

## Oxidation and apoptosis are induced by CLA-t10c12 similar to DHA in 3T3 adipocyte cells.

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### ABSTRACT

**Background:** Commercial conjugated linoleic acid (CLA) dietary supplements contain an equal mixture of the C18:2 isomers, cis-9trans-11 and trans-10cis-12. Predominantly, CLA-c9t11 occurs naturally in meat and dairy products at ~ 0.5% of total fat, whereas CLA-t10c12 occurs at > 0.1%. Recent studies show that CLA-c9t11 generally promotes lipid accumulation but CLA-t10c12 may inhibit lipid accumulation and may also promote inflammation. The omega-3 fatty acids  $\alpha$ -linolenic acid (C18:3n-3) and docosahexaenoic acid (DHA) have also been observed to inhibit lipid accumulation and effect inflammation; therefore we examined the effects of the two main isomers of CLA and omega -3 fatty acids C18:3n-3 and DHA at the molecular level to determine if they are causing similar oxidative stresses.

**Methods:** Purified CLA-c9t11 and CLA-t10c12 were added to 3T3 cells induced into mature adipocyte cultures at 100uM concentrations and compared with 100uM C18:3n-3 ( $\alpha$ -linolenic acid) and 50uM docosahexaenoic acid (DHA) to observe their effect on growth, gene transcription and general oxidation. The results of multiple separate trials were averaged and compared for significance at levels of  $P < 0.05$ , using one way ANOVA and *Student's t-test*.

**Results:** C18:3n-3, DHA and CLA-t10c12 inhibited 3T3 adipose cell growth and caused a significant increase in lipid hydro peroxide activity. CLA-t10c12 and c9t11 increased AFABP, FAS and ACOX1 mRNA gene expression but DHA and C18:3n-3 decreased the same mRNAs. CLA-c9t11 but not the t10c12 stimulated adipoQ expression even though; CLA-c9t11 had only a slightly greater affinity for PPAR $\gamma$  than CLA- t10c12, according to TR-FRET assays. The expression of the xenobiotic metabolism genes, aldo-keto reductase

1c1 (akr1c1), superoxide dismutase (SOD) and inflammation chemokine secretions of eotaxin (CCL11), Rantes (CCL5), MIG (CCL9) and MCP-1 were increased by DHA, C18:3n-3 and CLA-t10c12 but not CLA-c9t11. This correlated with an increase in apoptosis factors, caspase 3, Bcl-2 and BAXs. Apoptosis factors were partially reduced by co-treatment with lipophilic anti-oxidant  $\alpha$ -tocopherol.

**Conclusions:** CLA-t10c12 stimulated the production of more reactive oxygen species (ROS) than CLA-c9t11. In response, cascades of genes are activated in detoxification, inflammation or apoptosis, to deal with the potentially damaging effects of ROS including CCL5 and MCP-1. This was similar to treatment with the omega-3 fatty acids but the fat metabolic enzymes were generally inhibited by C18:3n-3 and DHA except CLA-t10c12 which did not stimulate adipoQ.

**Keywords:** CLA-t10c12, CLA-c9t11, gene expression, adipocyte lipid hydroperoxide, DHA, 3T3 adipocytes, apoptosis.

## INTRODUCTION

Conjugated linoleic acids (CLA) are a group of over 28 naturally occurring C18:2 linoleic acid isomers, containing a conjugated, instead of a methylene, interrupted double bond system. They are found naturally in meat and dairy products derived mainly from plant oil converted by ruminant bacteria [1]. The main natural isomer of CLA is *c9t11-18:2* (rumenic acid), accounting for over 75% of total CLAs in dairy or meat products and make up almost 0.5% of total fat in milk [2]. The next most common isomer in milk is CLA-t10c12 but levels typically only reach ~0.1% in milk (~10 mg/kg of fat). CLA-t10c12 can be increased to 60 mg/100g of fat in dairy cattle by feeding a high concentrate low forage diet, which also induces milk depression [3]. The principle dietary source of CLA-t10c12 is from commercial preparations of CLA that are chemically synthesized supplements containing equal mixtures of CLA-c9t11 and -t10,c12 isomers and are marketed (Trofocell® Hamburg, Germany)(Tonalin®, Hovdebygda, Norway) with the added health benefits of causing fat oxidation [4]. Feeding a synthetic mixture of 1:1, CLA-c9t11 to CLA-t10,c12 in swine has been shown to reduce fat accumulation and increase lean muscle mass [5, 6]. Evidence now shows that the two isomers of CLA, -c9t11 and -t10c12, may have different cellular and metabolic effects.

