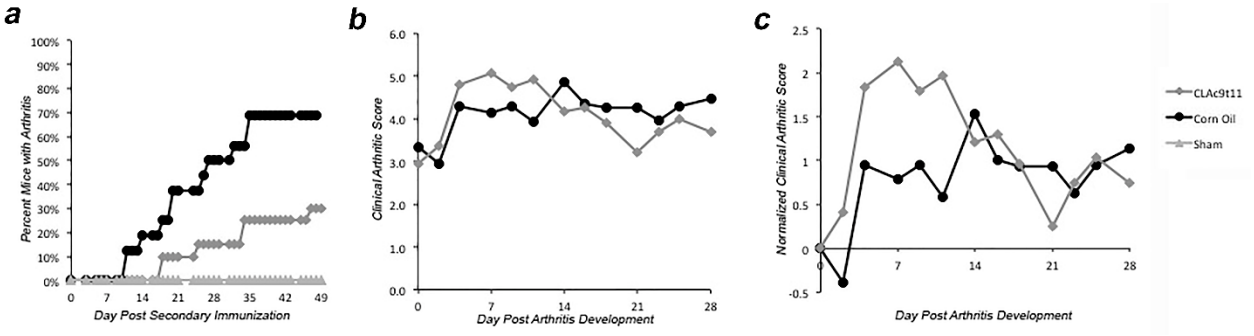

Figure 3: Experiment 2. Arthritis incidence and absolute or normalized arthritic score of arthritic mice fed diet supplemented with 1% CO or 0.5% dietary CLAc9t11 three weeks prior to immunization with chick collagen type II. The incidence of arthritis (a) in CO fed mice had a maximum incidence of 69%, while CLAc9t11 fed mice had a maximum incidence of 30%. The absolute clinical arthritic score (b) and the normalized clinical arthritic score (c) of both CO and CLAc9t11 fed mice was similar thought the study. See table 5 for summary of statistical data and error around the mean.

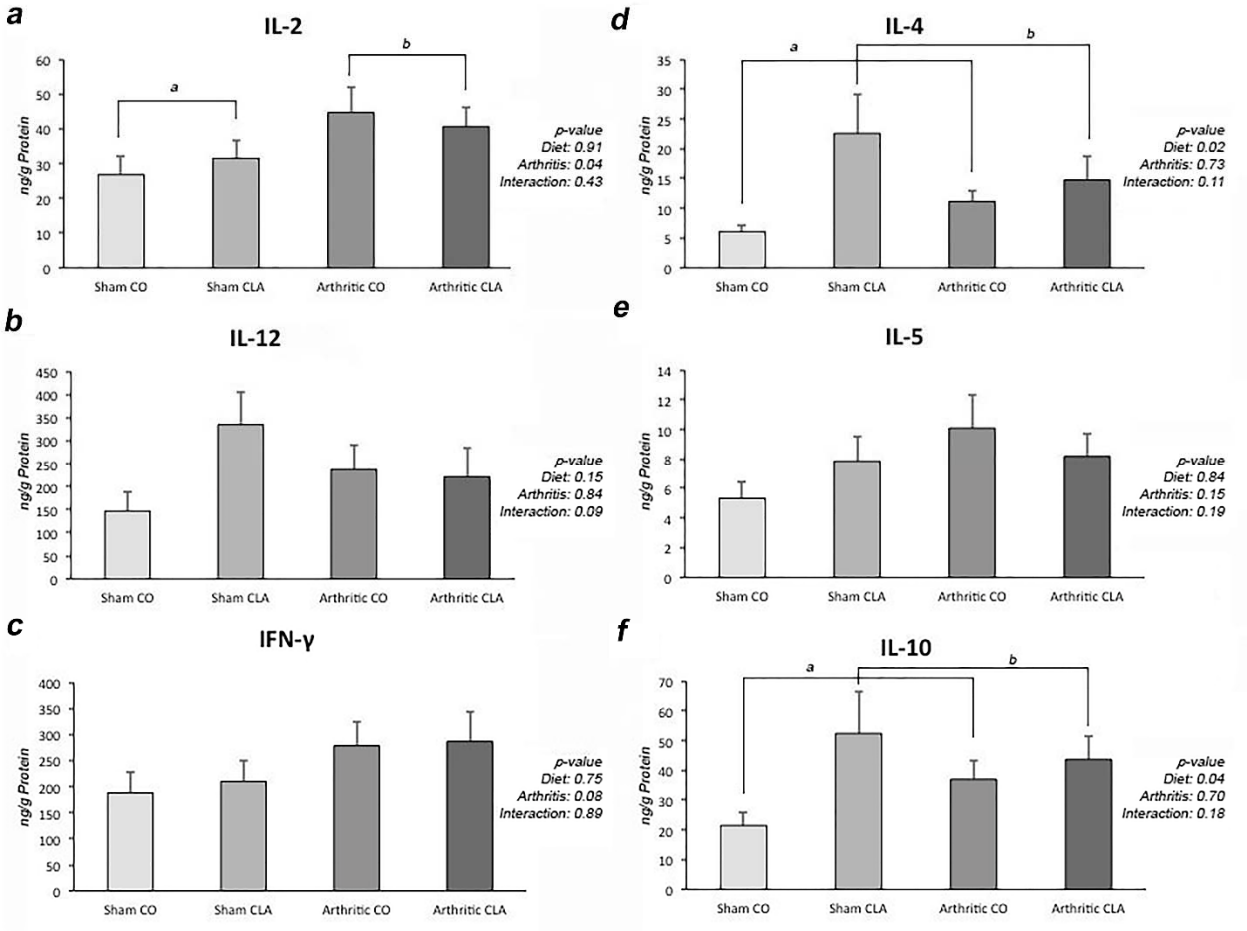

Figure 4: Paw extract concentrations of interleukin (IL)-2 (a), IL-12 (p70) (b), interferon (IFN)γ (c), IL-4 (d), IL-5 (e), and IL-10 (f) in ng/g protein of sham mice fed CO (n = 7) or CLAc9t11 fed mice (n = 6) and arthritic mice fed CO (n = 6) or CLAc9t11 (n = 6). Bars represent means + SEM. Means without a common letter differ by $P \leq 0.05$.

2.9 References

1. Kim JH, Kim Y, Kim YJ, Park Y (2016) Conjugated Linoleic Acid: Potential Health Benefits as a Functional Food Ingredient. Annu Rev Food Sci Technol 7:221–244. doi: 10.1146/annurev-food-041715-033028

2. Wahle KWJ, Heys SD, Rotondo D (2004) Conjugated linoleic acids: are they beneficial or detrimental to health? Prog Lipid Res 43:553–587. doi: 10.1016/j.plipres.2004.08.002

3. Whigham LD, Cook ME, Atkinson RL (2000) Conjugated linoleic acid: implications for human health. Pharmacol Res 42:503–510. doi: http://dx.doi.org/10.1006/phrs.2000.0735

4. Reaney MJT, Lui Y-D, Westcott ND (1999) Commercial Production of Conjugated Linoleic Acid. In: Yurawecz MP, Mossoba MM, Kramer JK., et al (eds) Adv. Conjug. Linoleic Acid Res. Vol. 1. AOCS Press, Champaign, Illinois, pp 39–54

5. Parodi PW (2003) Conjugated Linoleic Acid in Food. In: Sébédio J-L, Christie WW, Adolf R (eds) Adv. Conjug. Linoleic Acid Res. Vol. 2. AOCS Press, Champaign, Illinois, pp 101–122

6. Ha YL, Grimm NK, Pariza MW (1987) Anticarcinogens from fried ground beef: heat-altered derivatives of linoleic acid. Carcinogenesis 8:1881–1887.

7. Nicolosi RJ, Rogers EJ, Kritchevsky D, et al (1997) Dietary conjugated linoleic acid reduces plasma lipoproteins and early aortic atherosclerosis in hypercholesterolemic hamsters. Artery 22:266–277.

8. Cook ME, Miller CC, Park Y, Pariza M (1993) Immune modulation by altered nutrient metabolism: nutritional control of immune-induced growth depression. Poult Sci 72:1301–1305.

9. Lee KN, Kritchevsky D, Parizaa MW (1994) Conjugated linoleic acid and atherosclerosis in rabbits. Atherosclerosis 108:19–25. doi: 10.1016/0021-9150(94)90034-5

10. Yang M, Pariza MW, Cook ME (2000) Dietary Conjugated Linoleic Acid Protects Against End Stage Disease of Systemic Lupus Erythematosus in the NZB/W F1 Mouse. Immunopharmacol Immunotoxicol 22:433–449.

11. Yang M, Cook ME (2003) Dietary CLA decreased weight loss and extended survival following the onset of kidney failure in NZB/W F1 mice. Lipids 38:21–24. doi: 10.1007/s11745-003-1026-8

12. Whigham LD, Cook EB, Stahl JL, et al (2001) CLA reduces antigen-induced histamine and PGE2 release from sensitized guinea pig tracheae. Am J Physiol Regul Integr Comp Physiol 49:R908–R912.

13. Bassaganya-Riera J, Reynolds K, Martino-Catt S, et al (2004) Activation of PPAR γ and δ by conjugated linoleic acid mediates protection from experimental inflammatory bowel disease. Gastroenterology 127:777–791. doi: 10.1053/j.gastro.2004.06.049

14. Huebner SM, Campbell JP, Butz DE, et al (2010) Individual Isomers of Conjugated Linoleic Acid Reduce Inflammation Associated with Established Collagen-Induced Arthritis in DBA/1 Mice. J Nutr 140:1454–1461. doi: 10.3945/jn.109.120527

15. Butz DE, Li G, Huebner SM, Cook ME (2007) A mechanistic approach to understanding conjugated linoleic acid's role in inflammation using murine models of rheumatoid arthritis. Am J Physiol Regul Integr Comp Physiol 293:R669–R676. doi: 10.1152/ajpregu.00005.2007

16. Yang M, Cook ME (2003) Dietary Conjugated Linoleic Acid Decreased Cachexia,

Macrophage Tumor Necrosis Factor-α Production, and Modifies Splenocyte Cytokines Production. Exp Biol Med Diet 228:51–58.

17. Cook ME, Butz D, Li G, et al (2003) Conjugated Linoleic Acid Enhances Immune Responses but Protects Against the Collateral Damage of Immune Events. In: Sébédio J-L, Christie WW, Adolf R (eds) Adv. Conjug. Linoleic Acid Res. Vol. 2. AOCS Press, Champaign, Illinois, pp 283–291

18. Sugano M, Tsujita A, Yamasaki M, et al (1998) Conjugated linoleic acid modulates tissue levels of chemical mediators and immunoglobulins in rats. Lipids 33:521–527.

19. de Roos B, Rucklidge G, Reid M, et al (2005) Divergent mechanisms of cis9, trans11- and trans10, cis12-conjugated linoleic acid affecting insulin resistance and inflammation in apolipoprotein E knockout mice: a proteomics approach. FASEB J 19:1746–8. doi: 10.1096/fj.05-3953fje

20. Park Y, Yang M, Storkson JM, et al (2007) Effects of conjugated linoleic acid isomers on serum tumor necrosis factor-a concentration in mice. J Food Biochem 31:252–265.

21. Li G, Barnes D, Butz D, et al (2005) 10t,12c-Conjugated Linoleic Acid Inhibits Lipopolysaccharide-Induced Cyclooxygenase Expression in Vitro and in Vivo. J Lipid Res 46:2134–2142. doi: 10.1194/jlr.M500064-JLR200

22. Reynolds CM, Loscher CE, Moloney AP, et al (2008) Cis-9, trans-11-conjugated linoleic acid but not its precursor trans-vaccenic acid attenuate inflammatory markers in the human colonic epithelial cell line Caco-2. Br J Nutr 100:13–17. doi: 10.1017/S0007114508894329

23. Loscher CE, Draper E, Leavy O, et al (2005) Conjugated linoleic acid suppresses NF-

kappa B activation and IL-12 production in dendritic cells through ERK-mediated IL-10 induction. J Immunol 175:4990–4998. doi: 10.4049/JIMMUNOL.175.8.4990

24. Arbonés-Mainar JM, Navarro MA, Acín S, et al (2006) Trans-10, cis-12- and cis-9, trans-11-conjugated linoleic acid isomers selectively modify HDL-apolipoprotein composition in apolipoprotein E knockout mice. J Nutr 136:353–359.

25. Jaudszus A, Foerster M, Kroegel C, et al (2005) Cis-9,Trans-11-CLA exerts anti-inflammatory effects in human bronchial epithelial cells and eosinophils: Comparison to Trans-10,Cis-12-CLA and to linoleic acid. Biochim Biophys Acta - Mol Cell Biol Lipids 1737:111–118. doi: 10.1016/j.bbalip.2005.11.001

26. Tricon S, Burdge GC, Kew S, et al (2004) Opposing effects of cis-9,trans-11 and trans-10,cis-12 conjugated linoleic acid on blood lipids in healthy humans. Am J Clin Nutr 80:614–620.

27. Park Y, Storkson JM, Albright KJ, et al (1999) Evidence that the trans-10,cis-12 isomer of conjugated linoleic acid induces body composition changes in mice. Lipids 34:235–241. doi: 10.1007/s11745-999-0358-8

28. Brand DD, Latham KA, Rosloniec EF (2007) Collagen-induced arthritis. Nat Protoc 2:1269–1275. doi: 10.1038/nprot.2007.173

29. Kramer JKG, Blackadar CB, Zhou J (2002) Evaluation of two GC columns (60-m SUPELCOWAX 10 and 100-m CP sil 88) for analysis of milkfat with emphasis on CLA, 18:1, 18:2 and 18:3 isomers, and short- and long-chain FA. Lipids 37:823–835. doi: 10.1007/s11745-002-0967-2

30. Huebner SM, Olson JM, Campbell JP, et al (2014) Dietary trans-10,cis-12 CLA reduces

murine collagen-induced arthritis in a dose-dependent manner. J Nutr 144:177–184. doi: 10.3945/jn.113.182550

31. Lu LD, Stump KL, Seavey MM (2010) Novel method of monitoring trace cytokines and activated STAT molecules in the paws of arthritic mice using multiplex bead technology. BMC Immunol 11:55. doi: 10.1186/1471-2172-11-55

32. Folch J, Lees M, Sloane Stanley GH (1957) A simple method for the isolation and purification of total lipides from animal tissues. J Biol Chem 226:497–509.

33. Stoffel W, Insull W, Ahrens EH (1958) Gas-liquid chromatography of highly unsaturated fatty acid methyl esters. Proc Soc Exp Biol Med 99:238–241.

34. Yeonhwa Park, Karen J. Albright, Zu Y. Cai A, Pariza MW (2001) Comparison of Methylation Procedures for Conjugated Linoleic Acid and Artifact Formation by Commercial (Trimethylsilyl)diazomethane. J Agric Food Chem 49:1158–1164. doi: 10.1021/JF001209Z

35. Butz DE (2005) Conjugated Linoleic Acid Reduces Inflammation In Animal Models: Evidence of a Molecular Mechanism.

36. Huebner SM, Olson JM, Campbell JP, et al (2016) Low Dietary c9t11-Conjugated Linoleic Acid Intake from Dairy Fat or Supplements Reduces Inflammation in Collagen-Induced Arthritis. Lipids 51:807–819. doi: 10.1007/s11745-016-4163-8

37. Mauri C, Williams RO, Walmsley M, Feldmann M (1996) Relationship between Th1/Th2 cytokine patterns and the arthritogenic response in collagen-induced arthritis. Eur J Immunol 26:1511–1518. doi: 10.1002/eji.1830260716

38. Luross JA, Williams NA (2001) The genetic and immunopathological processes

underlying collagen-induced arthritis. Immunology 103:407–416. doi: 10.1046/j.1365-2567.2001.01267.x

39. Watson WC, Townes AS (1985) Genetic susceptibility to murine collagen II autoimmune arthritis. Proposed relationship to the IgG2 autoantibody subclass response, complement C5, major histocompatibility complex (MHC) and non-MHC loci. J Exp Med 162:1878–1891.

40. Thornton S, Duwel LE, Boivin GP, et al (1999) Association of the course of collagen-induced arthritis with distinct patterns of cytokine and chemokine messenger RNA expression. Arthritis Rheum 42:1109–1118. doi: 10.1002/1529-0131(199906)42:6<1109::AID-ANR7>3.0.CO;2-7

41. Segal R, Bermas BL, Dayan M, et al (1997) Kinetics of cytokine production in experimental systemic lupus erythematosus: involvement of T helper cell 1/T helper cell 2-type cytokines in disease. J Immunol 158:3009–3016.

42. Enghard P, Langnickel D, Riemekasten G (2006) T cell cytokine imbalance towards production of IFN-γ and IL-10 in NZB/W F1 lupus-prone mice is associated with autoantibody levels and nephritis. Scand J Rheumatol 35:209–216. doi: 10.1080/03009740500417791

43. Datta SK, Patel H, Berry D (1987) Induction of a cationic shift in IgG anti-DNA autoantibodies. Role of T helper cells with classical and novel phenotypes in three murine models of lupus nephritis. J Exp Med 165:1252–1268.

44. Jacob CO, van der Meide PH, McDevitt HO (1987) In vivo treatment of (NZB X NZW)F1 lupus-like nephritis with monoclonal antibody to gamma interferon. J Exp Med

166:798–803.

45. Yamasaki M, Chujo H, Hirao A, et al (2003) Immunoglobulin and cytokine production from spleen lymphocytes is modulated in C57BL/6J mice by dietary cis-9, trans-11 and trans-10, cis-12 conjugated linoleic acid. J Nutr 133:784–788.

46. Cooper SM, Sriram S, Ranges GE (1988) Suppression of murine collagen-induced arthritis with monoclonal anti-Ia antibodies and augmentation with IFN-gamma. J Immunol 141:1958–1962.

47. Kaplan C, Valdez JC, Chandrasekaran R, et al (2002) Th1 and Th2 cytokines regulate proteoglycan-specific autoantibody isotypes and arthritis. Arthritis Res 4:54. doi: 10.1186/ar383

48. Crombie DE, Turer M, Zuasti BB, et al (2005) Destructive effects of murine arthritogenic antibodies to type II collagen on cartilage explants in vitro. Arthritis Res Ther 7:R927–R937. doi: 10.1186/ar1766

49. Svensson L, Jirholt J, Holmdahl R, Jansson L (1998) B cell-deficient mice do not develop type II collagen-induced arthritis (CIA). Clin Exp Immunol 111:521–6. doi: 10.1046/j.1365-2249.1998.00529.x

50. Stuart JM, Townes AS, Kang AH (1982) Nature and specificity of the immune response to collagen in type II collagen-induced arthritis in mice. J Clin Invest 69:673–683. doi: 10.1172/jci110495

51. Li G (2004) Modulation of Cyclooxygenase and Other NF-κgB Regulated Gene Products by Conjugated Fatty Acids.

52. Bessis N, Boissier MC, Ferrara P, et al (1996) Attenuation of collagen-induced arthritis in

mice by treatment with vector cells engineered to secrete interleukin-13. Eur J Immunol 26:2399–2403. doi: 10.1002/eji.1830261020

53. James MJ, Gibson RA, Cleland LG (2000) Dietary polyunsaturated fatty acids and inflammatory mediator production. Am J Clin Nutr 71:343S–8S.

54. Simopoulos AP (2002) Omega-3 fatty acids in inflammation and autoimmune diseases. J Am Coll Nutr 21:495–505.

55. Canbay A, Bechmann L, Gerken G (2007) Lipid Metabolism in the Liver. Z Gastroenterol 45:35–41. doi: 10.1055/s-2006-927368

56. Belury MA, Kempa-Steczko A (1997) Conjugated linoleic acid modulates hepatic lipid composition in mice. Lipids 32:199–204. doi: 10.1007/s11745-997-0025-0

57. Choi Y, Kim YC, Han YB, et al (2000) The trans-10,cis-12 isomer of conjugated linoleic acid downregulates stearoyl-CoA desaturase 1 gene expression in 3T3-L1 adipocytes. J Nutr 130:1920–1924.

58. Park Y, Storkson JM, Ntambi JM, et al (2000) Inhibition of hepatic stearoyl-CoA desaturase activity by trans-10,cis-12 conjugated linoleic acid and its derivatives. Biochim Biophys Acta - Mol Cell Biol Lipids 1486:285–292. doi: 10.1016/S1388-1981(00)00074-3

59. Maslak E, Buczek E, Szumny A, et al (2015) Individual CLA Isomers, c9t11 and t10c12, Prevent Excess Liver Glycogen Storage and Inhibit Lipogenic Genes Expression Induced by High-Fructose Diet in Rats. Biomed Res Int. doi: 10.1155/2015/535982

60. Olson J (2016) The ruminant-derived fatty acids cis-9, trans-11 conjugated linoleic acid and trans-11 vaccenic acid are anti-inflammatory in the collagen-induced arthritis model:

Evidence of intestinal barrier dysfunction associated with arthritis-induced inflammation.

61. Chen C, Shah YM, Morimura K, et al (2008) Metabolomics Reveals that Hepatic Stearoyl-CoA Desaturase 1 Downregulation Exacerbates Inflammation and Acute Colitis. Cell Metab 7:135–147. doi: 10.1016/j.cmet.2007.12.003

Chapter 3:

Conjugated linoleic acid isomers *trans*-10, *cis*-12 and *cis*-9, *trans*-11 prevent collagen-induced arthritis in a direct comparison.

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3.1 Abstract

Mixed isomer conjugated linoleic acid (CLA) and the individual isomers, *trans*-10, *cis*-12 (t10c12) and *cis*-9, *trans*-11 (c9t11), decrease severity of collagen-induced arthritis (CA) when consumed after disease onset. Few studies have been conducted exploring the role of CLA in the prevention of autoimmune diseases. These studies suggest that isomer specific effects may be occurring; however, a direct comparison of CLAt10c12 and CLAc9t11 has yet to be conducted. A study to compare CLAt10c12's and CLAc9t11's ability to prevent CA and assess their effects on early inflammation was performed. DBA/1 mice were fed a semi-purified diet containing 6% corn oil (CO), 5.5% CO and 0.5% CLAt10c12, or 5.5% CO and 0.5% CLAc9t11 (n = 27 per diet) starting three weeks before CA primary immunization. Effects on disease incidence and severity, anti-collagen antibodies, plasma and paw cytokines, and hepatic fatty acids were measured. Arthritis incidence was reduced by a minimum of 34 % in mice fed either CLA isomer compared to those fed CO diet ($P = 0.06$). In mice that did develop arthritis (n = 9-12 mice per treatment), CLAt10c12 reduced arthritic severity to a greater extent than CLAc9t11 and CO ($P = 0.03$). CLA isomer treatment attenuated the increased hepatic arachidonic acid (20:4n-6) observed with arthritis at one-week post-onset ($P = 0.03$), while no differences in anti-collagen antibodies or cytokines were observed between dietary treatments. These results suggest that CLA isomers may be effective at preventing specific immune mediated inflammatory diseases, in part, through modulation of the arachidonic acid cascade.

3.2 Introduction

Approximately 32% of the United States population suffers from chronic inflammation, characterized by non-resolving activation of the immune system [1]. This unresolved inflammation (e.g., asthma, ulcerative colitis, atherosclerosis, sarcoidosis, rheumatoid arthritis) can be detrimental to the body's ability to maintain homeostasis [2]. Immune-mediated inflammatory diseases (IMID), which include autoimmunity, type I-IV hypersensitivities, and inflammatory bowel disease, are a class of chronic inflammatory diseases in which both the innate and adaptive immune systems propagate disease. The autoimmune condition rheumatoid arthritis (RA) affects about 2% of North American adults, and is often used to investigate novel methods to treat or prevent autoimmunity [3–5].

To decrease the need for potentially harmful and costly pharmaceuticals, dietary interventions for IMID are being explored. Using the murine CA model, dietary mixed-isomer conjugated linoleic acid (CLA) and the individual CLA isomers, *trans*-10, *cis*-12 (t10c12) and *cis*-9*, trans*-11 (c9t11), have been shown to reduce inflammation compared to a corn oil (CO) control diet when provided after the onset of arthritis [6–9]. Furthermore, CLAc9t11 treatment at 0.5% of the diet was equivalent to a 1.5mg/kg/day dose of celecoxib, a cyclooxygenase (COX)-2 inhibitor commonly prescribed as an anti-inflammatory [9]. CLA's anti-inflammatory properties have been shown in a variety of other animal disease models, including type I hypersensitivity [10–12], immune-induced cachexia [13, 14], and inflammatory bowel disease [15–17].

With IMID it is difficult to control the disease stimuli; therefore, treatments have focused on controlling progression instead of restoration or prevention. Anti-inflammatory research involving CLA has also historically focused on disease treatment; however, the high cost and rising incidence of chronic inflammatory diseases has created an increasing demand to combat

prevalence. Rula *et al.* [18] estimated that preventative treatments for chronic inflammatory diseases, health promotion, and chronic care management could save Medicare between $650 billion and $1.43 trillion dollars in the next ten years.

Studies that explore the preventative effects of CLA have typically used synthetic preparations containing CLAt10c12 and CLAc9t11 in equal amounts, which does not represent the CLA isomer ratio obtainable in the diet without additional supplementation. CLA is most commonly consumed with the intake of ruminant lipids, which contain CLAt10c12 at 1.4 - 3.5% and CLAc9t11 at 96.5 - 98.6% of total CLA [19]. It is valuable to study the CLA isomers individually, as isomer-specific effects have been shown. For example, CLAt10c12 is anti-adipogenic [20] and atherogenic [21, 22], while CLAc9t11 has no adipogenic effects [20], and is anti-atherogenic [21, 23].

A previous study by Muhlenbeck *et al*. [24] examined the preventative effects of the individual CLA isomers on arthritis incidence and reported a 39% decrease in incidence of mice fed CLAc9t11 compared to a CO fed control group. Isomer specific differences between CLAc9t11 and CLAt10c12 were also suggested in this study; however, the treatment initiation point and dose differed between CLAt10c12 and CLAc9t11, preventing a direct comparison of their effects on arthritis incidence. The study presented here was conducted to assess the effects of CLAt10c12 and CLAc9t11, fed individually and prior to any collagen immunization, on the incidence of arthritis in the CA model.

3.3 Materials and Methods

Experiments were performed in accordance with an animal protocol, A005438, approved by the College of Agricultural and Life Sciences Animal Care and Use Committee.

Collagen-induced arthritis model.

Four-week-old DBA/1 male mice were purchased from Harlan (Indianapolis, IN). Mice were housed in shoebox units (3 mice per box) and maintained on a 12:12 h light-dark cycle. After an acclimation period of one week on chow diet, all mice were fed a 99% complete, casein-based, semi-purified diet (containing 5% CO, TD94060; Harlan-Teklad, Madison, WI) supplemented with 1% CO, 0.5% CLAt10c12 (91% pure containing no detectable CLAc9t11, Nu-Chek Prep, Waterville, MN) and 0.5% CO, or 0.5 % CLAc9t11 (90% pure containing 5% CLAt10c12, Nu-Chek) and 0.5% CO (n = 27 per diet). The 5% CLAt10c12 present in the 90% pure CLAc9t11 was not expected to affect active inflammation as it comprised 0.025% of the diet, below the reported minimum effective dose of 0.125% [7]. After three weeks on treatment diets, mice (grouped by shoebox units) were randomly assigned to receive a 100µl intradermal tail injection of either 100µg chick collagen type II (CII) in 0.05 M acetic acid (Chondrex, Redmound, WA, n = 18 mice per diet) or a sham injection of 0.05 M acetic acid (n = 9 mice per diet) emulsified in a 1:1 ratio with complete Freund's adjuvant (4mg/ml heat-killed *Mycobacterium tuberculosis*; Chondrex) [24, 25]. The date of primary injection was designated as primary day 0. On primary day 21, mice received a 100µl intraperitoneal secondary injection of either 100µg chick CII in 0.05M acetic acid or a sham injection of 0.05M acetic acid emulsified in a 1:1 ratio with Freund's incomplete adjuvant (Chondrex). Following the secondary immunization, mice were monitored daily for clinical signs of arthritis (see Clinical Arthritic Score).

Clinical arthritic score.

As previously described, mice were examined by a trained observer blinded to treatments [6, 7, 24–26]. After the secondary immunization at primary day 21, mice were monitored daily for clinical signs of arthritis until primary day 63. Primary day 63 was chosen as the end point of the study to avoid the risk of older mice developing spontaneous arthritis [25, 27, 28]. Mice were scored upon arthritis development and prior to their euthanasia at arthritic day (AD) 7. Briefly, individual paws were assigned an inflammation severity score between 0 and 4 based on edema, erythema, joint involvement, and mobility. Individual paw scores were summed for an overall clinical arthritic score (CAS) with a maximum of 16 [25]. Normalized arthritic score was calculated by subtracting CAS at AD 0 from the CAS at AD 7.

Paw thickness measurements.

Upon arthritis development (AD 0) and at AD 7 paw thickness of each individual mouse paw was measured using a pressure-sensitive caliper (SPI, Garden Grove, CA). This provided an objective measure of arthritic severity. Measurements of individual mouse paws were summed to provide the overall paw thickness measurement for each mouse. Normalized paw thickness was calculated by subtracting the thickness at AD 0 from paw thickness measured at AD 7.

Plasma collection and preparation.

Blood was collected from the retroorbital venous plexus using heparinized capillary tubes (Midwest Veterinary Supply, Sun Prairie, WI) and transferred into ethylenediaminetetraacetic acid (EDTA) tubes (BD Biosciences, San Jose, CA), while mice were under isoflurane

anesthesia at AD 7 or at the end of the study period for sham mice. Plasma was separated by centrifugation at 3000xg for 10 minutes and stored at -80°C until analyzed.

Plasma anti-chick CII antibody ELISA.

Plasma samples were analyzed as previously described for total concentration of mouse anti-chick CII immunoglobulin (Ig)G_1 and IgG_{2a} [26]. In this experiment, ELISA plates were blocked using 1% dry milk (w/v, ConAgra Foods, Omaha, NE). Anti-chick collagen IgG_1 and IgG_{2a} levels were quantified with respective anti-chick CII monoclonal antibody standard (Millipore and Chondrex).

Paw tissue collection and cytokine extraction.

After mice were euthanized by exsanguination (AD 7 for arthritic mice and primary day 63 days for sham mice), arthritic paws of arthritic mice and all paws of sham mice were collected. Paws were stored at -80°C until cytokine analysis. Prior to preforming the paw cytokine assay, paws were weighed and crushed under liquid nitrogen and total protein was extracted through tissue homogenization in cell lysing solution (Bio-Rad Laboratories, Hercules, CA) containing phenylmethylsulfonyl fluoride, as previously described (MP Biomedicals, Santa Ana, CA) [7]. Tissue extracts were incubated at -80°C for one hour, then centrifuged at 3000xg for 10 minutes at 4°C. Supernatant was collected and stored at -80°C until analysis. Samples were diluted to 1 mg protein/ml in phosphate buffered saline following bicinchoninic acid protein assay results (Thermo Scientific, Waltham, MA) before final cytokine quantification [29].

Cytokine analysis.

Paw and plasma cytokine concentrations of interleukin (IL)-2, interferon (IFN)γ, tumor necrosis factor (TNF)α, and IL-10 were quantified using Bio-Plex Pro T helper cell (Th) 1/Th2 mouse cytokine assays according to manufacturer's instructions (Bio-Rad Laboratories). Fluorescence was measured using Luminex 100 system (Bio-Rad Laboratories), and results were analyzed using Bio-Plex Manager software (Bio-Rad Laboratories).

Hepatic fatty acid determination.

Total lipids of 0.2g liver samples were extracted using chloroform:methanol (2:1, v/v) according to the methods of Folch *et al*. [30]. Fatty acid methyl esters (FAME) were prepared by acid-catalyzed methylation [31, 32]. Relative percentages of liver FAME were determined using an Agilent 6890N gas chromatograph (Agilent Technologies Santa Clara, CA) equipped with a 100 m biscyanopropyl polysiloxane column (Restek, Bellefonte, PA). FAME were identified using a custom qualitative FAME standard (Matreya LLC, Pleasant Gap, PA, #SPL4833). The analytical lower-limit for individual peak assignment was 0.1% of the total peak area of each sample.

Statistical analysis.

Incidence data were analyzed using the SAS procedure LIFETEST (SAS Institute, Cary, NC) to determine difference due to dietary treatment overtime. Development of arthritis was considered a fail, non-survival. The SAS procedure FREQ (SAS Institute) was used to perform a Fisher Exacts Test on the proportions of arthritic mice in each treatment group at a specified time point. The dependent variables arthritic score, antibodies, cytokines, and fatty acid were analyzed using the SAS procedure MIXED (SAS Institute) to determine difference due to the fixed effects of

dietary treatment. When effects were significant ($P \leq 0.05$), ANOVA analysis was followed by a post hoc Fischer's Least Significant Difference test to identify individual differences among groups. A Levene's test was performed to detect unequal variance between groups. If a $P \leq 0.05$ was detected, a Welch's test was used to test for differences between groups. Differences were considered significant at $P \leq 0.05$ and a trend at $P \leq 0.10$. Values are displayed as means ± standard error of the mean (SEM).

3.4 Results

Collagen-induced arthritis incidence.

Maximum disease incidence occurred at primary day 63 and reached 80% in CO fed mice. Both CLAt10c12 and CLAc9t11 decreased the incidence of arthritis, 33% and 38% respectively, over the study period compared to CO fed mice ($P = 0.06$, **Table 1**). CLAt10c12 fed mice had significantly reduced incidence (52%) compared to CO fed mice between AD 56 and 59, as did CLAc9t11 fed mice (79%) on days AD 43 and 44 ($P \leq 0.05$, **Figure 1**). Although not significant, CLAt10c12 treatment resulted in an earlier appearance of arthritic symptoms by four days compared to CO fed mice, while CLAc9t11 treatment delayed symptom onset by seven days compared to CO. Arthritis symptoms were not observed in sham injected mice of any diet.

Arthritic severity.

No differences in CAS were observed at the day of arthritis onset between treatment groups (average severity was 3.0 ± 0.14, $P = 0.55$). At AD 7 CLAt10c12 fed mice had a 37% reduction in CAS compared to mice fed CO ($P = 0.03$, **Table 1**). When CAS at AD 7 was normalized to AD 0, arthritis severity decreased in the CLAt10c12 group over time (-0.33), while severity

increased in the CO and CLAc9t11 groups (+1.21 and +1.86, respectively; $P = 0.01$). Paw thickness measurements reflected the same severity reduction for CLAt10c12 fed mice ($P \leq 0.05$). There were no significant differences between CO and CLAc9t11 fed mice in any severity measurements.

Collagen IgG antibodies.

Plasma from mice did not contain any detectable level of anti-CII IgG_1 or IgG_{2a} prior to primary immunization, regardless of dietary treatment. Antibody levels of both subclasses increased post primary immunization ($P < 0.001$). Although not significant, CLAt10c12 decreased IgG_1 11% and IgG_{2a} 56%, while CLAc9t11 decreased IgG_1 35% and IgG_{2a} 30%, compared to CO fed arthritic mice (**Table 2**). Plasma from sham immunized mice did not contain any detectable anti-CII IgG_1 or IgG_{2a}.

Plasma cytokines.

Plasma sampled at AD 7 (or primary day 63 for shams) showed a trend of increased IFNγ and TNFα in arthritic mice compared to sham mice ($P \leq 0.10$, **Figure 2b** and **2c**). No differences in plasma cytokine levels were observed between dietary treatment groups at AD 7.

Paw cytokines.

At AD 7, there was significantly more IL-2, IFNγ, TNFα, and IL-10 in paws from arthritic mice compared to sham mice ($P \leq 0.03$, **Figure 3**). A trend toward reduced IL-2 levels was observed in paws of CLAt10c12 fed mice regardless of whether mice were arthritic or sham ($P = 0.08$).

Hepatic fatty acids.

No CLAt10c12 or CLAc9t11 was detected in the livers of CO fed mice (**Table 3**). Regarding mice fed individual CLA isomer diets, only the sole expected CLA isomer was detected in the livers of mice fed each respective diet. The 16:0 to 16:1c9 ratio was significantly increased in CLAt10c12 fed mice compared to CO and CLAc9t11 fed mice ($P = 0.01$). 18:1c9 was significantly increased in CLAt10c12 fed mice compared to CO and CLAc9t11 fed mice ($P < 0.0001$). There was a significant interaction between diet and arthritis for 18:3n-6, 20:3n-6, and 20:4n-6, which were decreased in CLAt10c12 fed mice ($P < 0.05$).

3.5 Discussion

When fed at 0.5% of the diet, both CLAt10c12 and CLAc9t11 reduced the incidence of arthritis in the CA model compared to a diet containing CO as the principal fat source. In agreement with Muhlenbeck *et al.*, mice fed 0.5% CLAc9t11 had at least a 37% decrease in incidence of arthritis compared to mice fed CO [24]. As CLAc9t11 is naturally abundant and can be enriched up to 6% in dairy fat [33], the practical intake of an effective preventative CLAc9t11 dose for humans should be further investigated. Human intake of 0.5% (w/w of diet) CLAc9t11 from naturally enriched sources is impractical (estimated at > 150g dairy fat per day); however, dietary intake less than 0.5% per day was previously effective over time in reducing arthritic inflammation in the CA model [8]. A dose-response experiment investigating the preventative effects of CLAc9t11 on CA would be necessary to determine the practicality of obtaining preventative amounts through consumption of animal fats, including dairy.

A previous study investigated the effects of CLAt10c12 on incidence of autoimmune arthritis in the CA model [24, 26]; however, this is the first experiment to feed high-purity

CLAt10c12 prior to the primary immunization with subsequent evaluation of arthritis incidence. CLAt10c12 reduced incidence (33% compared to CO) similar to CLAc9t11, but also demonstrated anti-inflammatory activity in mice that developed arthritis. CLAt10c12 is not naturally abundant in foods, but may be attainable through supplementation. A dietary level of 0.125% CLAt10c12 (about 3g/day in humans) was effective at reducing existing arthritic inflammation [7], and may be an effective dose for autoimmune arthritis prevention.

Autoimmune arthritis in the CA model progresses via the interactions of multiple Th subsets (i.e. Th1, Th2, Th17, γ/δ T-cells), with Th1 being a major initiator of joint disease [34, 35]. It is unclear how CLAc9t11 and CLAt10c12 mechanistically decrease the onset of autoimmunity in the CA model, although there is evidence that each isomer has differential effects on T cell subset development [24, 36]. CLAc9t11 has been shown to inhibit T-cell differentiation into Th1 or Th17 [12, 36–38]. In agreement with these reports, CLAc9t11, in a previous arthritis prevention study, potentiated the adaptive immune response toward a Th2 subset in non-developed mice [24]. Previous cytokine and antibody evidence suggests that the CLAt10c12 isomer may promote proliferation of T cells and differentiation to Th1 type responses during the induction of arthritis in the CA model [24, 36, 39, 40]. An early polarization toward a Th1 type response may be protective against the incidence of autoimmune arthritis by decreasing Th17 and γ/δ T cell differentiation and/or the respective cytokine repertoire [41–45]. Continued investigation into the effects of CLA isomers on Th subset differentiation in the context of autoimmunity is required.

In mice that developed arthritis, CLAc9t11 treatment had no effect on disease severity compared to CO, supporting results seen in Muhlenbeck *et al*. [24]. In this study CLAt10c12 had significant anti-inflammatory effect on CAS and paw thickness one week after disease onset

compared to CLAc9t11 and CO treatments suggesting additional anti-inflammatory benefit beyond incidence prevention; although, it is predicted CLAc9t11 would also decrease arthritis severity with more time post disease onset [6, 8, 9]. A significant increase in IL-2, IFNγ, TNFα, and IL-10 was observed in arthritic paw tissue compared to shams supporting CA as a Th1 mediated disease [46, 47]. For both CLA isomers no significant difference in anti-collagen IgG_1 and IgG_{2a} levels compared to corn oil treatment was detected. Antibody and cytokine responses were measured in arthritic mice only one week after disease onset, which may make detecting differences due to dietary treatment confounded by the developing pro-inflammatory state [48, 49].

As would be anticipated based on CLAt10c12's inhibitory activity on the SCD-1 enzyme [50, 51], CLAt10c12 increased the hepatic 16:0/16:1c9 ratio at AD 7; however, the 18:0/18:1c9 ratio was unexpectedly decreased. CLAt10c12 increased hepatic 18:1c9 levels by greater than 40 % in mice compared to other diets. In a previous CLAt10c12 dose-response study in the CA model, increasing dietary CLAt10c12 was also associated with increased hepatic 18:1c9 [7]. The elongation product of 18:1c9 is 20:1c11, which was also significantly increased in livers of arthritic and sham mice fed CLAt10c12. An increase in 18:1c9 and 20:1c11 would not be expected due to dietary differences in these fatty acids.

Hepatic fatty acid profiles from sham and arthritic mice fed CO revealed a significant increase in arachidonic acid (AA; 20:4n-6) in arthritic mice, while an arthritis-induced increase in AA did not occur in mice fed CLAt10c12 or CLAc9t11. AA modulates immune and inflammatory effects via its downstream products (e.g. prostaglandins, thromboxanes, and leukotrienes) [52]. In both CA and human RA, prostaglandin-E_2, produced by the AA cascade, promotes Th17 cell differentiation, expansion, and ultimately disease onset, while inhibition of

the AA cascade and certain Th cytokines (e.g. IL-17) results in impaired development of arthritis [53–56]. The metabolic flux from linoleic acid (LA, 18:2n-6) to AA involves the intermediate di-homo gamma linolenic acid (DGLA, 20:3n-6). Both DGLA and AA were increased in CO arthritic mice compared to shams, which is likely associated with the developing inflammatory response. The arthritis-associated increase in LA-AA metabolism was attenuated in CLAc9t11 fed arthritic mice and was strongly inhibited in CLAt10c12 fed arthritic mice. For both CLAt10c12 and CLAc9t11, preventing an initial increase in AA during the earliest activation of the immune response may be one shared mechanism by which both isomers prevent autoimmune arthritis incidence. While significant treatment effects on plasma cytokines were not observed at AD 7, this is not the only mechanism by which attenuation of the AA cascade may provide anti-inflammatory benefits. For example, fatty acid-derived oxylipins are potent immune and inflammatory modulators [57]. Paw and plasma oxylipins were not analyzed or compared here; therefore, we cannot speculate on the specific contribution that attenuating the AA cascade had on the observed inhibition of arthritis. Future work would be benefited by measurement of paw cytokines along with paw fatty acid contents and oxylipins, ideally from the same specimen, to understand the relationship between dietary fatty acid changes and tissue specific outcomes on inflammation. The effect of reduced dietary LA intake on reduced hepatic LA abundance in CLA-fed groups is possible; however, this and previous feeding trials suggest that similar dietary fat replacement with CLAc9t11 does not significantly reduce hepatic LA over 4, 10, or 12 weeks [8, 24].

Collectively, our evidence suggests that both CLAt10c12 and CLAc9t11 prevent the onset of arthritis in the CA model. The mechanisms by which each isomer prevents disease onset requires further investigation, although a mechanism may involve the interplay between isomer

specific polarization of T cell mediated immunity and attenuation of the AA cascade during early development.