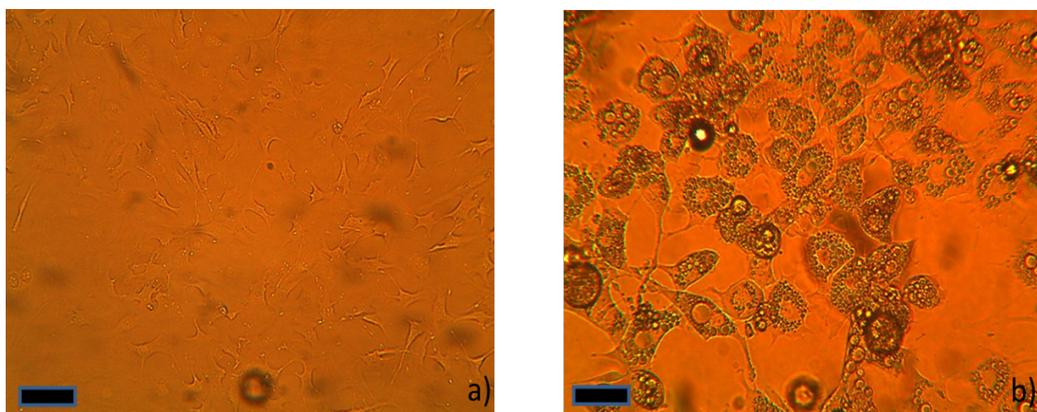
The effects of dietary CLAs were thought to act mainly through the peroxisome proliferator activated receptor (PPAR) family of transcription factors. Biological activity of CLA-c9t11 is associated with reducing fat, improving insulin sensitivity and possible have anti-carcinogenic effects [7]. CLA-t10c12 treatment is associated with substantial lipid oxidation and peroxidation which may induce the inflammatory NF- $\kappa$ B and mitogen-activated protein kinase (MAPK) signaling factors [8, 9]. In mouse liver [10] and human adipocytes, CLA-c9t11 binds with peroxisome proliferator activated receptor (PPAR) which increases fat metabolic enzymes including, acetyl CoA carboxylase (ACC), fatty acid synthase (FAS) and stearoyl coA delta-9 desaturase (SCD1). CLA-t10c12 is also thought to bind with PPAR but

prolonged treatment reduces fat metabolic enzymes and increased inflammation markers, tumour necrosis factor alpha (TNF $\alpha$ ) and cyclooxygenase-2 gene (COX-2) [11]. This divergent effect of the two major CLA isomers could be due to effects of their actions as ligands for the PPAR nuclear receptors or to their respective metabolic products acting downstream to influence expression.

Based on this evidence, CLA-t10c12 may promote more reactive oxygen species (ROS) than CLA-c9t11. In response to ROS, a cascade of genes may be activated to either deal with detoxification to prevent potentially damaging effects of ROS or to call out for assistance by the inflammasome [12]. Too much inflammation will also result in damage, leading to apoptosis activation, necrosis and cell death [13]. Similar mechanism dealing with oxidative stress has been speculated to occur with the omega-3 fatty acid, C18:3n-3 ( $\alpha$ -linolenic acid) and to a much greater effect by the long chain omega-3 fatty acids C20:5n-3 eicosapentaenoic acid (EPA) and C22:6n-3 docosahexaenoic acid (DHA) [14, 15]. The oxidative effects of DHA are partially attenuated by co-treatment with the anti-oxidative vitamin E ( $\alpha$ -tocopherol) [16]. The objective of the present study was to investigate the high dose effects of CLA-c9t11 and CLA-t10c12 and compare them with omega-3 fatty acids, C18:3(n-3), DHA and DHA with vitamin E treatments, on the stress response in 3T3 mature adipocytes.

## MATERIALS and METHODS

*Cell treatment in culture.* Mouse derived 3T3-L1 cells (ATCC CR-173, Manassas, VA, USA) were used to characterise the effects of the free fatty acids. The 3T3-L1 cells in their pre-adipocyte stage were grown in Dulbecco modified eagle's media (DMEM) + 10% fetal calf serum (FCS) + 10 U/ml penicillin/streptomycin (ThermoFisher Life Technologies, Burlington, ON, Canada). Induction of 3T3 cells to mature adipocytes began after the cultures reached >90% confluence. 3T3 cells were induced into terminally differentiated mature adipocyte by treatment with 10  $\mu$ g/ml insulin + 0.1  $\mu$ M 3-isobutyl-1-methylxanthine (IBMX) and 0.5 mM dexamethasone in DMEM (Life technologies, Burlington, ON Canada) + 10% fetal bovine serum (fbs) for 48h and then prolonged incubation in by DMEM + 10% fbs + 10  $\mu$ g/ml insulin (cat# I9278, Sigma-Aldrich Canada, Oakville, ON, Canada) . Mature adipocyte cultures ( $n= 6$ ) were visually assessed by the appearance of lipid droplets in the cells which became apparent under 10X magnification after 4 to 7 d in culture. [Figure 1] Treatments of the 3T3 mature adipocytes with the free fatty acids were complexed with 7.5% fatty acid free bovine serum albumin (BSA) at 4mM to insure solubility. All fatty acid solutions were initially solubilized in 100 % dimethyl sulfoxide (DMSO) then diluted to the appropriate concentrations in growth media. CLA-c9t11 (9Z,11E-octadeca-dienoic acid) , CLA-t10c12 (10E,12Z-octadeca-dienoic acid), C18:3(n-3) (all-cis-9,12,15-octadecatrienoic acid), C22:6n-3 (all-cis- 4,7,10,13,16,19 docosa-hexa-enoic acid, DHA) and linoleic acid (18:2, all-cis-9,12-octadecadienoic acid) were purchased from Nu-Chek Prep Inc. (Elysian, MN, USA). DL-all-rac-Alpha-tocopherol acetate was purchased from Sigma cat# T3251 (Sigma Aldrich Canada, Oakville, ON,Canada).



**Figure 1. Phase microscope 10X image of 3T3 cells.** a) pre-adipocyte form and as b) mature adipocytes after 7 days treatment and incubation in culture media DMEM + 10% fbs with 10 ug/ml insulin. Note the appearance of lipid droplets in the mature adipocyte cells. Scale bar equals 100  $\mu$ m.

*Assessment of cell viability with Thiazolyl Blue Tetrazolium Bromide (TBTB/MTT).* Cell viability was assessed using 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay [17]. Essentially, the cells were incubated with 10mg/ml of TBTB/MTT in the growth media for 4hours. The metabolic activity of cells was measured by the conversion of yellow TBTB/MTT into blue MTT-formazan by NADPH dependent mitochondrial dehydrogenase [18]. The adherent cells were rinsed with phosphate buffered salt solution and then colour (purple) was developed with 100% DMSO for 12hr. Absorbance was read at 570nm.

*In vitro competitive binding studies with PPAR $\gamma$ .* Competitive binding assays were performed in vitro using LanthaScreen time-resolved fluorescence resonance energy transfer (TR-FRET) method (Invitrogen, Carlsbad, CA). The fluormone Pan-PPAR green ligand competed with unlabelled CLA-c9t11, CLA-t10,c12, linoleic acid (LA ~18:2-cis9, cis12) and GW1929 (ToCris Biosciences, Bistol,UK), which is a known PPAR $\gamma$  agonist, for the binding site of a recombinant human PPAR $\gamma$  – GST protein which was monitored with a terbium-labelled anti-GST antibody. The half-maximal inhibitory concentration (IC<sub>50</sub>) value of each test compound was assessed using a sigmoidal dose-response equation (GraphPad Prism 5.0, GraphPad Software, La Jolla, CA, USA).