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3.7 Tables

Table 1 Maximum arthritis incidence 63 days after primary immunization and clinical arthritic score, normalized score, paw thickness, and normalized paw thickness at arthritic day 7 for mice fed 1% corn oil, 0.5% CLAt10c12, or 0.5% CLAc9t11 diets. Arthritic score and paw thickness values include only mice that developed arthritis (n = 12 corn oil, n = 9 CLAt10c12, and n = 9 CLAc9t11)[1]

	Corn oil	CLAt10c12	CLAc9t11	*P*-value
Maximum Incidence	80%	53%	50%	0.06
Clinical Arthritic Score	4.29 ± 0.45 [a]	2.69 ± 0.52 [b]	4.61 ± 0.52 [a]	0.03
Normalized Score	1.21 ± 0.43 [a]	-0.33 ± 0.50 [b]	1.86 ± 0.50 [a]	0.01
Paw Thickness (mm)	7.53 ± 0.15 [ab]	7.13 ± 0.18 [b]	7.80 ± 0.18 [a]	0.04
Normalized Paw Thickness (mm)	0.48 ± 0.17 [a]	-0.07 ± 0.20 [b]	0.64 ± 0.20 [a]	0.04

[1] Values are means ± SEM. Means within a row without a common letter differ by $P \leq 0.05$. CLA = conjugated linoleic acid and SEM = standard error of the mean

Table 2 Anti-collagen type II antibody titers of arthritic mice at arthritic day 7 (n = 12 corn oil, n = 9 CLAt10c12, and n = 9 CLAc9t11)[1]

µg/ml	Corn oil	CLAt10c12	CLAc9t11	*P*-Value
IgG_1	72 ± 18	64 ± 15	47 ± 15	0.56
IgG_{2a}	3581 ± 973	1565 ± 484	2546 ± 531	0.16

[1] Values are means ± SEM. CLA = conjugated linoleic acid and SEM = standard error of the mean

Table 3 Hepatic fatty acid profiles of arthritic mice at arthritic day 7 and of sham mice at the end of the study period, 63 days after primary immunization (for sham mice n = 9 per diet and for arthritic mice n = 12 corn oil, n = 9 CLAt10c12, and n = 9 CLAc9t11)[1]

	Sham			Arthritic			SEM[2]	*P*-value		
g/100g	Corn oil	CLAt10c12	CLAc9t11	Corn Oil	CLAt10c12	CLAc9t11		Type	Diet	Interaction
14:0	7.02^{a}	3.98^{b}	4.46^{b}	5.13^{b}	5.22^{b}	4.39^{b}	0.22	0.55	0.003	0.01
16:0	28.85	26.62	24.80	27.28	27.44	26.57	0.40	0.67	0.06	0.23
16:1c9	2.12	1.03	1.72	1.52	1.29	1.57	0.09	0.31	0.004	0.10
18:0	8.47	8.97	8.98	10.05	9.20	9.38	0.31	0.26	0.98	0.67
18:1c9	19.35	24.27	18.35	15.83	25.11	15.73	0.80	0.17	<0.0001	0.34
18:1c11	2.92	2.81	3.00	2.56	3.11	2.65	0.13	0.61	0.78	0.51
18:2n-6	17.51	15.97	21.08	19.96	14.03	20.01	0.51	0.81	<0.0001	0.07
20:0	0.37	0.40	0.35	0.37	0.36	0.33	0.01	0.35	0.36	0.79
18:3n-6	0.1^{abc}	n/d	0.15^{a}	0.1^{ab}	0.04^{cd}	0.07^{bc}	0.01	0.64	0.0003	0.04
20:1c11	0.51	0.62	0.57	0.52	0.65	0.52	0.02	0.95	0.01	0.55
18:3n-3	0.17	0.36	0.16	0.11	0.13	0.18	0.04	0.24	0.54	0.37
18:2c9t11	n/d	n/d	1.24	n/d	n/d	1.14	0.08	0.23	<0.0001	0.23
18:2t10c12	n/d	0.34	n/d	n/d	0.27	n/d	0.02	0.25	<0.0001	0.25
20:2n-6	0.27	0.36	0.34	0.35	0.32	0.36	0.02	0.54	0.54	0.34
20:3n-6	0.63^{b}	0.47^{c}	0.75^{a}	0.8^{a}	0.38^{c}	0.73^{ab}	0.02	0.80	<0.0001	0.01
20:4n-6	7.54^{cd}	8.76^{bcd}	9.99^{abc}	11 ^{ab}	7.09^{d}	11.44^{a}	0.41	0.22	0.01	0.03
22:6n-3	1.45	1.62	2.10	2.20	1.36	2.47	0.11	0.15	0.005	0.11
Unknown	2.78	3.41	1.99	2.79	4.05	2.46	0.18	0.25	0.001	0.73
16:0 / 16:1c9	14.10	35.45	17.28	21.23	29.51	18.14	2.38	0.88	0.01	0.52
18:0 / 18:1c9	0.45	0.41	0.53	0.71	0.46	0.63	0.04	0.11	0.28	0.58

[1] Values are means. Labeled means within a row without a common letter differ by $P \leq 0.05$. CLA = conjugated linoleic acid and SEM = standard error of the mean, and n/d = not detected

[2] Pooled SEM

3.8 Figures

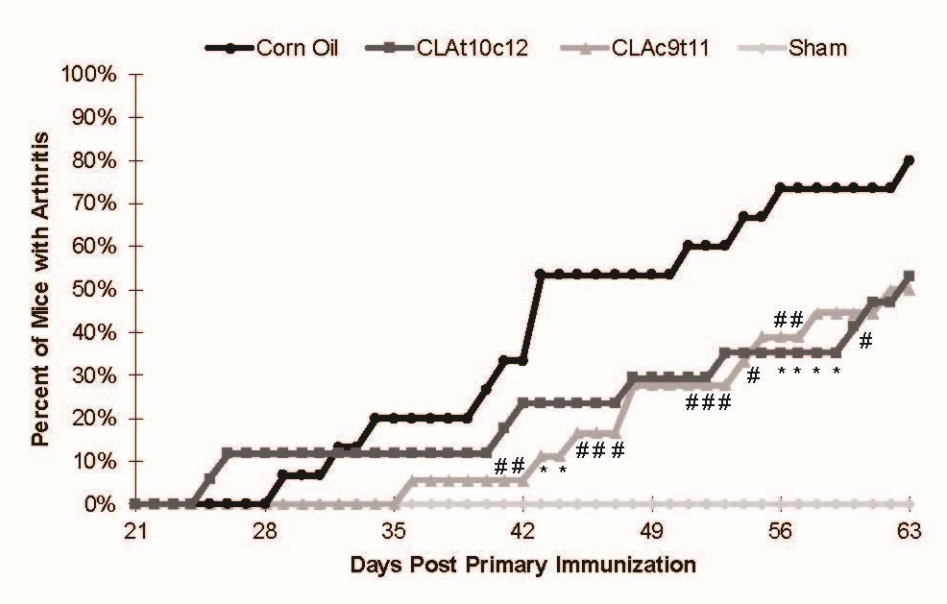

Figure 1: Arthritis incidence of mice fed diets supplemented with 1% corn oil, 0.5% conjugated linoleic acid (CLA) t10c12, or 0.5% CLAc9t11 starting three weeks prior to immunization with chick collagen type II. The maximum incidence of arthritis was 80% for corn oil (n = 12), 53% for CLAt10c12 (n = 9), and 50% for CLAc9t11 fed mice (n = 9). Sham values are representative of all three dietary treatments. Labeled measures indicate a significant or trending difference compared to corn oil fed mice at the specific day, * represents $P \leq 0.05$ and $^{\#}$ represents $0.10 \geq P > 0.05$. See table 1 for statistical analysis over time

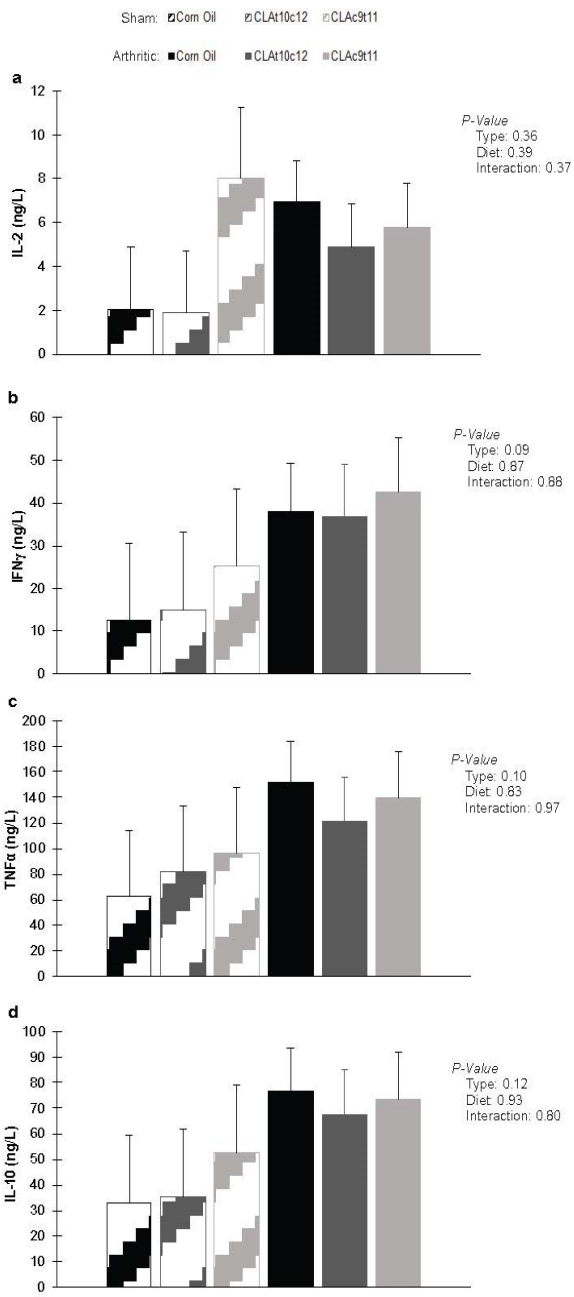

Figure 2: Plasma concentrations of interleukin (IL)-2 (a), interferon (IFN)γ (b), tumor necrosis factor (TNF)α (c), and IL-10 (d) in ng/L of sham (n = 4 per diet) and arthritic mice (n = 9 per diet) fed 1% corn oil, 0.5% CLAt10c12, or 0.5% CLAc9t11 three weeks prior to immunization with chick collagen type II. Plasma was collected from arthritic mice one week after arthritis onset and from sham mice at the end of the study period (63 days after primary immunization). Bars represent means + SEM

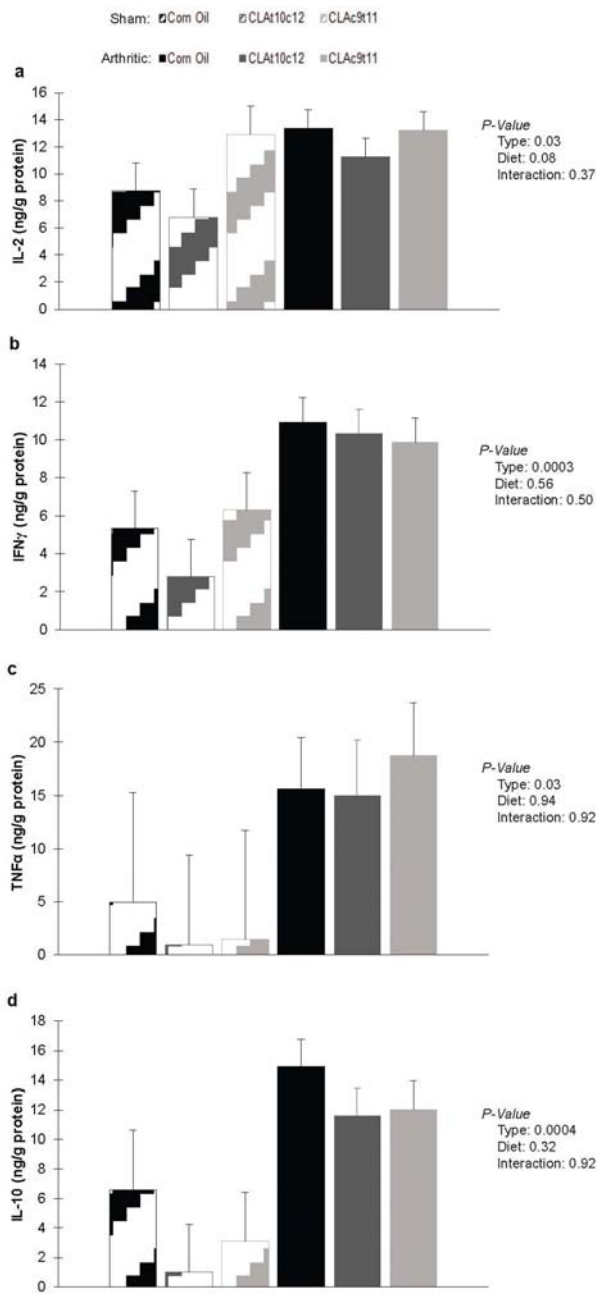

Figure 3: Paw extract concentrations of interleukin (IL)-2 (a), interferon (IFN)γ (b), tumor necrosis factor (TNF)α (c), and IL-10 (d) in ng/g protein from sham (n = 4 per diet) and arthritic mice (n = 9 per diet) fed 1% corn oil, 0.5% CLAt10c12, or 0.5% CLAc9t11 three weeks prior to immunization with chick collagen type II. Paws were collected from arthritic mice one week after arthritis onset and from sham mice at the end of the study period (63 days after primary immunization). Bars represent means + SEM

3.9 References

1. Ward BW, Schiller JS, Goodman RA (2014) Multiple Chronic Conditions Among US Adults: A 2012 Update. Prev Chronic Dis 11:130389. doi: 10.5888/pcd11.130389

2. Straub RH, Schradin C (2016) Chronic inflammatory systemic diseases: An evolutionary trade-off between acutely beneficial but chronically harmful programs. Evol Med public Heal 2016:37–51. doi: 10.1093/emph/eow001

3. Jacobs P, Bissonnette R, Guenther LC (2011) Socioeconomic burden of immune-mediated inflammatory diseases--focusing on work productivity and disability. J Rheumatol Suppl 88:55–61. doi: 10.3899/jrheum.110901

4. Hootman JM, Helmick CG, Barbour KE, et al (2016) Updated Projected Prevalence of Self-Reported Doctor-Diagnosed Arthritis and Arthritis-Attributable Activity Limitation Among US Adults, 2015-2040. Arthritis Rheumatol 68:1582–1587. doi: 10.1002/art.39692

5. Kaplan C, Valdez JC, Chandrasekaran R, et al (2002) Th1 and Th2 cytokines regulate proteoglycan-specific autoantibody isotypes and arthritis. Arthritis Res 4:54. doi: 10.1186/ar383

6. Huebner SM, Campbell JP, Butz DE, et al (2010) Individual Isomers of Conjugated Linoleic Acid Reduce Inflammation Associated with Established Collagen-Induced Arthritis in DBA/1 Mice. J Nutr 140:1454–1461. doi: 10.3945/jn.109.120527

7. Huebner SM, Olson JM, Campbell JP, et al (2014) Dietary trans-10,cis-12 CLA reduces murine collagen-induced arthritis in a dose-dependent manner. J Nutr 144:177–184. doi: 10.3945/jn.113.182550

8. Huebner SM, Olson JM, Campbell JP, et al (2016) Low Dietary c9t11-Conjugated Linoleic Acid Intake from Dairy Fat or Supplements Reduces Inflammation in Collagen-Induced Arthritis. Lipids 51:807–819. doi: 10.1007/s11745-016-4163-8

9. Olson JM, Haas AW, Lor J, et al (2017) A Comparison of the Anti-Inflammatory Effects of Cis-9, Trans-11 Conjugated Linoleic Acid to Celecoxib in the Collagen-Induced Arthritis Model. Lipids 52:151–159. doi: 10.1007/s11745-016-4228-8

10. Whigham LD, Cook EB, Stahl JL, et al (2001) CLA reduces antigen-induced histamine and PGE2 release from sensitized guinea pig tracheae. Am J Physiol Regul Integr Comp Physiol 49:R908–R912.

11. Jaudszus A, Mainz JG, Pittag S, et al (2016) Effects of a dietary intervention with conjugated linoleic acid on immunological and metabolic parameters in children and adolescents with allergic asthma--a placebo-controlled pilot trial. Lipids Health Dis 15:21. doi: 10.1186/s12944-016-0187-6

12. Turpeinen AM, Ylönen N, von Willebrand E, et al (2008) Immunological and metabolic effects of cis-9, trans-11-conjugated linoleic acid in subjects with birch pollen allergy. Br J Nutr 100:112–119. doi: 10.1017/S0007114507886326

13. Yang M, Cook ME (2003) Dietary Conjugated Linoleic Acid Decreased Cachexia, Macrophage Tumor Necrosis Factor-α Production, and Modifies Splenocyte Cytokines Production. Exp Biol Med Diet 228:51–58.

14. Cook ME, Miller CC, Park Y, Pariza M (1993) Immune modulation by altered nutrient metabolism: nutritional control of immune-induced growth depression. Poult Sci 72:1301–1305.

15. Bassaganya-Riera J, Reynolds K, Martino-Catt S, et al (2004) Activation of PPAR γ and δ by conjugated linoleic acid mediates protection from experimental inflammatory bowel disease. Gastroenterology 127:777–791. doi: 10.1053/j.gastro.2004.06.049

16. Hontecillas R, Wannemeulher MJ, Zimmerman DR, et al (2002) Nutritional regulation of porcine bacterial-induced colitis by conjugated linoleic acid. J Nutr 132:2019–27.

17. Yuan G, Chen X, Li D (2015) Modulation of Peroxisome Proliferator-Activated Receptor gamma (PPAR γ) by Conjugated Fatty Acid in Obesity and Inflammatory Bowel Disease. J Agric Food Chem 63:1883–1895. doi: 10.1021/jf505050c

18. Rula EY, Pope JE, Hoffman JC (2011) Potential Medicare savings through prevention and risk reduction. Popul Health Manag 14 Suppl 1:S35-44. doi: 10.1089/pop.2010.0063

19. Parodi PW (2003) Conjugated Linoleic Acid in Food. In: Sébédio J-L, Christie WW, Adolf R (eds) Adv. Conjug. Linoleic Acid Res. Vol. 2. AOCS Press, Champaign, Illinois, pp 101–122

20. Park Y, Storkson JM, Albright KJ, et al (1999) Evidence that the trans-10,cis-12 isomer of conjugated linoleic acid induces body composition changes in mice. Lipids 34:235–241. doi: 10.1007/s11745-999-0358-8

21. Tricon S, Burdge GC, Kew S, et al (2004) Opposing effects of cis-9,trans-11 and trans-10,cis-12 conjugated linoleic acid on blood lipids in healthy humans. Am J Clin Nutr 80:614–620.

22. Arbonés-Mainar JM, Navarro MA, Acín S, et al (2006) Trans-10, cis-12- and cis-9, trans-11-conjugated linoleic acid isomers selectively modify HDL-apolipoprotein composition

in apolipoprotein E knockout mice. J Nutr 136:353–359.

23. Li J, Hu S, He Y, et al (2018) 9c11tCLA modulates 11t18:1 and 9t18:1 induced Inflammations Differently in Human Umbilical Vein Endothelial Cells. Sci Rep 1–27. doi: 10.1038/s41598-018-19729-9

24. Muhlenbeck JA, Butz DE, Olson JM, et al (2017) Dietary Conjugated Linoleic Acid-c9t11 Prevents Collagen-Induced Arthritis, Whereas Conjugated Linoleic Acid-t10c12 Increases Arthritic Severity. Lipids 1–12. doi: 10.1007/s11745-017-4241-6

25. Brand DD, Latham KA, Rosloniec EF (2007) Collagen-induced arthritis. Nat Protoc 2:1269–1275. doi: 10.1038/nprot.2007.173

26. Butz DE, Li G, Huebner SM, Cook ME (2007) A mechanistic approach to understanding conjugated linoleic acid's role in inflammation using murine models of rheumatoid arthritis. Am J Physiol Regul Integr Comp Physiol 293:R669–R676. doi: 10.1152/ajpregu.00005.2007

27. Nordling C, Karlsson-Parra A, Jansson L, et al (1992) Characterization of a spontaneously occurring arthritis in male DBA/1 mice. Arthritis Rheum 35:717–722. doi: 10.1002/art.1780350619

28. Holmdahl R, Jansson L, Andersson M, Jonsson R (1992) Genetic, hormonal and behavioural influence on spontaneously developing arthritis in normal mice. Clin Exp Immunol 88:467–472. doi: 10.1111/j.1365-2249.1992.tb06473.x

29. Lu LD, Stump KL, Seavey MM (2010) Novel method of monitoring trace cytokines and activated STAT molecules in the paws of arthritic mice using multiplex bead technology.

BMC Immunol 11:55. doi: 10.1186/1471-2172-11-55

30. Folch J, Lees M, Sloane Stanley GH (1957) A simple method for the isolation and purification of total lipides from animal tissues. J Biol Chem 226:497–509.

31. Stoffel W, Insull W, Ahrens EH (1958) Gas-liquid chromatography of highly unsaturated fatty acid methyl esters. Proc Soc Exp Biol Med 99:238–241.

32. Yeonhwa Park, Karen J. Albright, Zu Y. Cai A, Pariza MW (2001) Comparison of Methylation Procedures for Conjugated Linoleic Acid and Artifact Formation by Commercial (Trimethylsilyl)diazomethane. J Agric Food Chem 49:1158–1164. doi: 10.1021/JF001209Z

33. Shingfield KJ, Reynolds CK, Hervás G, et al (2006) Examination of the Persistency of Milk Fatty Acid Composition Responses to Fish Oil and Sunflower Oil in the Diet of Dairy Cows. J Dairy Sci 89:714–732. doi: 10.3168/jds.S0022-0302(06)72134-8

34. Germann T, Hess H, Szeliga J, Rude E (1996) Characterization of the Adjuvant Effect of IL-12 and Efficacy of IL-12 Inhibitors in Type II Collagen-Induced Arthritis. Ann N Y Acad Sci 795:227–240. doi: 10.1111/j.1749-6632.1996.tb52672.x

35. Ito Y, Usui T, Kobayashi S, et al (2009) Gamma/delta T cells are the predominant source of interleukin-17 in affected joints in collagen-induced arthritis, but not in rheumatoid arthritis. Arthritis Rheum 60:2294–2303. doi: 10.1002/art.24687

36. Loscher CE, Draper E, Leavy O, et al (2005) Conjugated linoleic acid suppresses NF-kappa B activation and IL-12 production in dendritic cells through ERK-mediated IL-10 induction. J Immunol 175:4990–4998. doi: 10.4049/JIMMUNOL.175.8.4990

37. Draper E, DeCourcey J, Higgins SC, et al (2014) Conjugated linoleic acid suppresses dendritic cell activation and subsequent Th17 responses. J Nutr Biochem 25:741–749. doi: 10.1016/j.jnutbio.2014.03.004

38. Jaudszus A, Krokowski M, Möckel P, et al (2008) Cis-9,trans-11-conjugated linoleic acid inhibits allergic sensitization and airway inflammation via a PPARgamma-related mechanism in mice. J Nutr 138:1336–42.

39. Cook ME, Butz D, Li G, et al (2003) Conjugated Linoleic Acid Enhances Immune Responses but Protects Against the Collateral Damage of Immune Events. In: Sébédio J-L, Christie WW, Adolf R (eds) Adv. Conjug. Linoleic Acid Res. Vol. 2. AOCS Press, Champaign, Illinois, pp 283–291

40. Yang M, Cook ME (2003) Dietary CLA decreased weight loss and extended survival following the onset of kidney failure in NZB/W F1 mice. Lipids 38:21–24. doi: 10.1007/s11745-003-1026-8

41. Sarkar S, Cooney LA, White P, et al (2009) Regulation of pathogenic IL-17 responses in collagen-induced arthritis: roles of endogenous interferon-gamma and IL-4. Arthritis Res Ther 11:R158. doi: 10.1186/ar2838

42. Matthys P, Vermeire K, Mitera T, et al (1998) Anti-IL-12 antibody prevents the development and progression of collagen-induced arthritis in IFN-gamma receptor-deficient mice. Eur J Immunol 28:2143–51. doi: 10.1002/(SICI)1521-4141(199807)28:07<2143::AID-IMMU2143>3.0.CO;2-C

43. Vermeire K, Heremans H, Vandeputte M, et al (1997) Accelerated collagen-induced arthritis in IFN-gamma receptor-deficient mice. J Immunol 158:5507–13.

44. Manoury-Schwartz B, Chiocchia G, Bessis N, et al (1997) High susceptibility to collagen-induced arthritis in mice lacking IFN-gamma receptors. J Immunol 158:5501–6.

45. Chu C-Q, Swart D, Alcorn D, et al (2007) Interferon-γ regulates susceptibility to collagen-induced arthritis through suppression of interleukin-17. Arthritis Rheum 56:1145–1151. doi: 10.1002/art.22453

46. Mauri C, Williams RO, Walmsley M, Feldmann M (1996) Relationship between Th1/Th2 cytokine patterns and the arthritogenic response in collagen-induced arthritis. Eur J Immunol 26:1511–1518. doi: 10.1002/eji.1830260716

47. Luross JA, Williams NA (2001) The genetic and immunopathological processes underlying collagen-induced arthritis. Immunology 103:407–416. doi: 10.1046/j.1365-2567.2001.01267.x

48. Kim H-Y, Kim W-U, Cho M-L, et al (1999) Enhanced T cell proliferative response to type II collagen and synthetic peptide CII (255-274) in patients with rheumatoid arthritis. Arthritis Rheum 42:2085–2093. doi: 10.1002/1529-0131(199910)42:10<2085::AID-ANR8>3.0.CO;2-Z

49. Kim WU, Yoo WH, Park W, et al (2000) IgG antibodies to type II collagen reflect inflammatory activity in patients with rheumatoid arthritis. J Rheumatol 27:575–81.

50. Park Y, Storkson JM, Ntambi JM, et al (2000) Inhibition of hepatic stearoyl-CoA desaturase activity by trans-10,cis-12 conjugated linoleic acid and its derivatives. Biochim Biophys Acta - Mol Cell Biol Lipids 1486:285–292. doi: 10.1016/S1388-1981(00)00074-3

51. Choi Y, Kim YC, Han YB, et al (2000) The trans-10,cis-12 isomer of conjugated linoleic acid downregulates stearoyl-CoA desaturase 1 gene expression in 3T3-L1 adipocytes. J Nutr 130:1920–1924.

52. James MJ, Gibson RA, Cleland LG (2000) Dietary polyunsaturated fatty acids and inflammatory mediator production. Am J Clin Nutr 71:343S–8S.

53. Boniface K, Bak-Jensen KS, Li Y, et al (2009) Prostaglandin E2 regulates Th17 cell differentiation and function through cyclic AMP and EP2/EP4 receptor signaling. J Exp Med 206:535–48. doi: 10.1084/jem.20082293

54. Anderson GD, Keys KL, De Ciechi PA, Masferrer JL (2009) Combination therapies that inhibit cyclooxygenase-2 and leukotriene synthesis prevent disease in murine collagen induced arthritis. Inflamm Res 58:109–17. doi: 10.1007/s00011-009-8149-3

55. Chen Q, Muramoto K, Masaaki N, et al (2010) A novel antagonist of the prostaglandin E(2) EP(4) receptor inhibits Th1 differentiation and Th17 expansion and is orally active in arthritis models. Br J Pharmacol 160:292–310. doi: 10.1111/j.1476-5381.2010.00647.x

56. Ye L, Jiang B, Deng J, et al (2015) IL-37 Alleviates Rheumatoid Arthritis by Suppressing IL-17 and IL-17-Triggering Cytokine Production and Limiting Th17 Cell Proliferation. J Immunol 194:5110–9. doi: 10.4049/jimmunol.1401810

57. Gabbs M, Leng S, Devassy JG, et al (2015) Advances in Our Understanding of Oxylipins Derived from Dietary PUFAs. Adv Nutr 6:513–40. doi: 10.3945/an.114.007732

Chapter 4:

Trans-11 vaccenic acid decreases the severity of murine autoimmune arthritis, in part due to its conversion to conjugated linoleic acid *cis*-9, *trans*-11.

4.1 Abstract

Autoimmune disease prevalence, in the United States, is projected to increase to 49% of the population by 2040. The rise in disease incidence will coincided with an increase in the already large economic strain attributed to autoimmune diseases. While pharmaceuticals are generally the first resources for disease care, dietary treatments are being increasingly looked upon as cost-effective alternatives with less negative side effects. Dairy fats, including *trans*-11 vaccenic acid (TVA) and its desaturation product conjugated linoleic acid (CLA) *cis*-9, *trans*-11 (c9t11) are known for their anti-inflammatory properties; however, more exploration into the minimally effective dose and mechanism of action is required for these fatty acids to be adopted as autoimmune disease treatment. DBA/1 male mice (n = 162) were fed diets containing 0, 0.25, 0.5, or 1% TVA, with a fat background that was intended to either allowed (corn oil, CO) or inhibited (cottonseed oil, CSO) TVA's conversion to CLAc9t11, for nine weeks after onset of collagen-induced arthritis. Arthritic severity and hepatic fatty acid profiles were compared between dietary treatment groups. A trend toward decreased clinical arthritic severity in mice fed a CSO containing diet, regardless of TVA dose, was observed ($P = 0.06$). At the end of the study, 63 days after arthritis onset, the 0.25% TVA + 5.75% CO diet showed the largest decrease (91%) in arthritic severity compared to the 6% CO control diet. No difference in the TVA to CLAc9t11 conversion was observed between CO and CSO diets, with an average conversion of 42% ($P = 0.12$). The minimal effective anti-inflammatory dose of TVA was lower than previously suggested, and although CSO did not inhibit the TVA to CLAc9t11 conversion as predicted, CSO may have additional anti-inflammatory benefits of its own.

4.2 Introduction

Over 52.5 million Americans suffer from an autoimmune disease [1]. Of autoimmune conditions, rheumatoid arthritis (RA) is extremely common, affecting 1.5 million people across the United States in 2007 [2]. In RA, the immune system targets the synovial joints resulting in severe pain and swelling [3]. The pain increases the sufferer's reliance on pharmaceutical drugs as treatments, which can be expensive and lead to harmful side effects [4]. Understanding alternative treatment options, like dietary intervention, for autoimmune disease can reduce provide a much needed cost and health savings [5]. One such dietary treatment option being explored is dairy fat, specifically the fatty acids conjugated linoleic acid (CLA) isomer *cis*-9, *trans*-11 (c9t11) and *trans*-11 vaccenic acid (TVA), the metabolic precursor of CLAc9t11 [6–8].

CLAc9t11 is known to provide several anti-inflammatory health effects in inflammatory bowel disease [9, 10], allergy [11], and RA [7, 12]. Suggested mechanisms of action for these anti-inflammatory effects include the regulation of proliferator-activated nuclear receptor (PPAR)γ [9, 13, 14], reducing tumor necrosis factor (TNF)α [15, 16], increasing interleukin (IL)-10 [12, 17, 18], and replacing arachidonic acid (AA) [7, 12, 19]. TVA is a metabolic precursor to CLAc9t11, and gets converted to CLAc9t11 through the delta-9 desaturase, stearoyl-CoA desaturase (SCD)-1 at approximately 19% in humans [20]. More research is needed to understand this conversion fully, as studies have suggested a much higher hepatic conversion of CLAc9t11 to TVA at 48% [8]. TVA has been shown to reduce cardiovascular disease [21–23], anti-inflammatory mediators [24], metabolic syndrome [25], and arthritis [19].

Research to determine if TVA's anti-inflammatory effects are due to TVA itself or dependent on the conversion to CLAc9t11, has been limited and varying. TVA treatment i*n vitro* caused the reduction of IL-2 and TNFα production through direct activation of PPARγ, independent of its conversion to CLAc9t11 [26, 27]. The anti-carcinogenic effects of TVA on mammary tumors was attenuated when the conversion of TVA to CLAc9t11 was partially blocked by sterculic acid, a delt-9 desaturase inhibitor [28]. TVA and CLAc9t11 may also have synergistic anti-inflammatory effects, as demonstrated by a rodent allergic airway inflammation model in which milk fat reduced inflammatory leukocyte infiltration in airways and plasma only when TVA and CLAc9t11 were fed in combination [29].

To study the anti-inflammatory effects of both TVA independently and after conversion to CLAc9t11, mice, with collagen-induced arthritis (CA), were fed diets supplemented with 0, 0.25, 0.5, or 1% TVA on either a corn oil (CO) or cottonseed oil (CSO) background (up to 6% of the diet). CSO contains the cyclopropenoid fatty acids malvalic, sterculic, and dihydrosterculic acid, which are linked to inhibition of delta-9 desaturase activity [30–32]. In mice, studies showed a 10% inhibition of SCD-1 activity [30], and in a preliminary study by Cook laboratory a 10% decrease in the hepatic conversion of TVA to CLAc9t11 was observed when mice were fed 0.5% TVA + 0.5% CSO for three weeks. CSO has been used as a control diet in the CA model, and showed no change in rheumatoid arthritis severity in a clinical trial [33, 34]. This study was designed to assess the anti-inflammatory effects of TVA independently and due to its conversion to CLAc9t11, while further assessing a minimum effective anti-inflammatory TVA dose.

4.3 Materials and Methods

Experiments were conducted in accordance with an animal protocol, A005438, approved by the College of Agricultural and Life Sciences Animal Care and Use Committee.

Collagen-induced arthritis model.

Four-week-old DBA/1 male mice (n = 162) purchased from Harlan (Indianapolis, IN), were housed in shoebox units (3 mice per box), and maintained on a 12:12 h light-dark cycle. After a one-week acclimation period on chow diet, mice were switched to a 94% complete semi-purified diet (n = 81 mice per diet, TD10053 Harlan-Teklad, Madison, WI) supplemented with either 6% CO or 6% CSO (CocoJoJo, Santa Ana, CA) (**Table 1**). After three weeks on initial treatment diets, mice (grouped by shoebox units) were randomly assigned to receive a 100µl intradermal tail injection of either 100µg chick collagen type II (CII) (Chondrex, Redmound, WA, n = 72 mice per diet) in 0.05M acetic acid or a sham injection of 0.05M acetic acid emulsified in a 1:1 ratio with complete Freund's adjuvant (4mg/ml heat-killed *Mycobacterium tuberculosis*; Chondrex, n = 9 mice per diet) [35, 36]. Twenty-one days after the primary injection, mice received a 100µl intraperitoneal secondary injection of either 100µg chick CII in 0.05M acetic acid or a sham injection of 0.05M acetic acid emulsified in a 1:1 ratio with Freund's incomplete adjuvant (Chondrex). Following the secondary immunization, mice were monitored daily for clinical signs of arthritis (see Clinical Arthritic Score).

Clinical arthritic score.

Mice were examined by a trained observer, blinded to treatments. After the secondary immunization, mice were monitored daily for clinical signs of arthritis for nine weeks. Nine weeks post-secondary injection was chosen to avoid the risk of older mice spontaneous developing arthritis [37]. Arthritis onset was defined as clinical arthritic score (CAS) of greater than two for two consecutive days. As previously described, individual paws were assigned an inflammation severity score between 0 and 4 based on edema, erythema, joint involvement, and mobility [12, 36]. Individual paw scores were then summed for an overall CAS with a maximum of 16. The date of arthritis development was recorded as arthritic day (AD) 0. Upon development of arthritis, mice were scored three times per week for nine weeks. Normalized arthritic score was calculated by subtracting CAS at AD 0 from the CAS any given AD.

Dietary treatment.

Once a mouse was declared arthritic (CAS of two or greater for two consecutive days), it was moved to a new shoebox, started on one of eight dietary treatments, and remained on that diet until the end of the study period, AD 63. Mice remained on their respective CO or CSO, pre-arthritis development, background fat source (up to 6%), with the additional supplementation of either 0, 0.25, 0.5, or 1 % TVA (**Table 1**). To minimize the stress of individually housed mice, when mice from the same shoebox unit as an already arthritic mouse became arthritic, they were assigned to the same diet and placed in the same shoebox as the original mouse.

Paw thickness measurements.

Once weekly, between AD 0 and AD 63, paw thickness of each individual mouse paws were measured using a pressure-sensitive caliper (SPI, Garden Grove, CA). Measurements of individual mouse paws were summed to provide the overall paw thickness measurement for each mouse. Normalized paw thickness was calculated by subtracting the paw thickness at AD 0 from paw thickness measured at a given AD.

Hepatic fatty acid determination.

After mice were euthanized by exsanguination on AD 63, liver samples were collected and stored at -80°C until analyzed. Total lipids of 0.2g liver samples were extracted using chloroform:methanol (2:1, v/v) according to the methods of Folch *et al*. [38]. Fatty acid methyl esters (FAME) were prepared by acid-catalyzed methylation [39, 40]. Relative percentages of liver FAME were determined using an Agilent 6890N GC (Agilent Technologies Santa Clara, CA) equipped with a 100m CP-Sil 88 column (Varian Palo Alto, CA) [41]. FAME were identified by comparing retention times against a custom qualitative FAME standard (Matreya, LLC, Pleasant Gap, PA, #SPL4833). The conversion of hepatic TVA to CLAc9t11 was calculated by the net gain of CLAc9t11 as a percentage of TVA equivalents (TVA + CLAc9t11) [8, 20]. This approach represents conversion based on retained TVA.

Statistical analysis.

The SAS procedure FREQ (SAS Institute, Cary, NC) was used to perform a Fisher Exacts Test on the proportions of arthritic mice in each treatment group at a specified time point. SAS procedure MIXED (SAS Institute, Cary, NC) to determine difference due to the fixed effects of dietary treatment and time. In order to account for repeated measures of score across time, a

REPEATED statement was employed with the SAS procedure MIXED with a first order autoregressive covariance structure. Additionally, each dependent variable was tested utilizing unequal variance among groups and a Levene's test was performed determine the appropriate model ($P \leq 0.05$). When dependent variables are identified to have a significant diet, arthritis, time, and/or interaction ($P \leq 0.05$) ANOVA analysis was followed by post hoc least squared difference test to identify individual differences among groups. Differences were considered significant at $P \leq 0.05$ and a trend at $P \leq 0.10$. Values are displayed as means ± standard error of the mean (SEM).

4.4 Results

Collagen-induced arthritis incidence.

Overall arthritis incidence was 47% in mice fed 6% CO diet and 53% in mice fed 6% CSO diet prior to arthritis onset ($P = 0.64$). Sham immunized mice of either diet showed no signs of arthritis.

Body weight.

No difference in body weight was observed between dietary treatment groups of arthritic mice ($P = 0.76$, **Table 2**). The overall mean body weight of all arthritic mice between AD 0 and 63 was 27.4g ± 0.15. Although a significant change in body weight was observed over the nine-week study ($P = <0.0001$), there was no interaction between dietary treatment and time ($P = 0.63$).

Arthritic severity.

Normalized clinical arthritic score at AD 7 showed a trend toward reduced severity of all CSO background fed treatment groups (*P = 0.08*, **Table 3**). A trend toward an interaction between diet and day was also identified for normalized CAS (*P = 0.06*). Generally, decreased severity was observed in mice fed CSO when compared to the equivalent TVA dose with a CO background. No significant differences were identified between treatment groups or over time when comparing mean normalized paw thickness (*P = 0.11,* **Table 4**). Decreased severity was again observed in mice fed CSO when compared the equivalent TVA dose on a CO background.

Hepatic fatty acids.

The level of hepatic CLAc9t11 increased with increasing dietary TVA dose (*P = <0.0001*, **Table 4**). The TVA to CLAc9t11 conversion ratio almost reflected the increase in dietary TVA; however, the 0.5% TVA + CSO treatment had the lowest conversion at 36% (*P = 0.12*). The regression of hepatic CLAc9t11 and arthritic severity at AD 63 showed no significant correlation ($R^2 = 0.001$, **Figure 1**). Regardless of TVA dose, mice fed diets with a CSO background had an 83% decrease in 22:3n-6 compared to mice fed a CO background (*P = <0.0001*). Again regardless of TVA dose, there was a 48% increase in the amount of unidentified hepatic fatty acids in all CSO fed mice compared to mice fed CO (*P = <0.0001*). CLAt10c12 was not detected in any dietary treatment group. There was no difference in the 16:0 to16:1c9 or 18:0 to18:1c9 ratio between dietary treatments. (*P = 0.55* and *P = 0.63*, respectively).

4.5 Discussion

Mice fed 0.25% TVA + 5.75% CO had a 91% decrease in normalized arthritic severity compared to a 6% CO control diet. This dietary dose resulted in 0.36% hepatic CLAc9t11, which was similar to the 0.25% TVA + 5.75% CSO diet. The previously identified minimum effective TVA dose, in the CA model, was 0.375% of diet [8]. The previous identified minimum effective dose for CLAc9t11, 0.125% of diet, resulted in a 75% decrease in severity compared to CO control diet, and a hepatic CLAc9t11 level of 0.34% with no detected TVA [7]. Comparing these studies suggests that TVA may provide anti-inflammatory effects below the previously predicted minimally effective dose. It may also support that TVA can provide anti-inflammatory benefits, beyond its conversion to CLAc9t11; however, doses below 0.125% CLAc9t11 need to be explored.

As dietary TVA dose increased hepatic CLAc9t11 followed, with an average conversion, of TVA to CLAc9t11, of 43% for mice fed a CO background. The average TVA to CLAc9t11 conversion was 40% for mice fed a CSO background diet. These was no statistically significant difference between these groups. Inhibition of SCD-1 and the TVA to CLAc9t11 conversion was anticipated at a minimum of 10% [30–32]. There was also no significant difference between CO and CSO treatments in the 16:0/16:1c9 and 18:0/18:1c9 ratios, which suggests no SCD-1 inhibition was induced. The 59% increase in 16:0 and 39% decrease in 18:1c9 in dietary CSO compared to CO may skew these ratios. The lack of SCD-1 inhibition may be a result of low statically power in the study; it may also suggest that the unrefined CSO used is not the best way to inhibit the TVA to CLAc9t11 conversion. Options to explore in the future include: quantification of the cyclopropenoid fatty acid levels, supplementation with isolated sterculic oil, pharmaceuticals SCD-1 inhibition, or gene modification to induce inhibition [42–45].

Mice fed a diet of 6% CSO had a 56% reduction in arthritic severity at the end of the study period compared to mice fed 6% CO diets. This suggests that CSO may have some anti-inflammatory benefit of its own, and was not anticipated as CSO has been used as a control diet the CA model previously and associated with more severe inflammation [34]. In the CA model of arthritis CSO fed mice had 78% arthritis incidence, highly inflamed cartilage, increased level of TNFα, IL-1β, IL-17, IL-21, and IL-6, and decreased IL-10 [34]. In a human clinical trial, control group patients who received 6g/day CSO showed no arthritis improvement over a 24 week treatment period [33]. The increase in hepatic 22:6n-3 (docosahexaenoic acid, DHA) levels in CO fed mice compared to CSO fed mice is also interesting, as this fatty acid is typically considered to be anti-inflammatory [46]. The anti-inflammatory benefit of CSO may be associated with the unidentified hepatic fatty acids, which were increased compared to CO fed mice. Future CSO studies would be benefited by a standard to quantify cyclopropenoid fatty acids, and quantification of inflammatory responses through protein and RNA markers. Inflammation severity scores for all dietary treatments containing CSO could be further normalized to the baseline effects of the 6% CSO diet; however, further research is required for analysis in this manner to be justified.

Results in this study varied greatly from the predicted negative sloped line demonstrating increased hepatic CLAc9t11 levels correlated with decreased arthritic severity. Mice fed 1% TVA + 5% CO only had an 11% decrease in arthritic severity. Interestingly, this dietary dose resulted in the highest amount of hepatic CLAc9t11 (1.5%). Paw thickness and severity score generally agreed, demonstrating that the effects observed were unlikely due to qualitative scoring errors. The hepatic TVA and CLAc9t11 levels within individual dietary treatment groups following predicted values confirms that proper dietary treatments were fed to each group

throughout the study. The study's unpredicted results may be due to low power in the study or variation in the collagen-induced arthritis model [12, 36]. Further analysis into the results observed in this study, and the mechanism by which they occur may provide more clarification into the observed effects.

Overall, this study suggests that TVA has anti-inflammatory properties below the previously anticipated minimum effective level and that these effects are due to both TVA itself and its conversion to CLAc9t1. Further comparisons are still required to support this hypothesis and identify the mechanisms responsible for these effects. The anti-inflammatory benefit of both CLAc9t11 and TVA provides value to dietary supplementation with diary fat enriched for these fatty acids as an anti-inflammatory treatment.

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4.7 Tables

Table 1 Fatty acid composition of dietary lipid sources

Fatty Acid (g/100g)	Corn oil	CSO	TVA
14:0	n/d	0.71	n/d
16:0	12.8	20.3	n/d
16:1c9	n/d	0.43	n/d
18:0	2.4	1.99	n/d
TVA	n/d	n/d	99.0
18:1c9	29.1	17.7	n/d
18:1c11	0.8	1.0	n/d
18:2n-6	53.2	57.0	n/d
18:2c9t11	n/d	n/d	n/d
18:2t10c12	n/d	n/d	n/d
20:0	0.5	0.18	n/d
Unknown	1.2	0.69	1.0

CSO = cottonseed oil, TVA = *trans*-11 vaccenic acid, n/d= not detected

Table 2 Mean body weight of arthritic mice, by dietary treatment group, from arthritic day 0 to 63 in grams

Arthritic Day (grams)	**0**	**7**	**14**	**21**	**28**	**35**	**42**	**49**	**56**	**63**	**SEM[1]**
CSO	29.5	27.8	28.4	28.6	30.0	29.4	29.1	29.4	29.6	29.6	0.15
Corn Oil	25.6	25.3	25.5	25.5	25.6	26.0	26.1	26.4	26.0	26.7	
0.25% TVA + CSO	28.3	26.4	27.0	26.8	27.1	27.7	27.5	28.0	27.8	28.0	
0.25% TVA + Corn Oil	27.7	26.4	26.5	26.7	26.5	27.0	26.8	27.3	27.2	27.7	
0.5% TVA + CSO	27.7	27.2	26.4	27.7	27.2	27.3	27.2	27.6	27.4	28.6	
0.5% TVA + Corn Oil	27.1	26.9	26.6	26.8	26.8	28.0	27.0	27.1	27.3	27.5	
1% TVA + CSO	29.2	27.9	28.3	28.4	28.4	28.5	28.4	28.3	28.3	29.3	
1% TVA + Corn Oil	27.1	25.3	25.2	25.8	25.7	25.7	26.5	26.8	26.7	27.4	
***P*-Value**	0.61	0.85	0.66	0.63	0.63	0.61	0.79	0.83	0.68	0.82	

CSO = Cottonseed oil, TVA = *trans*-11 vaccenic acid, and SEM = Standard error of the mean pooled for all samples on all dates

n Values: CSO = 9, Corn oil = 8, 0.25% TVA + CSO = 9, 0.25% TVA + Corn Oil = 9, 0.5% TVA + CSO = 10, 0.5% TVA + Corn Oil = 10, 1% TVA + CSO = 10, 1% TVA + Corn oil = 7.

Overall *P*-Value

Diet	Day	Diet*Day
0.76	<0.0001	0.63

Table 3 Mean normalized clinical arthritic score, by dietary treatment group, from arthritic day 0 to 63[1]

Arthritic Day	**7**	**14**	**21**	**28**	**35**	**42**	**49**	**56**	**63**	**SEM[1]**
CSO	0.44	0.28	-0.06	-0.67	-0.28	0.97	0.53	0.75	0.94	0.09
Corn oil	0.89	0.72	0.44	0.90	1.50	1.13	1.41	2.16	2.16	
0.25% TVA + CSO	1.03	0.97	0.33	1.06	0.86	0.83	1.56	1.47	1.22	
0.25% TVA + Corn oil	-0.28	0.64	0.67	0.50	0.11	0.19	1.11	1.11	0.19	
0.5% TVA + CSO	0.00	0.33	0.93	0.25	1.18	0.75	1.73	0.95	0.92	
0.5% TVA + Corn oil	0.48	0.75	1.30	0.93	1.03	1.36	0.50	1.25	1.36	
1% TVA + CSO	0.80	1.25	0.58	0.60	1.10	1.10	1.03	1.18	0.68	
1% TVA + Corn oil	2.21	2.82	2.18	2.50	1.57	1.61	0.89	1.07	1.93	
***P*-Value**	0.08	0.34	0.78	0.35	0.67	0.96	0.95	0.97	0.80	

[1]Normalized score is defined as the clinical arthritic score on arthritic day 0 subtracted from the clinical arthritic score on a given day.
CSO = Cottonseed oil, TVA = *trans*-11 vaccenic acid, and SEM = Standard error of the mean pooled for all samples on all dates
n Values: CSO = 9, Corn oil = 8, 0.25% TVA + CSO = 9, 0.25% TVA + Corn oil = 9, 0.5% TVA + CSO = 10, 0.5% TVA + Corn oil = 10, 1% TVA + CSO = 10, 1% TVA + Corn oil = 7.

Overall *P*-Value

Diet	Day	Diet*Day
0.84	0.14	0.06

Table 4 Mean normalized paw thickness, by dietary treatment group, from arthritic day 0 to 63[1]

Arthritic Day	**7**	**14**	**21**	**28**	**35**	**42**	**49**	**56**	**63**	**SEM[1]**
CSO	0.34	0.25	0.09	0.30	0.25	0.55	0.55	0.51	0.52	
Corn oil	0.33	0.20	0.22	0.46	0.46	0.53	0.43	0.78	0.76	
0.25% TVA + CSO	-0.01	-0.12	-0.20	-0.02	-0.08	0.03	0.07	-0.15	0.01	
0.25% TVA + Corn oil	0.03	0.17	0.14	0.19	0.18	0.02	0.19	0.28	0.18	
0.5% TVA + CSO	0.36	0.17	0.17	0.27	0.36	0.28	0.40	0.43	0.37	0.03
0.5% TVA + Corn oil	0.40	0.41	0.30	0.05	0.25	0.42	0.31	0.33	0.43	
1% TVA + CSO	0.44	0.37	0.23	0.21	0.45	0.52	0.40	0.26	0.42	
1% TVA + Corn oil	0.80	0.85	0.89	0.84	0.83	0.85	0.71	0.84	1.01	
***P*-Value**	0.22	0.21	0.34	0.33	0.26	0.30	0.69	0.11	0.18	

[1]Normalized paw thickness is defined as the sum of all 4 individual paw thicknesses (mm) on arthritic day 0 from the sum of all 4 individual paw thicknesses (mm) at a given day.
CSO = Cottonseed oil, TVA = *trans*-11 vaccenic acid, and SEM = Standard error of the mean pooled for all samples on all dates
n Values: CSO = 9, Corn oil = 8, 0.25% TVA + CSO = 9, 0.25% TVA + Corn oil = 9, 0.5% TVA + CSO = 10, 0.5% TVA + Corn oil = 10, 1% TVA + CSO = 10, 1% TVA + Corn oil = 7.

Overall *P*-Value

Diet	Day	Diet*Day
0.11	0.11	0.94

Table 5 Hepatic fatty acids of arthritic mice at end of the study period (arthritic day 63, nine weeks after initiation of TVA treatment diets)

Fatty acid (g/100g)	CSO	Corn oil	0.25% TVA + CSO	0.25% TVA + Corn oil	0.5% TVA + CSO	0.5% TVA + Corn oil	1% TVA + CSO	1% TVA + Corn oil	SEM[1]	P-Value
14:0	0.46	0.65	0.66	0.49	0.97	1.62	1.68	1.55	0.24	0.45
16:0	29.1^{ab}	27.8^{bc}	28.1^{abc}	27.6^{bc}	29.7^{a}	26.7^{c}	27.5^{c}	26.6^{c}	0.20	0.005
16:1c9	1.83	1.91	1.80	2.12	2.31	1.95	2.31	2.16	0.07	0.35
18:0	10.3	9.93	9.83	9.08	9.51	9.54	8.79	8.49	0.17	0.11
TVA	n/d^{e}	n/d^{e}	0.49^{d}	0.49^{d}	0.87^{c}	0.96^{c}	1.60^{b}	1.83^{a}	0.07	<0.0001
18:1c9	16.0	18.0	16.8	19.6	17.8	17.9	17.6	18.3	0.32	0.31
18:1c11	2.88	2.62	2.77	3.07	2.94	3.05	3.19	2.86	0.06	0.62
18:2n-6	16.2^{bcd}	18.2^{a}	17.4^{ab}	17.1^{abc}	15.5^{cd}	15.4^{d}	15.4^{d}	16.4^{abcd}	0.22	0.009
20:0	0.39	0.44	0.43	0.42	0.46	0.39	0.38	0.40	0.01	0.54
18:3n-6	0.14	0.18	0.18	0.17	0.15	0.14	0.15	0.14	0.01	0.31
20:1c11	0.59^{bc}	0.70^{ab}	0.59^{bc}	0.77^{a}	0.59^{bc}	0.72^{a}	0.57^{c}	0.68^{abc}	0.01	0.006
18:3n-3	0.20^{d}	0.38^{a}	0.20^{d}	0.35^{a}	0.21c^{d}	0.31^{abc}	0.22^{bcd}	0.32^{ab}	0.01	0.003
CLAc9t11	n/d^{d}	n/d^{d}	0.33^{c}	0.36^{c}	0.58^{b}	0.66^{b}	1.40^{a}	1.5^{a}	0.06	<0.0001
CLAt10c12	n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d	-	-
20:2n-6	0.39^{ab}	0.38^{abc}	0.43^{a}	0.34^{bcd}	0.33^{cd}	0.32^{cd}	0.33^{cd}	0.30^{d}	0.01	0.001
20:3n-6	0.80	0.87	0.83	0.84	0.79	0.82	0.78	0.82	0.01	0.70
20:4n-6	12.3	11.1	11.4	10.6	11.4	11.5	10.8	10.6	0.23	0.41
22:6n-3	0.50^{b}	3.56^{a}	0.54^{b}	3.48^{a}	0.80^{b}	3.20^{a}	0.45^{b}	3.37^{a}	0.17	<0.0001
Unknown	7.80^{a}	3.28^{d}	7.54^{ab}	3.18^{d}	6.14^{b}	4.83^{c}	6.83^{ab}	3.68^{cd}	0.22	<0.0001
TVA to CLAc9t11	-	-	0.40	0.43	0.36	0.40	0.47	0.45	0.01	0.12
16:0 to 16:1c9	17.3	15.8	15.9	14.4	14.8	14.5	13.2	11.7	0.57	0.55
18:0 to 18:1c9	0.67	0.57	0.60	0.49	0.60	0.56	0.53	0.49	0.02	0.63

[1] Pooled SEM. CSO = Cottonseed oil, TVA = *trans*-11 vaccenic acid, CLA = conjugated linoleic acid, SEM = Standard error of the mean

n Values: CSO = 9, Corn oil = 8, 0.25% TVA + CSO = 9, 0.25% TVA + Corn oil = 9, 0.5% TVA + CSO = 10, 0.5% TVA + Corn oil = 10, 1% TVA + CSO = 10, 1% TVA + Corn oil = 7.

4.8 Figures

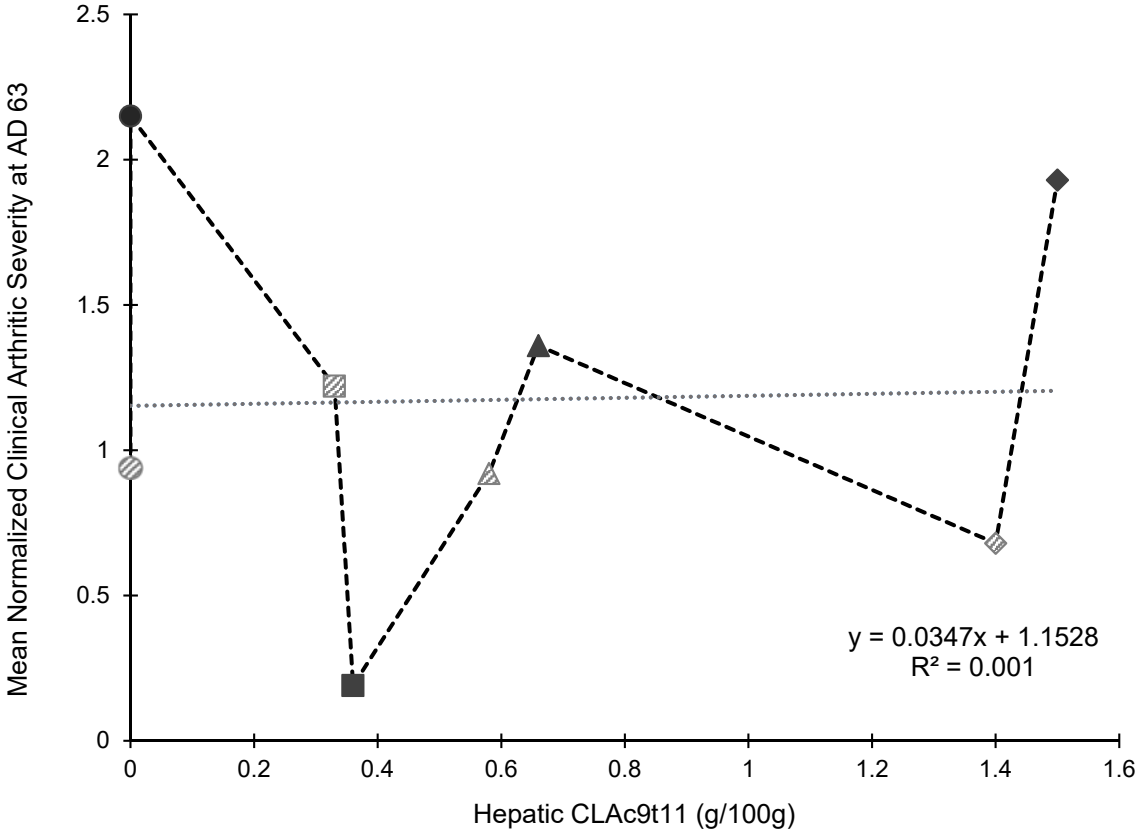

Figure 1: Regression of mean normalized clinical arthritic score at arthritic day (AD) 63 and hepatic CLAc9t11 (R^2 = 0.001). Symbols representing dietary groups are as follows: 6% cottonseed oil (CSO), 6% corn oil (CO), 0.25% *trans* -11 vaccenic acid (TVA) + 5.75% CSO, 0.25% TVA + 5.75% CO, 0.5% TVA + 5.5% CSO, 0.5% TVA + 5.5% CO, 1% TVA + 5% CSO, 1% TVA + 5% CO.

4.9 References

1. Hootman JM, Helmick CG, Barbour KE, et al (2016) Updated Projected Prevalence of Self-Reported Doctor-Diagnosed Arthritis and Arthritis-Attributable Activity Limitation Among US Adults, 2015-2040. Arthritis Rheumatol 68:1582–1587. doi: 10.1002/art.39692

2. Myasoedova E, Crowson CS, Kremers HM, et al (2010) Is the incidence of rheumatoid arthritis rising?: results from Olmsted County, Minnesota, 1955-2007. Arthritis Rheum 62:1576–82. doi: 10.1002/art.27425

3. Heidari B (2011) Rheumatoid Arthritis: Early diagnosis and treatment outcomes. Casp J Intern Med 2:161–70.

4. Sundy JS (2001) COX-2 inhibitors in rheumatoid arthritis. Curr Rheumatol Rep 3:86–91.

5. Rula EY, Pope JE, Hoffman JC (2011) Potential Medicare savings through prevention and risk reduction. Popul Health Manag 14 Suppl 1:S35-44. doi: 10.1089/pop.2010.0063

6. Shingfield KJ, Reynolds CK, Hervás G, et al (2006) Examination of the Persistency of Milk Fatty Acid Composition Responses to Fish Oil and Sunflower Oil in the Diet of Dairy Cows. J Dairy Sci 89:714–732. doi: 10.3168/jds.S0022-0302(06)72134-8

7. Huebner SM, Olson JM, Campbell JP, et al (2016) Low Dietary c9t11-Conjugated Linoleic Acid Intake from Dairy Fat or Supplements Reduces Inflammation in Collagen-Induced Arthritis. Lipids 51:807–819. doi: 10.1007/s11745-016-4163-8

8. Olson J (2016) The ruminant-derived fatty acids cis-9, trans-11 conjugated linoleic acid and trans-11 vaccenic acid are anti-inflammatory in the collagen-induced arthritis model:

Evidence of intestinal barrier dysfunction associated with arthritis-induced inflammation.

9. Bassaganya-Riera J, Reynolds K, Martino-Catt S, et al (2004) Activation of PPAR γ and δ by conjugated linoleic acid mediates protection from experimental inflammatory bowel disease. Gastroenterology 127:777–791. doi: 10.1053/j.gastro.2004.06.049

10. Yuan G, Chen X, Li D (2015) Modulation of Peroxisome Proliferator-Activated Receptor gamma (PPAR γ) by Conjugated Fatty Acid in Obesity and Inflammatory Bowel Disease. J Agric Food Chem 63:1883–1895. doi: 10.1021/jf505050c

11. Turpeinen AM, Ylönen N, von Willebrand E, et al (2008) Immunological and metabolic effects of cis-9, trans-11-conjugated linoleic acid in subjects with birch pollen allergy. Br J Nutr 100:112–119. doi: 10.1017/S0007114507886326

12. Muhlenbeck JA, Butz DE, Olson JM, et al (2017) Dietary Conjugated Linoleic Acid-c9t11 Prevents Collagen-Induced Arthritis, Whereas Conjugated Linoleic Acid-t10c12 Increases Arthritic Severity. Lipids. doi: 10.1007/s11745-017-4241-6

13. Yu Y, Correll PH, Vanden Heuvel JP (2002) Conjugated linoleic acid decreases production of pro-inflammatory products in macrophages: evidence for a PPAR gamma-dependent mechanism. Biochim Biophys Acta 1581:89–99.

14. Marion-Letellier R, Savoye G, Ghosh S (2016) Fatty acids, eicosanoids and PPAR gamma. Eur J Pharmacol 785:44–49. doi: 10.1016/j.ejphar.2015.11.004

15. Kuhnt K, Kraft J, Vogelsang H, et al (2007) Dietary supplementation with trans-11- and trans-12-18 : 1 increases cis-9, trans-11-conjugated linoleic acid in human immune cells, but without effects on biomarkers of immune function and inflammation. Br J Nutr

97:1196. doi: 10.1017/S0007114507685183

16. O'Shea M, Bassaganya-Riera J, Mohede IC (2004) Immunomodulatory properties of conjugated linoleic acid. Am J Clin Nutr 79:1199S–1206S. doi: 10.1093/ajcn/79.6.1199S

17. Pariza MW, Park Y, Cook ME (2008) Mechanisms of Action of Conjugated Linoleic Acid: Evidence and Speculation. Proc Soc Exp Biol Med 223:8–13. doi: 10.1111/j.1525-1373.2000.22302.x

18. Loscher CE, Draper E, Leavy O, et al (2005) Conjugated linoleic acid suppresses NF-kappa B activation and IL-12 production in dendritic cells through ERK-mediated IL-10 induction. J Immunol 175:4990–4998. doi: 10.4049/JIMMUNOL.175.8.4990

19. Olson JM, Haas AW, Lor J, et al (2017) A Comparison of the Anti-Inflammatory Effects of Cis-9, Trans-11 Conjugated Linoleic Acid to Celecoxib in the Collagen-Induced Arthritis Model. Lipids 52:151–159. doi: 10.1007/s11745-016-4228-8

20. Santora JE, Palmquist DL, Roehrig KL (2000) Trans-vaccenic acid is desaturated to conjugated linoleic acid in mice. J Nutr 130:208–15.

21. Tyburczy C, Major C, Lock AL, et al (2008) Individual Trans Octadecenoic Acids and Partially Hydrogenated Vegetable Oil Differentially Affect Hepatic Lipid and Lipoprotein Metabolism in Golden Syrian Hamsters. J Nutr 139:257–263. doi: 10.3945/jn.108.098004

22. Rice BH, Kraft J, Destaillats F, et al (2012) Ruminant-produced trans-fatty acids raise plasma HDL particle concentrations in intact and ovariectomized female Hartley guinea pigs. J Nutr 142:1679–83. doi: 10.3945/jn.112.160077

23. Wang Y, Jacome-Sosa MM, Ruth MR, et al (2009) Trans-11 Vaccenic Acid Reduces

Hepatic Lipogenesis and Chylomicron Secretion in JCR:LA-cp Rats. J Nutr 139:2049–2054. doi: 10.3945/jn.109.109488

24. Sun X, Zhang J, MacGibbon AKH, et al (2011) Bovine milk fat enriched in conjugated linoleic and vaccenic acids attenuates allergic dermatitis in mice. Clin Exp Allergy 41:729–738. doi: 10.1111/j.1365-2222.2011.03723.x

25. Blewett HJ, Gerdung CA, Ruth MR, et al (2009) Vaccenic acid favourably alters immune function in obese JCR:LA-cp rats. Br J Nutr 102:526–36. doi: 10.1017/S0007114509231722

26. Jaudszus A, Jahreis G, Schlörmann W, et al (2012) Vaccenic acid-mediated reduction in cytokine production is independent of c9,t11-CLA in human peripheral blood mononuclear cells. BBA - Mol Cell Biol Lipids 1821:1316–1322. doi: 10.1016/j.bbalip.2012.06.010

27. Jaudszus A, Krokowski M, Möckel P, et al (2008) Cis-9,trans-11-conjugated linoleic acid inhibits allergic sensitization and airway inflammation via a PPARgamma-related mechanism in mice. J Nutr 138:1336–42.

28. Lock AL, Corl BA, Barbano DM, et al (2004) The anticarcinogenic effect of trans-11 18:1 is dependent on its conversion to cis-9, trans-11 CLA by delta9-desaturase in rats. J Nutr 134:2698–704.

29. Kanwar RK, MacGibbon AK, Black PN, et al (2007) Bovine milk fat enriched in conjugated linoleic and vaccenic acids attenuates allergic airway disease in mice. Clin Exp Allergy 0:071115150625003–??? doi: 10.1111/j.1365-2222.2007.02868.x

30. Paton, Chad M.; Vaughan, Roger A.; Selen Alpergin, Ebru S.; Assadi-Porter F (2017) Dihydrosterculic acid from cottonseed oil suppresses desaturase activity and improves liver metabolomic profiles of high-fat–fed mice. Nutr Res 45:52–62. doi: 10.1016/J.NUTRES.2017.06.008

31. Griinari JM, Corl BA, Lacy SH, et al (2000) Conjugated Linoleic Acid Is Synthesized Endogenously in Lactating Dairy Cows by Δ9-Desaturase. J Nutr 130:2285–2291. doi: 10.1093/jn/130.9.2285

32. Roehm JN, Lee DJ, Wales JH, et al (1970) The effect of dietary sterculic acid on the hepatic lipids of rainbow trout. Lipids 5:80–84. doi: 10.1007/BF02531099

33. Leventhal LJ, Boyce EG, Zurier RB (1993) Treatment of rheumatoid arthritis with gammalinolenic acid. Ann Intern Med 119:867–73.

34. Park M-K, Jhun J-Y, Lee S-Y, et al (2012) Retinal attenuates inflammatory arthritis by reciprocal regulation of IL-17-producing T cells and Foxp3+ regulatory T cells and the inhibition of osteoclastogenesis. Immunol Lett 148:59–68. doi: 10.1016/j.imlet.2012.05.008

35. Butz DE, Li G, Huebner SM, Cook ME (2007) A mechanistic approach to understanding conjugated linoleic acid's role in inflammation using murine models of rheumatoid arthritis. Am J Physiol Regul Integr Comp Physiol 293:R669–R676. doi: 10.1152/ajpregu.00005.2007

36. Brand DD, Latham KA, Rosloniec EF (2007) Collagen-induced arthritis. Nat Protoc 2:1269–1275. doi: 10.1038/nprot.2007.173

37. Nordling C, Karlsson-Parra A, Jansson L, et al (1992) Characterization of a spontaneously occurring arthritis in male DBA/1 mice. Arthritis Rheum 35:717–722. doi: 10.1002/art.1780350619

38. Folch J, Lees M, Sloane Stanley GH (1957) A simple method for the isolation and purification of total lipides from animal tissues. J Biol Chem 226:497–509.

39. Stoffel W, Insull W, Ahrens EH (1958) Gas-liquid chromatography of highly unsaturated fatty acid methyl esters. Proc Soc Exp Biol Med 99:238–241.

40. Yeonhwa Park, Karen J. Albright, Zu Y. Cai A, Pariza MW (2001) Comparison of Methylation Procedures for Conjugated Linoleic Acid and Artifact Formation by Commercial (Trimethylsilyl)diazomethane. J Agric Food Chem 49:1158–1164. doi: 10.1021/JF001209Z

41. Kramer JKG, Blackadar CB, Zhou J (2002) Evaluation of two GC columns (60-m SUPELCOWAX 10 and 100-m CP sil 88) for analysis of milkfat with emphasis on CLA, 18:1, 18:2 and 18:3 isomers, and short- and long-chain FA. Lipids 37:823–835. doi: 10.1007/s11745-002-0967-2

42. Ortinau LC, Pickering RT, Nickelson KJ, et al (2012) Sterculic Oil, a Natural SCD1 Inhibitor, Improves Glucose Tolerance in Obese ob/ob Mice. ISRN Endocrinol 2012:947323. doi: 10.5402/2012/947323

43. Krugner-Higby L, Brown R, Rassette M, et al (2012) Ulcerative dermatitis in C57BL/6 mice lacking stearoyl CoA desaturase 1. Comp Med 62:257–63.

44. Paton CM, Ntambi JM (2010) Loss of stearoyl-CoA desaturase activity leads to free

cholesterol synthesis through increased Xbp-1 splicing. Am. J. Physiol. - Endocrinol. Metab. 299:

45. Miyazaki M, Ntambi JM (2003) Role of stearoyl-coenzyme A desaturase in lipid metabolism. Prostaglandins, Leukot Essent Fat Acids 68:113–121. doi: 10.1016/S0952-3278(02)00261-2

46. Wall R, Ross RP, Fitzgerald GF, Stanton C (2010) Fatty acids from fish: the anti-inflammatory potential of long-chain omega-3 fatty acids. Nutr Rev 68:280–289. doi: 10.1111/j.1753-4887.2010.00287.x

Chapter 5:

Trans-11 vaccenic acid prevents collagen-induced arthritis.

5.1 Abstract

Trans-11 vaccenic acid (TVA) is converted by stearoyl-CoA desaturase-1, a delta-9 desaturase, to conjugated linoleic acid (CLA) isomer *cis*-9, *trans*-11 (c9t11). TVA and CLAc9t11 both decrease the severity of collagen-induced arthritis (CA) when consumed after disease onset. TVA has its own anti-inflammatory properties, but its effects on CA are also linked to its conversion to CLAc9t11. CLAc9t11 reduces the incidence of CA when provided prior to CA immunization; however, TVA's ability to prevent inflammatory disease has yet to be examined. DBA/1 male mice (n = 54) were fed a 99% complete semi-purified diet supplemented with 1% corn oil (CO) or 0.5% TVA + 0.5% CO starting three weeks before CA immunization. Dietary effects on disease CA incidence and severity were monitored; changes in anti-collagen antibodies and hepatic fatty acids were also measured. Overall incidence of arthritis was reduced 35% in mice fed TVA compared to a CO control diet ($P = 0.11$). In mice that developed arthritis, TVA had no effect on early disease severity or anti-collagen antibody levels. The conversion of TVA to CLAc9t11 increased 29% in arthritic mice. This reduced arthritis incidence supports the continued exploration into the use of TVA and dairy fat as preventative treatments for inflammatory disease.

5.2 Introduction

Trans-11 vaccenic acid (TVA), a precursor to conjugated linoleic acid (CLA) *cis*-9, *trans*-11 (c9t11), is abundant in dairy fat at levels up to 14% of total fatty acids [1]. Approximately 12% (mice) [2] to 19% (humans) [3] of dietary TVA is converted to CLAc9t11 by stearoyl-CoA desaturase (SCD)-1, a delta-9 desaturase. CLAc9t11 known for its anti-inflammatory properties, particularly in immune mediated inflammatory diseases (IMID) including arthritis [4–8]. TVA also has anti-inflammatory actions in cardiovascular disease [9–11], allergic dermatitis [12], metabolic syndrome [13], and arthritis [14].

The rising cost, prevalence, and destructive effects of IMID and their pharmacological treatments supports the need for investigation into dietary anti-inflammatories like CLAc9t11 and TVA as cost effective and safe alternatives to current practices [15, 16]. Using the collagen-induced arthritis (CA) model of rheumatoid arthritis (RA), dietary treatment with 0.5% CLAc9t11 was equivalent to a 1.5mg/kg dose of celecoxib, a commonly prescribed anti-inflammatory with many side effects [14, 17–19]. Olson [7] fed mice with CA 0.375, 0.75, or 1.5% TVA and observed a 21, 42, and 65% decrease in arthritic severity, respectively, compared to a corn oil (CO) control diet. TVA's anti-inflammatory properties in CA are likely linked to its conversion to CLAc9t11 [20, 21]. Currently, the minimum effective anti-inflammatory dose of CLAc9t11 is 0.125% and 0.25% for TVA [6]. The simplest method to receive the anti-inflammatory benefit of CLAc9t11 TVA is dietary supplementation with dairy fat. Dairy fat can be enriched to contain CLAc9t11 at 6 % of total fatty acids [1, 22] and TVA up to 14% [1]. Fed to mice at 6% of the diet, dairy fat reduced the severity of CA [6]. The amount of CLAc9t11 and TVA in maximally enriched dairy fat could allow humans to receive an anti-inflammatory

benefit through the consumption of dairy fat below the recommended intake limit of 30g/day set by the United Sates Department of Agriculture [23].

Safer cost effective treatment options, like dairy fat, for inflammatory disease is beneficial. However, low cost preventative therapies can be an even more beneficial option. Preventative treatments for chronic inflammatory diseases could save Medicare between $650 billion and $1.43 trillion in the next ten years [24]. Fed prior to any collagen immunization, a 0.5% dose of CLAc9t11 reduced CA incidence by 39% [8]. TVA has been shown to have similar anti-inflammatory effects as CLAc9t11 [7]; therefore, the study in this chapter was conducted to assess the ability of dietary supplementation with 0.5% TVA to prevent CA and explore its effects on early stage disease severity.

5.3 Materials and Methods

Experiments were conducted in accordance with an animal protocol, A005438, approved by the College of Agricultural and Life Sciences Animal Care and Use Committee.

Collagen-induced arthritis model.

Four-week-old DBA/1 mice were purchased from Harlan (Indianapolis, IN) and housed in shoebox units (3 mice per box) and maintained on a 12:12 h light-dark cycle. After an acclimation period of one week on chow diet, all mice were fed a 99% complete diet, casin-based, semi-purified diet (containing 5% CO, TD94060; Harlan-Teklad, Madison, WI) supplemented with 1% CO or 0.5% TVA (99% pure, Nu-Chek). After three weeks on treatment diets, mice (grouped by shoebox units) were randomly assigned to receive a 100µl intradermal injection, at the base of the tail, of 100µg chick collagen type II (CII) in 0.05M acetic acid

(Chondrex, Redmound, WA, n = 18 mice per diet) or a sham injection of 0.05M acetic acid (n = 9 mice per diet) emulsified in a 1:1 ratio with complete Freund's adjuvant (4mg/ml heat-killed *Mycobacterium tuberculosis*; Chondrex) [25, 26]. The date of primary injection was designated as primary day 0. On primary day 21, mice received a 100µl intraperitoneal secondary injection of either 100µg chick CII in 0.05M acetic acid or a sham injection of 0.05M acetic acid emulsified in a 1:1 ratio with Freund's incomplete adjuvant (Chondrex). Following the secondary immunization mice were monitored daily for clinical signs of arthritis (see arthritis assessment).

Arthritis assessment.

As previously described, mice were examined by a trained observer blinded to treatments [5, 8, 25–27]. After the secondary immunization, at primary day 21, mice were monitored daily for clinical signs of arthritis until primary day 63. Day 63 was chosen as the end point of the study to avoid the risk of older mice developing spontaneous arthritis [26, 28, 29]. Mice were scored upon arthritis development and prior to their euthanasia at arthritic day (AD) 7. Briefly, individual paws were assigned an inflammation severity score between 0 and 4 based on inflammation, redness, joint involvement, and mobility and summed for the clinical arthritic score (CAS) with a maximum of 16 [26]. Normalized arthritic score was calculated by subtracting CAS at AD 0 from a given AD score post AD 0.

Upon arthritis development (AD 0) and at AD 7 paw thickness of each individual mouse paw was measured using a pressure-sensitive caliper (SPI, Garden Grove, CA). This provided an objective measure of arthritic severity. Paw thickness measurements are represented as a sum of

all 4 paws of an individual mouse paws. Normalized paw thickness was calculated by subtracting the thickness at AD 0 from paw thickness measured at AD 7.

Plasma collection and preparation.

Blood was collected from the retroorbital venous plexus using heparinized capillary tubes (Midwest Veterinary Supply, Sun Prairie, WI) and transferred into ethylenediaminetetraacetic acid (EDTA) tubes (BD Biosciences, San Jose, CA), while mice were under isoflurane anesthesia at AD 7 or at the end of the study period for sham mice. Plasma was separated by centrifugation at 3000xg for 10 minutes and stored at -80°C until analyzed.

Plasma anti-CII antibody ELISA.

Plasma samples were analyzed as previously described for total concentration of mouse anti-chick CII immunoglobulin (Ig)G_1 and IgG_{2a} [25]. In this experiment, ELISA plates were blocked using 1% dry milk (w/v, ConAgra Foods, Omaha, NE) and anti-CII IgG_{2a} levels were quantified with respect to an anti-CII IgG_{2a} monoclonal antibody standard (Chondrex).

Hepatic fatty acid determination.

Total lipids of 0.2g liver samples were extracted using chloroform:methanol (2:1, v/v) according to the methods of Folch *et al*. [30]. Fatty acid methyl esters (FAME) were prepared by acid-catalyzed methylation [31, 32]. Relative percentages of liver FAME were determined using an Agilent 6890N GC (Agilent Technologies Santa Clara, CA) equipped with a 100m biscyanopropyl polysiloxane column (Restek, Bellefonte, PA) [33]. FAME were identified against a custom qualitative FAME standard (Matreya, LLC, Pleasant Gap, PA, #SPL4833) by

comparing retention times. The conversion of stored TVA to CLAc9t11 was calculated by the net gain of CLAc9t11 as a percentage of TVA equivalents (TVA + CLAc9t11) [3, 7]. This approach represents conversion based on retained TVA.

Statistical analysis.

Incidence data was analyzed using the SAS procedure LIFETEST (SAS Institute, Cary, NC) to determine difference due to dietary treatment overtime. The dependent variables, arthritic score and antibodies, were analyzed using the SAS procedure MIXED (SAS Institute) to determine difference due to the fixed effects of dietary treatment. When dependent variables were identified to have a significant type, diet, and/or interaction ($P \leq 0.05$) ANOVA analysis was followed by a post hoc Fischer's Least Significant Difference test to identify individual differences among groups. Differences were considered significant at $P \leq 0.05$ and a trend at $P \leq 0.10$. Values are displayed as means ± standard error of the mean (SEM).

5.4 Results

Collagen-induced arthritis incidence.

Maximum disease incidence occurred at day 61 post primary immunization, and reached 82% in CO treated mice. Maximum arthritis incidence for TVA fed mice was reached at day 53 post primary immunization, and decreased by 35% compared to CO fed mice ($P = 0.11$, **Figure 1** and **Table 1**). TVA treatment only delayed arthritis onset by one day compared to CO fed mice. No signs of arthritis were observed in sham immunized mice of either diet.

Arthritis severity.

At AD 7 no differences in CAS were observed between CO, 5.93 ± 0.83, and TVA, 4.50 ± 1.03, fed mice (*P = 0.29*, **Table 1**). Paw thickness measurements also reflected no differences in arthritis severity between CO and TVA fed mice (7.95 ± 0.21 and 7.80 ± 0.26, respectively, *P = 0.65*).

Collagen IgG antibodies.

Anti-CII IgG_1 levels were increased 36% in TVA compared to CO treated mice (*P = 0.09*, **Table 2**). Anti-CII IgG_{2a} levels on AD 7 were not significantly different between CO and TVA dietary treatments (1047 ± 221 and 1006 ± 275, respectively, *P = 0.91*). No anti-CII IgG_1 or IgG_{2a} was detected in sham immunized mice at any time, or in any mice prior to primary immunization.

Hepatic fatty acids.

The conversion of TVA to CLAc9t11 was increased 29% in arthritic mice compared to sham immunized mice (*P < 0.0001*, **Table 3**). 16:1c9 and 18:1c11 were increased 32% in arthritic TVA fed mice compared to their respective sham mice (*P = 0.05* and *0.04*, respectively). TVA and CLAc9t11 were not detected in CO fed mice, and CLAt10c12 was not detected in any samples.

5.5 Discussion

Fed at 0.5% of the diet, TVA reduced the incidence of arthritis in the CA model compared to a CO diet. TVA's anti-inflammatory properties are predicted to occur both through TVA itself and through its downstream metabolite, CLAc9t11 [20, 21]. CLAc9t11 is known to prevent CA at 0.5% of diet [8]. Humans can consume both TVA and CLAc9t11 through dairy fat, which, with

enrichment, can contain up to 14% TVA and 6% CLAc9t11 [1, 3]. A person would need to consume less than 3g/day of maximally enriched dairy fat to receive an anti-inflammatory based on these dosing levels, well above the recommended daily intake limit [1, 23]. A dose-response study is required to determine if lower doses of TVA and CLAc9t11 can be effective CA preventatives. Both TVA and CLAc9t11 have been shown to be anti-inflammatory in developed CA at as low as 0.25% and 0.125%, respectively [6, 7], and are hypothesized to be effective as preventatives as lower doses as well.

TVA fed mice with arthritis had a 29% increase in TVA to CLAc9t11 conversion compared to TVA fed sham mice. This may suggest that SCD-1 may play a role in modulating an inflammatory event by increasing its activity. This hypothesis is further supported in this study by the decrease in the 16:0/16:1c9 ratio because of decreased 16:1c9 in arthritic TVA fed mice. SCD-1 is known to play a complex role in regulating inflammation, and further investigation into this finding and the mechanism of SCD-1 is still required [34]. TVA supplementation also decreased the amount of the inflammatory fatty acid, arachidonic acid (AA; 20:4n-6), in mice compared to mice fed CO. Decreased AA levels with CLA exposure has been observed previously, and is a possible mechanism of action for CLA's anti-inflammatory effects [35–39].

In mice that developed arthritis, TVA had no effect on disease severity one week after onset. This was expected as TVAs effects are largely linked to its conversion to CLAc9t1, which shows similar post onset effects [8]. It is predicted that if feeding continued beyond one-week post CA onset, a decrease in arthritic severity may be observed, as both TVA and CLAc9t11 decrease severity nine weeks after disease onset [6, 7]. Although difficult to design, future TVA preventative studies should look to compare mice that do not develop arthritis with those with

the diseases. Overall, TVA can be an effective anti-inflammatory as both a treatment and a preventative.

5.6 Acknowledgments

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5.7 Tables

Table 1 Maximum arthritis incidence 63 days after primary immunization and clinical arthritic score, normalized score, paw thickness, and normalized paw thickness at arthritic day 7 for mice fed 1% corn oil or 0.5% TVA. Arthritic score and paw thickness values include only mice that developed arthritis (n = 14 corn oil, n = 9 TVA)[1]

	Corn oil	TVA	*P*-value
Maximum Incidence	82%	53%	0.11
Clinical Arthritic Score	5.93 ± 0.83	4.50 ± 1.03	0.29
Normalized Score	2.18 ± 0.75	1.31 ± 0.93	0.47
Paw Thickness (mm)	7.95 ± 0.21	7.80 ± 0.26	0.65
Normalized Paw Thickness (mm)	0.54 ± 0.21	0.53 ± 0.26	0.96

[1] Values are means ± SEM. TVA = *trans*-11 vaccenic acid and SEM = standard of the mean

Table 2 Anti-collagen type II antibody levels of arthritic mice at arthritic day 7[1]

μg/ml	Corn oil	TVA	*P*-Value
IgG_1	63 ± 13	99 ± 16	0.09
IgG_{2a}	1047 ± 221	1006 ± 275	0.91

[1] Values are means ± SEM. TVA = *trans*-11 vaccenic acid and SEM = standard of the mean

Table 3 Hepatic fatty acid profiles of arthritic mice at arthritic day 7 and of sham mice at the end of the study period, 63 days after primary immunization[1]

	Sham		Arthritic		SEM[1]	*P*-Value		
g/100g	Corn oil	TVA	Corn oil	TVA		Type	Diet	Interaction
14:0	6.65	5.82	7.61	4.85	0.33	0.98	0.004	0.11
16:0	24.48	26.44	24.53	26.35	0.32	0.97	0.0031	0.88
16:1c9	1.33^{ab}	1.11^{b}	1.26^{ab}	1.63^{a}	0.08	0.13	0.61	0.05
18:0	10.91	9.64	10.83	8.72	0.29	0.34	0.003	0.43
18:1t11	n/d^{c}	1.33^{a}	n/d^{c}	1.04^{b}	0.10	0.009	<0.0001	0.009
18:1c9	15.40	14.45	14.28	17.33	0.44	0.29	0.21	0.02
18:1c11	2.49^{a}	1.69^{b}	2.53^{a}	2.48^{a}	0.10	0.02	0.02	0.04
18:2n-6	19.63	22.05	19.27	20.48	0.31	0.07	0.001	0.25
20:0	0.31	0.41	0.29	0.33	0.02	0.16	0.02	0.33
18:3n-6	0.16	0.17	0.13	0.13	0.01	0.03	0.73	0.88
20:1c11	0.52	0.41	0.56	0.56	0.02	0.002	0.05	0.10
18:3n-3	0.16	0.11	0.18	0.16	0.01	0.11	0.08	0.39
18:2c9t11	n/d ^{c}	0.56 ^{b}	n/d^{c}	0.75^{a}	0.06	0.001	<0.0001	0.001
18:2t10c12	n/d	n/d	n/d	n/d	n/d	-	-	-
20:2n-6	0.36	0.33	0.41	0.31	0.01	0.35	0.0007	0.11
20:3n-6	0.77	0.69	0.76	0.69	0.02	0.97	0.04	0.87
20:4n-6	11.59	10.03	11.83	9.98	0.39	0.90	0.03	0.85
22:6n-3	2.40	1.94	2.73	2.16	0.10	0.13	0.007	0.76
Unknown	2.86^{a}	2.83^{a}	2.80^{a}	2.04^{b}	0.09	0.006	0.009	0.01
16:0 / 16:1c9	20.37^{ab}	24.49^{a}	22.32^{ab}	16.65^{b}	1.04	0.16	0.75	0.02
18:0 / 18:1c9	0.72	0.68	0.80	0.54	0.04	0.67	0.04	0.14
TVA to CLAc9t11	-	0.30^{b}	-	0.42^{a}	0.02	<0.0001	-	-

[1] Values are means. Means within a row without a common letter differ by $P \leq 0.05$. TVA = *trans*-11 vaccenic acid, CLA = conjugated linoleic acid, and SEM = standard of the mean, and n/d = not detected

[2] Pooled SEM

5.8 Figures

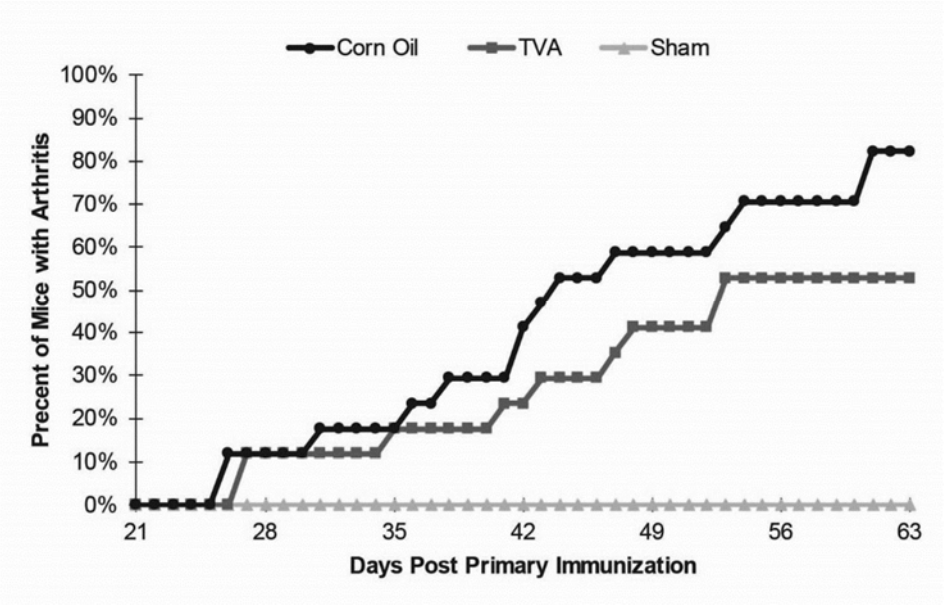

Figure 1: Arthritis incidence of mice fed diets supplemented with 1% corn oil (CO) or 0.5% *trans*-11 vaccenic acid (TVA) starting three weeks prior to immunization with chick collagen type II. The maximum incidence of arthritis was 82% for CO and 53% for TVA fed mice. Sham values are representative of all three dietary treatments. See table 1 for statistical analysis over time.

5.9 References

1. Shingfield KJ, Reynolds CK, Hervás G, et al (2006) Examination of the Persistency of Milk Fatty Acid Composition Responses to Fish Oil and Sunflower Oil in the Diet of Dairy Cows. J Dairy Sci 89:714–732. doi: 10.3168/jds.S0022-0302(06)72134-8
2. Turpeinen AM, Mutanen M, Aro A, et al (2002) Bioconversion of vaccenic acid to conjugated linoleic acid in humans. Am J Clin Nutr 76:504–10.
3. Santora JE, Palmquist DL, Roehrig KL (2000) Trans-vaccenic acid is desaturated to conjugated linoleic acid in mice. J Nutr 130:208–15.
4. Yang M, Pariza MW, Cook ME (2000) Dietary Conjugated Linoleic Acid Protects Against End Stage Disease of Systemic Lupus Erythematosus in the NZB/W F1 Mouse. Immunopharmacol Immunotoxicol 22:433–449.
5. Huebner SM, Campbell JP, Butz DE, et al (2010) Individual Isomers of Conjugated Linoleic Acid Reduce Inflammation Associated with Established Collagen-Induced Arthritis in DBA/1 Mice. J Nutr 140:1454–1461. doi: 10.3945/jn.109.120527
6. Huebner SM, Olson JM, Campbell JP, et al (2016) Low Dietary c9t11-Conjugated Linoleic Acid Intake from Dairy Fat or Supplements Reduces Inflammation in Collagen-Induced Arthritis. Lipids 51:807–819. doi: 10.1007/s11745-016-4163-8
7. Olson J (2016) The ruminant-derived fatty acids cis-9, trans-11 conjugated linoleic acid and trans-11 vaccenic acid are anti-inflammatory in the collagen-induced arthritis model: Evidence of intestinal barrier dysfunction associated with arthritis-induced inflammation.
8. Muhlenbeck JA, Butz DE, Olson JM, et al (2017) Dietary Conjugated Linoleic Acid-c9t11 Prevents Collagen-Induced Arthritis, Whereas Conjugated Linoleic Acid-t10c12 Increases Arthritic Severity. Lipids 1–12. doi: 10.1007/s11745-017-4241-6

9. Tyburczy C, Major C, Lock AL, et al (2008) Individual Trans Octadecenoic Acids and Partially Hydrogenated Vegetable Oil Differentially Affect Hepatic Lipid and Lipoprotein Metabolism in Golden Syrian Hamsters. J Nutr 139:257–263. doi: 10.3945/jn.108.098004

10. Rice BH, Kraft J, Destaillats F, et al (2012) Ruminant-produced trans-fatty acids raise plasma HDL particle concentrations in intact and ovariectomized female Hartley guinea pigs. J Nutr 142:1679–83. doi: 10.3945/jn.112.160077

11. Wang Y, Jacome-Sosa MM, Ruth MR, et al (2009) Trans-11 Vaccenic Acid Reduces Hepatic Lipogenesis and Chylomicron Secretion in JCR:LA-cp Rats. J Nutr 139:2049–2054. doi: 10.3945/jn.109.109488

12. Sun X, Zhang J, MacGibbon AKH, et al (2011) Bovine milk fat enriched in conjugated linoleic and vaccenic acids attenuates allergic dermatitis in mice. Clin Exp Allergy 41:729–738. doi: 10.1111/j.1365-2222.2011.03723.x

13. Blewett HJ, Gerdung CA, Ruth MR, et al (2009) Vaccenic acid favourably alters immune function in obese JCR:LA-cp rats. Br J Nutr 102:526–36. doi: 10.1017/S0007114509231722

14. Olson JM, Haas AW, Lor J, et al (2017) A Comparison of the Anti-Inflammatory Effects of Cis-9, Trans-11 Conjugated Linoleic Acid to Celecoxib in the Collagen-Induced Arthritis Model. Lipids 52:151–159. doi: 10.1007/s11745-016-4228-8

15. Helmick CG, Felson DT, Lawrence RC, et al (2008) Estimates of the prevalence of arthritis and other rheumatic conditions in the United States: Part I. Arthritis Rheum 58:15–25. doi: 10.1002/art.23177

16. Bloom BS (1988) Direct medical costs of disease and gastrointestinal side effects during treatment for arthritis. Am J Med 84:20–24. doi: 10.1016/0002-9343(88)90250-1

17. Silverstein FE, Faich G, Goldstein JL, et al (2000) Gastrointestinal Toxicity With Celecoxib vs Nonsteroidal Anti-inflammatory Drugs for Osteoarthritis and Rheumatoid Arthritis. JAMA 284:1247. doi: 10.1001/jama.284.10.1247

18. Schnitzer TJ (2001) Cyclooxygenase-2–specific inhibitors: are they safe? Am J Med 110:S46–S49. doi: 10.1016/S0002-9343(00)00650-1

19. Buttgereit F, Burmester GR, Simon LS (2001) Gastrointestinal toxic side effects of nonsteroidal anti-inflammatory drugs and cyclooxygenase-2–specific inhibitors. Am J Med 110:13–19. doi: 10.1016/S0002-9343(00)00728-2

20. Lock AL, Corl BA, Barbano DM, et al (2004) The anticarcinogenic effect of trans-11 18:1 is dependent on its conversion to cis-9, trans-11 CLA by delta9-desaturase in rats. J Nutr 134:2698–704.

21. Kanwar RK, MacGibbon AK, Black PN, et al (2007) Bovine milk fat enriched in conjugated linoleic and vaccenic acids attenuates allergic airway disease in mice. Clin Exp Allergy 0:071115150625003–??? doi: 10.1111/j.1365-2222.2007.02868.x

22. Parodi PW (2003) Conjugated Linoleic Acid in Food. In: Sébédio J-L, Christie WW, Adolf R (eds) Adv. Conjug. Linoleic Acid Res. Vol. 2. AOCS Press, Champaign, Illinois, pp 101–122

23. How Much Food from the Dairy Group is Needed Daily? In: US Dep. Agric. https://www.choosemyplate.gov/dairy. Accessed 6 Mar 2017

24. Rula EY, Pope JE, Hoffman JC (2011) Potential Medicare savings through prevention and risk reduction. Popul Health Manag 14 Suppl 1:S35-44. doi: 10.1089/pop.2010.0063

25. Butz DE, Li G, Huebner SM, Cook ME (2007) A mechanistic approach to understanding conjugated linoleic acid's role in inflammation using murine models of rheumatoid

arthritis. Am J Physiol Regul Integr Comp Physiol 293:R669–R676. doi: 10.1152/ajpregu.00005.2007

26. Brand DD, Latham KA, Rosloniec EF (2007) Collagen-induced arthritis. Nat Protoc 2:1269–1275. doi: 10.1038/nprot.2007.173

27. Huebner SM, Olson JM, Campbell JP, et al (2014) Dietary trans-10,cis-12 CLA reduces murine collagen-induced arthritis in a dose-dependent manner. J Nutr 144:177–184. doi: 10.3945/jn.113.182550

28. Nordling C, Karlsson-Parra A, Jansson L, et al (1992) Characterization of a spontaneously occurring arthritis in male DBA/1 mice. Arthritis Rheum 35:717–722. doi: 10.1002/art.1780350619

29. Holmdahl R, Jansson L, Andersson M, Jonsson R (1992) Genetic, hormonal and behavioural influence on spontaneously developing arthritis in normal mice. Clin Exp Immunol 88:467–472. doi: 10.1111/j.1365-2249.1992.tb06473.x

30. Folch J, Lees M, Sloane Stanley GH (1957) A simple method for the isolation and purification of total lipides from animal tissues. J Biol Chem 226:497–509.

31. Stoffel W, Insull W, Ahrens EH (1958) Gas-liquid chromatography of highly unsaturated fatty acid methyl esters. Proc Soc Exp Biol Med 99:238–241.

32. Yeonhwa Park, Karen J. Albright, Zu Y. Cai A, Pariza MW (2001) Comparison of Methylation Procedures for Conjugated Linoleic Acid and Artifact Formation by Commercial (Trimethylsilyl)diazomethane. J Agric Food Chem 49:1158–1164. doi: 10.1021/JF001209Z

33. Kramer JKG, Blackadar CB, Zhou J (2002) Evaluation of two GC columns (60-m SUPELCOWAX 10 and 100-m CP sil 88) for analysis of milkfat with emphasis on CLA,

18:1, 18:2 and 18:3 isomers, and short- and long-chain FA. Lipids 37:823–835. doi: 10.1007/s11745-002-0967-2

34. Liu X, Strable MS, Ntambi JM (2011) Stearoyl CoA Desaturase 1: Role in Cellular Inflammation and Stress. Adv Nutr 2:15–22. doi: 10.3945/an.110.000125

35. Liu K-L, Belury MA (1998) Conjugated linoleic acid reduces arachidonic acid content and PGE2 synthesis in murine keratinocytes. Cancer Lett 127:15–22. doi: 10.1016/S0304-3835(97)00479-5

36. Cook ME, Miller CC, Park Y, Pariza M (1993) Immune modulation by altered nutrient metabolism: nutritional control of immune-induced growth depression. Poult Sci 72:1301–1305.

37. Leslie CA, Gonnerman WA, Ullman MD, et al (1985) Dietary fish oil modulates macrophage fatty acids and decreases arthritis susceptibility in mice. J Exp Med 162:1336–49.

38. Park Y, Albright KJ, Liu W, et al (1997) Effect of conjugated linoleic acid on body composition in mice. Lipids 32:853–858. doi: 10.1007/s11745-997-0109-x

39. Lokesh BR, Hsieh HL, Kinsella JE (1986) Peritoneal Macrophages from Mice Fed Dietary (n-3) Polyunsaturated Fatty Acids Secrete Low Levels of Prostaglandins. J Nutr 116:2547–2552. doi: 10.1093/jn/116.12.2547

Concluding Remarks and Future Directions

Conjugated linoleic acid (CLA), *trans*-10, *cis*-12 (t10c12), and *cis*-9, *trans*-11 (c9t11) are effective preventative treatments for collagen induced arthritis (CA). Provided in diet at a 0.5% dose both CLA isomers resulted in a similar CA in incidence. In arthritic mice, CLAt10c12 treatment additional decreased disease severity compared to corn oil and CLAc9t11 treatment. The dietary precursor to CLAc9t11, *trans*-11 vaccenic acid (TVA), also prevented CA incidence at a 0.5% dietary dose with no effects on disease severity, similarly to CLAc9t11. Severity assessment in these studies occurred shortly after disease induction, and CLAc9t11 and TVA may just require additional time to provide an anti-inflammatory benefit.

Immune mediated inflammatory disease (IMID) modulation may occur through the ability of these fatty acids to regulate cytokine pathways and inflammatory fatty acid levels. TVA, specifically, may provide its anti-inflammatory effects through the conversion to and the downstream effects of CLAc9t11. Although this thesis supports the decrease in pro-inflammatory tumor necrosis factor α, interferon γ, and interleukin (IL)-2, increase in IL-10, regulation of arachidonic acid levels, and regulation of the intestinal barrier integrity as possible mechanisms of action; further research is required to show causation between these endpoints and the decrease in arthritis severity or prevention of disease.

Future studies regarding the anti-inflammatory effects of CLAt10c12, CLAc9t11, and TVA should include dose response and treatment timing analysis, mechanism investigation, whole dairy fat and enriched dairy fat treatment, other IMID models, and finally human clinical trials. This research can ultimately lead to treatment of IMID with dietary supplementation and nutritional recommendations toward the consumption of dairy fat for anti-inflammatory health benefits.

Appendix A:

Collagen-induced arthritis weakens the gastrointestinal barrier, while conjugated linoleic acid *cis*-9, *trans*-11 provides protection.

A.1 Abstract

Interest in the role of the gastrointestinal system in chronic inflammatory disease has grown tremendously over the last decade. Both the microbiome and gastrointestinal barrier integrity have been linked to inflammatory disease and provide a potential treatment target. A study was performed to assess the integrity of the gastrointestinal barrier after induction of collagen-induced arthritis in DBA/1 male mice. Using a 2x2 factorial design, mice (n = 54) were fed control diet containing 6% corn oil (CO) or treatment diet containing 5.5% CO and 0.5% conjugated linoleic acid (CLA) *cis*-9, *trans*-11 (c9t11), a fat known for its anti-inflammatory properties, and received either a sham or arthritis inducing immunization. Intestinal permeability was measured before arthritis onset and once per week for 4 weeks afterward, by plasma fluorescein isothyocyanate-conjugated dextran (FITC) and horseradish peroxidase (HRP) levels four hours after oral gavage. Periodic acid-Schiff Alcian Blue staining was used to quantify mucin levels in gastrointestinal sections on arthritic day 28. A significant increase in plasma FITC concentration was observed after arthritis onset ($P = 0.04$), and increased mucin production was observed in mice fed CLAc9t11 ($P = 0.07$). This study supports changes the intestinal barriers integrity during inflammatory disease and suggests dietary intervention may be a way to target these changes.

A.2 Introduction

The gastrointestinal barrier is a defense mechanism for the gut, protecting it from harmful bacterial and inflammatory responses to the gut flora [1]. Many chronic inflammatory diseases including celiac disease, type I diabetes, multiple sclerosis, and rheumatoid arthritis (RA) have been linked to defects in the gastrointestinal barrier [2–5]. The focus of this thesis has been on the role of dietary fatty acids to both treat and prevent collagen-induced arthritis (CA), and this appendix aims to explore the link between CA, dietary treatment, and the gastrointestinal barrier.

Both human and animal models of arthritis support a link between the disease and the intestine. In humans, juvenile-onset chronic arthritis was associated with increased intestinal permeability compared to healthy controls [6]. K/BxN arthritic mice, housed in germ free conditions, developed attenuated arthritis, but upon exposure to a single commensal microbe arthritic severity increased [7]. For RA specifically, bacterial components, identified in the synovial tissues of patients, may enhance inflammation [8]. When RA patients consumed whey protein concentrated with microbial antibodies, improvement of arthritis symptoms was observed [9, 10]. Dietary adjustments, including vegetarian and vegan diet, and changes in the fecal flora of RA patients were associated with symptoms improvement [11, 12].

Murine CA is a widely accepted model of RA that has been used in the development of treatments including anti-interleukin (IL)-1 and anti-tumor necrosis factor (TNF)α drugs [13–15]. Using the CA model of RA, Olson [16] measured intestinal barrier function post-arthritis challenge, and demonstrated that mice with a clinical arthritic score between 6 and 10 had increased levels of plasma endotoxin. After oral gavage containing fluorescein isothyocyanate-conjugated dextran (FITC), a marker for para-cellular permeability [17, 18], an increase in intestinal permeability was observed in arthritic mice [16]. Arthritic mice also had significantly

decreased levels of TNFα in the intestinal luminal fluid [16]. A single injection of lipopolysaccharide has been shown to increase anti-collagen antibody levels and the severity of CA [19, 20]. These results suggest a loss of the host's intestinal immune defenses during CA.

Conjugated linoleic acid (CLA) and two of its isomers, *cis*-9, *trans*-11 (c9t11) and *trans*-10, *cis*-12 (t10c12), effectively reduce CA inflammation and incidence [21–25]. CLA has been shown to modulate barrier function by acting as a metabolic precursor for microbes, like *Bifidobacterium breve*, leading to the production and utilization of CLA [26, 27]. CLA also acts as an anti-inflammatory agent through the activation of trefoil factor (TFF)3, a peptide essential for intestinal barrier strength, through peroxisome proliferator-activated receptor (PPAR)γ [28]. This appendix begins to assess the hypothesis that CLAc9t11, the primary isomer of CLA in dairy fat [29], regulates intestinal barrier function during early CA.

A.3 Materials and Methods

Collagen-induced arthritis model.

Experiments were conducted with the approval of the College of Agricultural and Life Sciences Animal Care and Use Committee. Studies presented here were performed in conjunction with the study performed in Muhlenbeck *et al*. 2017 [25], chapter two of this thesis. Briefly, four-week-old DBA/1 male mice were purchased from Harlan (Indianapolis, IN) and maintained on a 12:12 hour light-dark cycle. After one week on chow diet mice were switched to a 99% complete diet (5% CO, TD94060; Harlan-Teklad, Madison, W) supplemented with 1% CO or 0.5% CLAc9t11 (92% pure, containing no detectable CLAt10c12). Three weeks after diet initiation, mice (grouped by shoebox units) were randomly assigned to receive a 100µl intradermal injection, at the base of the tail, of 100µg chick collagen type II (CII) in 0.05M acetic acid (Chondrex,

Redmound, WA) or a sham injection of 0.05 M acetic acid emulsified in a 1:1 ratio with complete Freund's adjuvant (4mg/ml heat-killed *Mycobacterium tuberculosis*, Chondrex) [30]. The date of primary immunization was designated as day 0. On day 21, mice received a 100µl intraperitoneal booster injection of either 100µg chick CII in 0.05M acetic acid or a sham injection of 0.05M acetic acid emulsified in a 1:1 ratio with Freund's incomplete adjuvant (Chondrex). Mice were randomized in a 2x2 factorial arrangement of diet and arthritis (n = 18 per diet in chick CII immunized groups and n = 9 per diet in sham groups). Following the booster immunization mice were monitored daily for clinical signs of arthritis (see Clinical Arthritic Score).

Clinical arthritic score.

As previously described, mice were examined by a trained observer blinded to treatments [20–25, 30]. After booster immunization mice were monitored daily for clinical signs of arthritis, and after the first day post arthritis development (AD 0) mice were scored 3 times per week. Briefly, individual paws were assigned an inflammation severity score between 0 and 4 based on joint involvement, inflammation, redness, and swelling. Clinical arthritic score (CAS) was the sum of the 4 individual paw scores [30]. Normalized arthritic score was calculated by subtracting AD 0 from a given AD score post AD 0. Mice receiving a primary and secondary injection of CII, but never developing arthritis were designated as non-developed (ND). ND mice were monitored daily for signs of arthritis until primary day 70. Day 70 was chosen as the end point of the study to avoid the risk of older mice developing spontaneous arthritis [30–32].

FITC and horseradish peroxidase oral gavage.

At one week post primary immunization and AD 1, AD 8, AD 15, and AD 22 mice were orally gavage with FITC (440mg/kg bw, 4kDa; MilliporeSigma, St. Louis, MO) and horseradish peroxidase (20mg/kg bw, HRP, MilliporeSigma) and plasma was collected to measure intestinal permeability. Mice were fasted for 4 hours prior to FTIC/HRP oral gavage and deprived of feed for an additional 4 hours prior to blood collection. Blood was collected from the retroorbital venous plexus using heparinized capillary tubes, while mice were under light isoflurane anesthesia. Plasma was separated by centrifugation at 3000xg for 10 minutes and stored at -80°C until analyzed.

FITC measurement.

Plasma was diluted 1:2 and fluorescence was detected on a GloMax (Promega, Fitchburg, WI) spectrophotofluorometer at an excitation wavelength of 490nm and an emission wavelength of 530nm. The amount of FITC in each plasma sample (µg/ml) was obtained by comparison of optical density values of unknowns to a serially diluted 1mg/ml FTIC standard. Values reported are normalized to FITC levels one week after primary immunization to portray the change with arthritis onset.

HRP ELISA.

Plasma was diluted 1:2 and loaded to plates coated, overnight at 4°C, with rabbit IgG anti-HRP (Millipore Sigma) and blocked with protein free blocking buffer (PA1-26409, Thermo-Fisher Scientific, Waltham, MA). Plates were allowed to incubate for two hours at room temperature. Tetramethylbenzidine was added to wells and plates were incubated for 15 minutes at room

temperature, in the dark, before being stopped with 0.5M H_2SO_4. Pates were read at 450nm. The amount of HRP in each plasma sample (µg/ml) was obtained by comparison of optical density values of unknowns to a serially diluted 1mg/ml HRP standard. Values reported are normalized to HRP levels one week after primary immunization to portray the change with arthritis onset.

Intestine collection and Periodic Acid Schiff and Alcian Blue staining.

After mice were euthanized by exsanguination on AD 28, intestinal sections were collected and fixed in 10% buffered formalin (Millipore Sigma) for 48 hours. Samples were then moved to a 70% ethanol solution and stored at room temperature until staining. Intestinal sections were embedded in paraffin and 5µm sectioned samples were collected. Samples were deparaffinized in distilled water and stained with Alcian Blue (pH 2.5) for 30 minutes, 0.5% Periodic acid for 5 minutes, Schiff solution for 15 minutes, and Gill's Hematoxylin for 10 seconds (PAS/AB). Samples were then dehydrated in 100% ethanol and kept in xylene for 10 minutes. Coverslips were added and slides were stored at room temperature until viewing. Alcian Blue staining was used to identify acid mucins, stained a deep blue, and Periodic acid Schiff staining was used to identify neutral mucins, stained bright magenta. The combination of both acid and neutral mucins appear deep blue and purple.

PAS/AB image collection and analysis.

Brightfield images were captured using an Olympus BX51 Fluorescent Microscope at 20x magnification, encompassing the bottom of the basal membrane to the tops of the villi. Sample analysis was done using the Image J open source software. Both image collection and analysis were performed by a trained observer blinded to treatments. Images were converted to 8-bit

grayscale, and two, non-overlapping, 450x450 pixel sections were cropped out form each image for analysis. The color threshold was brought to 0 and increased until all PAS/AB stained areas were included. The fraction of the total image area included in the threshold area was recorded. Measurements are represented as an average percent area for the two sections analyzed per sample.

Statistical analysis.

Incidence data were analyzed using the SAS procedure LIFETEST (SAS Institute) to determine difference due to dietary treatment. Development of arthritis was considered a fail, non-survival, in this analysis. The dependent variables CAS, FITC, HRP, and area fraction were analyzed using the SAS procedure MIXED (SAS Institute) to determine difference due to the fixed effects of dietary treatment and time. In order to account for repeated measures across time, a REPEATED statement was employed with the SAS procedure MIXED with a first order autoregressive covariance structure. When dependent variables were identified to have a significant diet, arthritis, time, and/or interaction ($P \leq 0.05$) ANOVA analysis was followed by post hoc least squared difference test to identify individual differences among groups. Differences were considered significant at $P \leq 0.05$ and a trend at $P \leq 0.10$. Values are displayed as means ± standard error of the mean (SEM).

A.4 Results

Clinical arthritic score.

Arthritis incidence reached 69% in CO fed mice, and 30% in CLAc9t11 fed mice. This reduction was significant over the course of the study ($P = 0.01$). However, there were no significant

differences in CAS between CO and CLAc9t11 fed mice that developed arthritis throughout the course of this study (4.1 ± 0.1, **Table 1**).

Plasma FITC concentration.

There was a significant interaction across type, diet, and time for plasma FITC concentrations (*P = 0.04*, **Table 2**). The largest difference in plasma FIITC change, between arthritic and sham mice, was observed on AD 8 (0.066 and -0.083μg/ml, respectively, **Figure 1**). No correlation between plasma FITC concentration and CAS was observed.

Plasma HRP concentration.

No significant differences were observed for plasma HRP concentrations (*P = 0.91*, **Table 3** and **Figure 2**). On all days measured, sham and arthritic mice of both diets had decreased plasma HRP concentrations relative to one week post primary immunization.

PAS/AB image analysis.

A 5% increase in mucin stained area was observed in the colons of CLAc9t11 fed mice compared to mice fed CO (*P = 0.07*, **Table 4** and **Figure 3**). Arthritic mice fed CO had the lowest fraction of mucin stained area, 11.9%, compared to all other groups.

A.5 Discussion

Chronic inflammatory diseases continue to be linked to intestinal barrier function both in its onset and progression [1]. Understanding the intestinal barrier and its connection to inflammatory disease provides a route to discover new treatments. The objective of the study

was to determine the effects of CA induction on the gastrointestinal barrier, and determine if CLAc9t11 changes intestinal barrier function during arthritis onset and the four weeks following.

Previous research supports CA increasing intestinal permeability, demonstrated through increased concentrations of plasma endotoxin and FITC [16]. Permeability was measured in that study nine weeks after arthritis onset, when mice had prolonged inflammatory responses. To assess how intestinal permeability impacts early stage CA, barrier function was measured before arthritis onset and once per week for four weeks afterward. Results showed increased plasma FITC concentrations in arthritic mice compared to sham injected mice, with the largest difference observed one week after arthritis onset. This result supports a decrease in intestinal barrier integrity directly after the onset of CA. No correlation between FITC concentration and CA severity was observed. The increase in para-cellular permeability may occur through a breakdown in the seal of tight junctions, and requires further analysis. HRP was also given by oral gavage, as a marker for trans-cellular permeability [33, 34]. No changes in plasma HRP concentrations were observed with CA onset. This may indicate that no trans-cellular permeability changes are occurring during CA onset; however, further optimization of HRP dosing is necessary to confirm these results.

Anti-inflammatory treatments, especially dietary based ones, may attenuate inflammatory diseases by recovering the weakened intestinal barrier. CLA, which is known to decrease CA inflammation [21, 24], has been shown be anti-inflammatory due to an increase in intestinal TFF3, a protein linked to protection and restoration of the gut mucosa [28]. After PAS/AB image analysis, the colon of the CLAc9t11 treated mice showed significantly higher mucin levels. Mucin serves as the first line of defensive for the intestinal barrier, and an insufficient amount of mucin lining the gut may result in intestinal inflammation [35, 36]. Plasma FITC and HRP

concentrations were not affected by CLAc9t11 treatment, suggesting CLAc9t11 effects may not occur through the modulation of trans- or para-cellular intestinal permeability.

General growth and aging can have effects on the intestinal barrier [37]; therefore, for control measures across time are essential. Not all of CLA's suggested effects on gastrointestinal function are beneficial. When CLA enriched butter was fed to mice for 30 days before induction of mucositis, an increase in intestinal permeability and reduced secretory immunoglobulin A was observed compared to mice fed the control diet [38]. Further analysis of markers of intestinal integrity, both at a protein and histological level, is required to understand possible disease progression and treatment mechanisms. Results from these studies support the role of barrier function in immune mediated inflammatory disease (IMID) progression, and warrant the further study of the gastrointestinal barrier in relation to both dietary and pharmaceuticals treatments to IMID.

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A.7 Tables

Table 1 Clinical arthritic score of mice that developed arthritis (n = 11 CO and n = 6 CLAc9t11)[1]

Arthritic Day	Corn oil	CLAc9t11	P-value
0	2.96 ± 0.24	3.34 ± 0.31	0.65
7	5.08 ± 1.07	4.14 ± 0.94	0.54
14	4.17 ± 1.06	4.86 ± 0.90	0.64
21	3.21 ± 1.06	4.27 ± 0.80	0.44

[1] Values are means ± SEM. CLA = conjugated linoleic acid, and SEM = standard of the mean

Table 2 Plasma FITC concentration, normalized to pre-arthritis onset, for arthritic and sham mice fed 6% corn oil or 0.5% CLAc9t11 and 5.5% corn oil[1]

	Sham		Arthritic	
µg/ml	Corn oil	CLAc9t11	Corn oil	CLAc9t11
AD 1	-0.051 ± 0.02^{e}	-0.045 ± 0.02 ^{abcde}	0.027 ± 0.01^{abc}	-0.013 ± 0.02^{cde}
AD 8	-0.048 ± 0.02^{de}	-0.118 ± 0.02^{de}	0.067 ± 0.01^{abc}	0.065 ± 0.02^{ab}
AD 15	-0.035 ± 0.02^{de}	-0.053 ± 0.03 ^{abcde}	0.046 ± 0.01^{abc}	0.033 ± 0.02^{abc}
AD 22	-0.041 ± 0.02^{de}	0.014 ± 0.02^{abc}	0.047 ± 0.01^{ac}	0.044 ± 0.02^{abc}

[1] Values are means ± SEM. Interaction *P*-value for type, diet, and day = 0.04. Means in the table without a common letter differ by $P \leq 0.05$. FITC = fluorescein isothyocyanate-conjugated dextran, AD = arthritic day, CLA = conjugated linoleic acid, and SEM = standard of the mean

Table 3 Plasma HRP concentration, normalized to pre-arthritis onset, for arthritic and sham mice fed 6% corn oil or 0.5% CLAc9t11 and 5.5% corn oil[1]

	Sham		Arthritic	
µg/ml	Corn oil	CLAc9t11	Corn oil	CLAc9t11
AD 1	-0.174 ± 0.05	-0.385 ± 0.07	-0.018 ± 0.04	-0.002 ± 0.07
AD 8	-0.151 ± 0.05	-0.424 ± 0.07	-0.018 ± 0.04	-0.004 ± 0.07
AD 15	-0.151 ± 0.05	-0.385 ± 0.07	-0.018 ± 0.04	-0.001 ± 0.07
AD 22	-0.152 ± 0.05	-0.3412 ± 0.07	-0.016 ± 0.04	-0.0001 ± 0.07

[1] Values are means ± SEM. Interaction *P*-value for type, diet, and day = 0.91. No other significant differences were observed. HRP = horseradish peroxidase, AD = arthritic day, CLA = conjugated linoleic acid, and SEM = standard of the mean

Table 4 Mucin stained area of gastrointestinal colon sections, stained with PAS/AB, of arthritic and sham mice at the end of the study period[1]

% Total Area	Sham		Arthritic		*P*-Value		
	Corn oil	CLAc9t11	Corn oil	CLAc9t11	Type	Diet	Interaction
Colon	18.1 ± 0.03	20.7 ± 0.03	11.94 ± 0.02	19.6 ± 0.03	0.18	0.07	0.35

[1] Values are means ± SEM. PAS/AB = Periodic acid-Schiff/Alcian Blue, CLA = conjugated linoleic acid, and SEM = standard of the mean

A.8 Figures

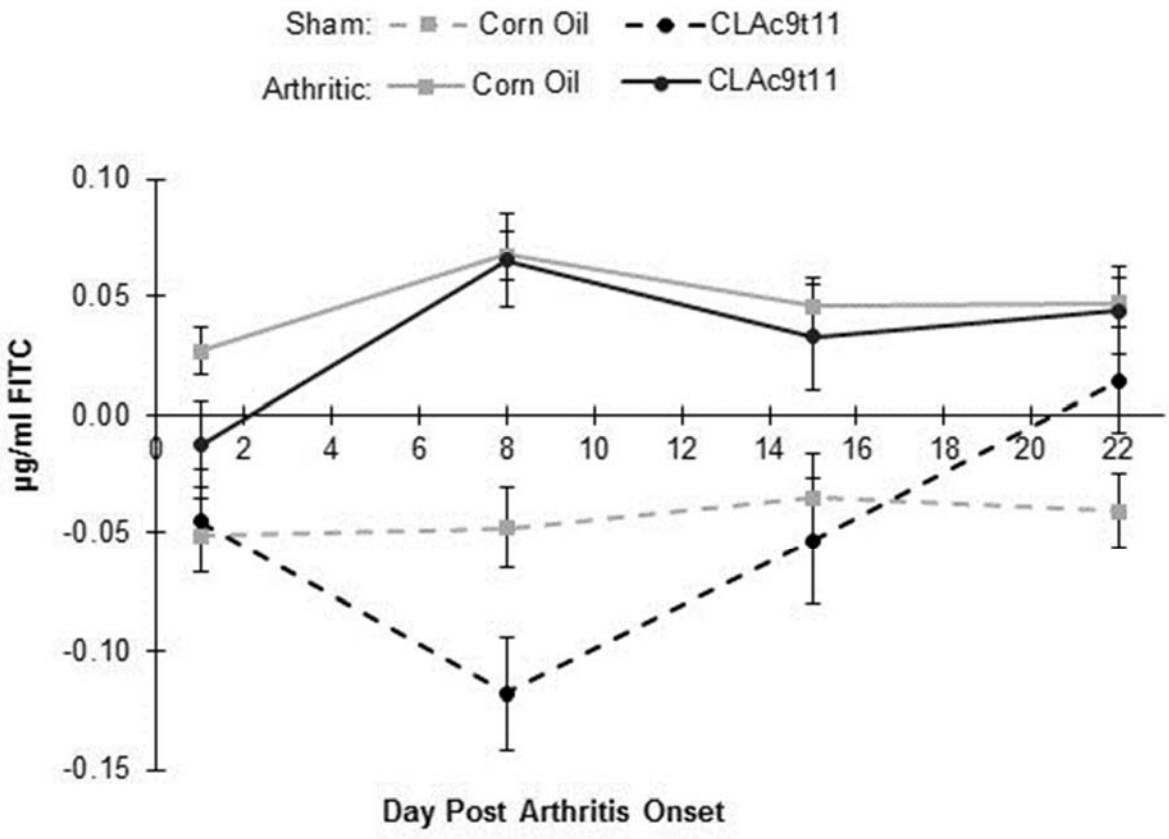

Figure 1: Plasma FITC concentration, normalized to pre-arthritis onset, for arthritic and sham mice fed 6% corn oil or 0.5% CLAc9t11 and 5.5% corn oil. See table 1 for statistical analysis.

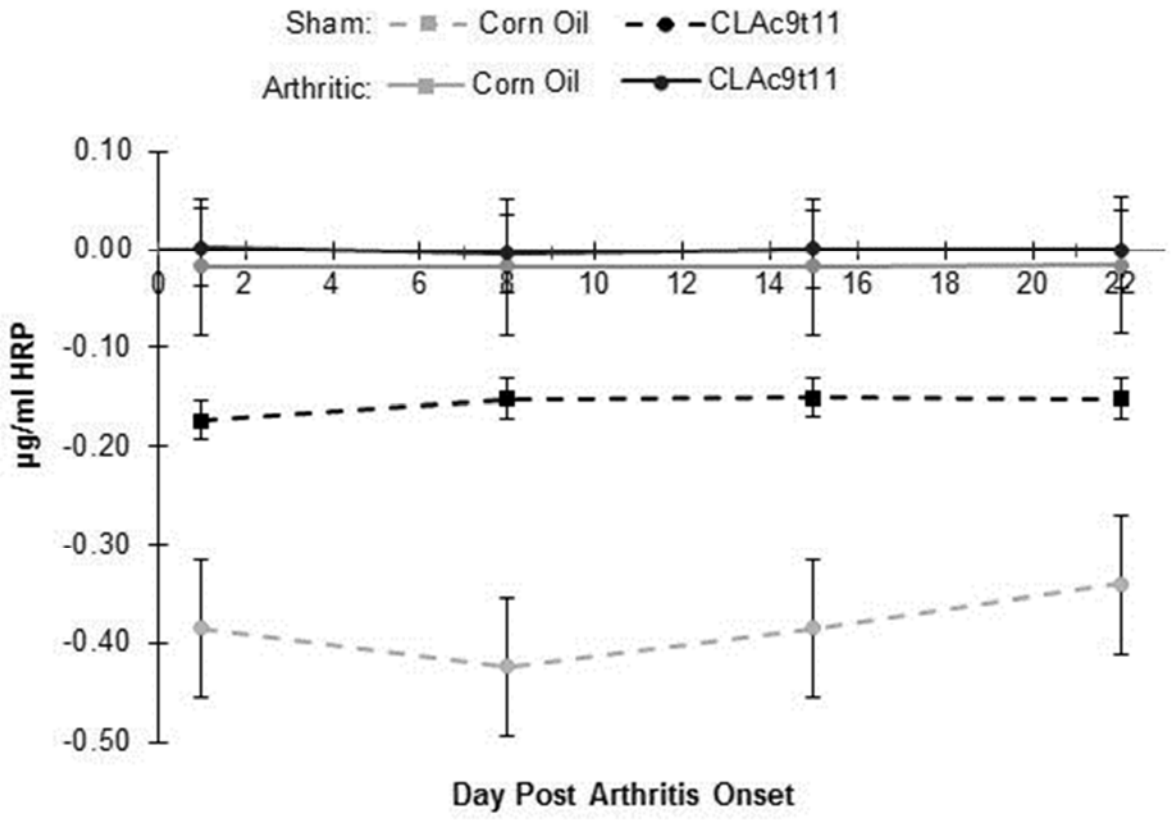

Figure 2: Plasma HRP concentration, normalized to pre-arthritis onset, for arthritic and sham mice fed 6% corn oil or 0.5% CLAc9t11 and 5.5% corn oil. See table 2 for statistical analysis.

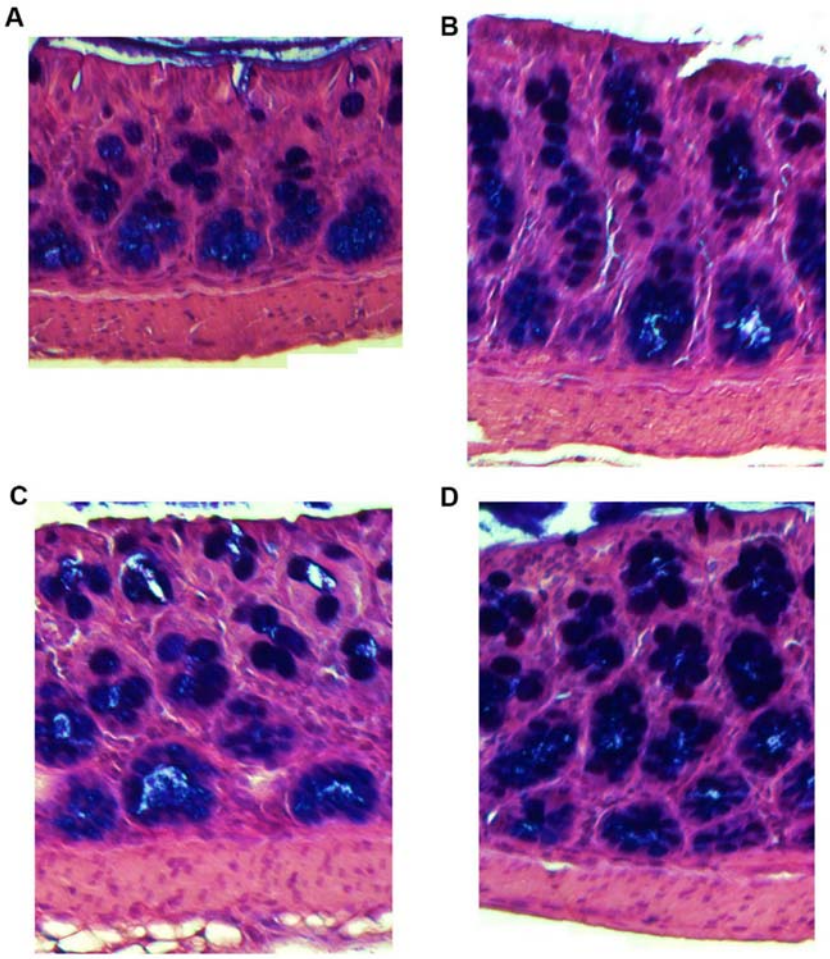

Figure 3: Periodic acid-Schiff/Alcian Blue stained gastrointestinal colon sections of arthritic and sham mice at the end of the study period. Sham immunized 6% corn oil diet (a), sham immunized 0.5% CLAc9t11 and 5.5% corn oil diet (b), arthritic corn oil diet (c), arthritic CLAc9t11 diet (d). See table 3 for statistical analysis.

A.9 References

1. Bischoff SC, Barbara G, Buurman W, et al Intestinal permeability – a new target for disease prevention and therapy. doi: 10.1186/s12876-014-0189-7
2. van Elburg RM, Uil JJ, Mulder CJ, Heymans HS (1993) Intestinal permeability in patients with coeliac disease and relatives of patients with coeliac disease. Gut 34:354–7.
3. Bosi E, Molteni L, Radaelli MG, et al (2006) Increased intestinal permeability precedes clinical onset of type 1 diabetes. Diabetologia 49:2824–2827. doi: 10.1007/s00125-006-0465-3
4. Yacyshyn B, Meddings J, Sadowski D, Bowen-Yacyshyn MB (1996) Multiple sclerosis patients have peripheral blood CD45RO+ B cells and increased intestinal permeability. Dig Dis Sci 41:2493–8.
5. Mielants H, De Vos M, Goemaere S, et al (1991) Intestinal mucosal permeability in inflammatory rheumatic diseases. II. Role of disease. J Rheumatol 18:394–400.
6. Picco P, Gattorno M, Marchese N, et al (2000) Increased gut permeability in juvenile chronic arthritides. A multivariate analysis of the diagnostic parameters. Clin Exp Rheumatol 18:773–8.
7. Wu H-J, Ivanov II, Darce J, et al (2010) Gut-residing segmented filamentous bacteria drive autoimmune arthritis via T helper 17 cells. Immunity 32:815–27. doi: 10.1016/j.immuni.2010.06.001
8. Van Der Heijden IM, Wilbrink B, Tchetverikov I, et al (2000) Presence of bacterial DNA and bacterial peptidoglycans in joints of patients with rheumatoid arthritis and other arthritides. Arthritis Rheum 43:593. doi: 10.1002/1529-0131(200003)43:3<593::AID-ANR16>3.0.CO;2-1

9. Katayama K, Matsuno T, Waritani T, et al (2011) Supplemental treatment of rheumatoid arthritis with natural milk antibodies against enteromicrobes and their toxins: results of an open-labelled pilot study. Nutr J 10:2. doi: 10.1186/1475-2891-10-2

10. Aoki S, Yoshikawa K, Yokoyama T, et al (1996) Role of enteric bacteria in the pathogenesis of rheumatoid arthritis: evidence for antibodies to enterobacterial common antigens in rheumatoid sera and synovial fluids. Ann Rheum Dis 55:363–9.

11. Peltonen R, Kjeldsen-Kragh J, Haugen M, et al (1994) Changes of faecal flora in rheumatoid arthritis during fasting and one-year vegetarian diet. Rheumatology 33:638–643. doi: 10.1093/rheumatology/33.7.638

12. Peltonen R, Nenonen M, Helve T, et al (1997) Faecal microbial flora and disease activity in rheumatoid arthritis during a vegan diet. Rheumatology 36:64–68. doi: 10.1093/rheumatology/36.1.64

13. Banni S (2002) Conjugated linoleic acid metabolism. Curr Opin Lipidol 13:261–266.

14. Williams RO, Feldmann M, Maini RN (1992) Anti-tumor necrosis factor ameliorates joint disease in murine collagen-induced arthritis. Proc Natl Acad Sci U S A 89:9784–8.

15. Joosten LA, Helsen MM, Saxne T, et al (1999) IL-1 alpha beta blockade prevents cartilage and bone destruction in murine type II collagen-induced arthritis, whereas TNF-alpha blockade only ameliorates joint inflammation. J Immunol 163:5049–55.

16. Olson J (2016) The ruminant-derived fatty acids cis-9, trans-11 conjugated linoleic acid and trans-11 vaccenic acid are anti-inflammatory in the collagen-induced arthritis model: Evidence of intestinal barrier dysfunction associated with arthritis-induced inflammation.

17. Short SS, Wang J, Castle SL, et al (2013) Low doses of Celecoxib attenuate gut barrier failure during experimental peritonitis. Lab Investig 93:1265–1275. doi:

10.1038/labinvest.2013.119

18. Chong BE, Wall DB, Lubman DM, Flynn SJ (1997) Rapid profiling ofE. coli proteins up to 500 kDa from whole cell lysates using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Rapid Commun Mass Spectrom 11:1900–1908. doi: 10.1002/(SICI)1097-0231(199711)11:17<1900::AID-RCM95>3.0.CO;2-K
19. Terato K, Harper DS, Griffiths MM, et al (1995) Collagen-induced arthritis in mice: synergistic effect of E. coli lipopolysaccharide bypasses epitope specificity in the induction of arthritis with monoclonal antibodies to type II collagen. Autoimmunity 22:137–47.
20. Butz DE, Li G, Huebner SM, Cook ME (2007) A mechanistic approach to understanding conjugated linoleic acid's role in inflammation using murine models of rheumatoid arthritis. Am J Physiol Regul Integr Comp Physiol 293:R669–R676. doi: 10.1152/ajpregu.00005.2007
21. Huebner SM, Campbell JP, Butz DE, et al (2010) Individual Isomers of Conjugated Linoleic Acid Reduce Inflammation Associated with Established Collagen-Induced Arthritis in DBA/1 Mice. J Nutr 140:1454–1461. doi: 10.3945/jn.109.120527
22. Huebner SM, Olson JM, Campbell JP, et al (2014) Dietary trans-10,cis-12 CLA reduces murine collagen-induced arthritis in a dose-dependent manner. J Nutr 144:177–184. doi: 10.3945/jn.113.182550
23. Huebner SM, Olson JM, Campbell JP, et al (2016) Low Dietary c9t11-Conjugated Linoleic Acid Intake from Dairy Fat or Supplements Reduces Inflammation in Collagen-Induced Arthritis. Lipids 51:807–819. doi: 10.1007/s11745-016-4163-8
24. Olson JM, Haas AW, Lor J, et al (2017) A Comparison of the Anti-Inflammatory Effects

of Cis-9, Trans-11 Conjugated Linoleic Acid to Celecoxib in the Collagen-Induced Arthritis Model. Lipids 52:151–159. doi: 10.1007/s11745-016-4228-8

25. Muhlenbeck JA, Butz DE, Olson JM, et al (2017) Dietary Conjugated Linoleic Acid-c9t11 Prevents Collagen-Induced Arthritis, Whereas Conjugated Linoleic Acid-t10c12 Increases Arthritic Severity. Lipids 1–12. doi: 10.1007/s11745-017-4241-6
26. Basson A, Trotter A, Rodriguez-Palacios A, Cominelli F (2016) Mucosal Interactions between Genetics, Diet, and Microbiome in Inflammatory Bowel Disease. Front Immunol 7:290. doi: 10.3389/fimmu.2016.00290
27. Bassaganya-Riera J, Viladomiu M, Pedragosa M, et al (2012) Probiotic Bacteria Produce Conjugated Linoleic Acid Locally in the Gut That Targets Macrophage PPAR γ to Suppress Colitis. PLoS One 7:e31238. doi: 10.1371/journal.pone.0031238
28. Borniquel S, Jadert C, Lundberg JO (2012) Dietary Conjugated Linoleic Acid Activates PPAR and the Intestinal Trefoil Factor in SW480 Cells and Mice with Dextran Sulfate Sodium-Induced Colitis. J Nutr 142:2135–2140. doi: 10.3945/jn.112.163931
29. Parodi PW (2003) Conjugated Linoleic Acid in Food. In: Sébédio J-L, Christie WW, Adolf R (eds) Adv. Conjug. Linoleic Acid Res. Vol. 2. AOCS Press, Champaign, Illinois, pp 101–122
30. Brand DD, Latham KA, Rosloniec EF (2007) Collagen-induced arthritis. Nat Protoc 2:1269–1275. doi: 10.1038/nprot.2007.173
31. Nordling C, Karlsson-Parra A, Jansson L, et al (1992) Characterization of a spontaneously occurring arthritis in male DBA/1 mice. Arthritis Rheum 35:717–722. doi: 10.1002/art.1780350619
32. Holmdahl R, Jansson L, Andersson M, Jonsson R (1992) Genetic, hormonal and

behavioural influence on spontaneously developing arthritis in normal mice. Clin Exp Immunol 88:467–472. doi: 10.1111/j.1365-2249.1992.tb06473.x

33. Ménard S, Cerf-Bensussan N, Heyman M (2010) Multiple facets of intestinal permeability and epithelial handling of dietary antigens. Mucosal Immunol 3:247–259. doi: 10.1038/mi.2010.5

34. Wang Q, Fang CH, Hasselgren P, et al (2013) Intestinal permeability is reduced and IL-10 levels are increased in septic IL-6 knockout mice Intestinal permeability is reduced and IL-10 levels are increased in septic IL-6 knockout mice. 0558:1013–1023.

35. Lievin-Le Moal V, Servin AL (2006) The Front Line of Enteric Host Defense against Unwelcome Intrusion of Harmful Microorganisms: Mucins, Antimicrobial Peptides, and Microbiota. Clin Microbiol Rev 19:315–337. doi: 10.1128/CMR.19.2.315-337.2006

36. Kim YS, Ho SB (2010) Intestinal goblet cells and mucins in health and disease: recent insights and progress. Curr Gastroenterol Rep 12:319–30. doi: 10.1007/s11894-010-0131-2

37. Man AL, Gicheva N, Nicoletti C (2014) The impact of ageing on the intestinal epithelial barrier and immune system. Cell Immunol 289:112–118. doi: 10.1016/j.cellimm.2014.04.001

38. Barros PAV de, Generoso S de V, Andrade MER, et al (2017) Effect of Conjugated Linoleic Acid-enriched Butter After 24 hours of Intestinal Mucositis Induction. Nutr Cancer 69:168–175. doi: 10.1080/01635581.2016.1225100