*Gene expression analysis of lipid metabolism, lipid oxidation and apoptosis.* Total RNA was isolated using TRIzol Reagent (Life Science Technologies, ON, Canada) and purified using the Aurum Total RNA kit (Bio-Rad Laboratories, Mississauga, ON, Canada). Total RNA 100 ng/ml was converted to cDNA using MMLV reverse transcriptase (BioBasic Inc, Markham, ON Canada) oligo-dT 12-18 at 500 ug/ml and random hexamers at 125 ug/ml. Gene expression levels of fat metabolic gene included PPAR $\gamma$ , fatty acid binding protein 4 (FABP4 also known as ~AFABP, aP2), fatty acid synthase (FASN), stearoyl CoA desaturase (SCD1), acyl CoA oxidase(Acox1), carnitine palmitoyltransferase 1A (cpt1a), CIDEA, and adipoQ (~adiponectin, Acrp30) transcripts, were compared using qPCR.  $\beta$ -actin, RP2A, HPRT and b-microglobulin were used as the reference genes [38]. Gene expression was

converted from their cycle threshold (Ct) by  $2^{-\Delta\Delta Ct}$  method [19].

Gene expression of fat oxidation biomarkers were initially determined by microarray analysis using expression macroarray panel PAMM-065Z (Qiagen Canada, Mississauga, ON). Total RNA was collected from the CLA-c9t11 and CLA-t10c12 treatments of 100uM for 48h and was converted to cDNA with a RT<sup>2</sup> First Strand Kit (Qiagen). Using the RT<sup>2</sup> SYBR Green polymerase chain reaction (PCR) Master mix according to manufacturer's recommendations on a Mx3000P QPCR real time PCR machine with SYBR Green detection (Agilent Technologies Canada Inc., Mississauga, ON). Quantitative gene analysis was based on comparative  $2^{-\Delta\Delta Ct}$  method [19] using the RT<sup>2</sup> profiler PCR data analysis program 3.5. (SABiosciences/ Qiagen, Mississauga, ON). A list of 5 genes identified by the array as being the significantly induced by CLA-t10c12 relative to the CLA-c9t11 treatment were further tested in an oxidation panel by qPCR along with the other cells treatments as previously noted. The expression of mRNA was apoptosis was also tested on the treated cells by qPCR. Primers (presented in Table #1) were checked for expression according to the minimal information for quantitative real-time PCR experiments (MIQE) guidelines [20]. Normalization of qPCR data [21] was done against the reference genes,  $\beta$ -actin, RP13A and HPRT and samples treatments were adjusted against the C18:2 treatments harvested at the same time.

**Table 1. Gene specific forward and reverse primers sequences of genes used for QPCR.**

Gene <sup>a</sup>	Forward primer 5' → 3'	Reverse Primer 5'→3'	Size (bp)	Source Genbank No.
RP13A	ggctgccgaagatggcggag	gccttcacagcgtacgaccac	130	NM_009438
$\beta$ -actin	cctctatgccaacacagtgc	cctgcttgcgatccacatc	185	NM_007393
HPRT	tgacactggcaaaacaatgca	aggctccttttcaccagcaagct	94	NM_013556
SOD3	atggagctaggacgacgaag	ctcaggtccccgaactcatg	229	NM_011435
AKR1c1	gcggcaggatctcttcattg	gttccatagccgtccaagtg	232	D32250
CCL5	atatggctcggacaccactc	cttctctctgggtggcac	163	NM_013653
MCP-1	agcaccagccaactctcac	tctggaccattccttcttg	300	NM_011333
CASPASE-3	aaggagcagctttgtgtgtg	ggcaggcctgaatgatgaag	160	NM_001284409
Bax	agatcatgaagacaggggccc	gtccacgtcagcaatcatcc	199	NM_007527
Bcl-2	aactcttcagggatggggtg	atcaaacagaggtcgcgatgc	226	NM_009741
PPAR $\gamma$	ctgtggggataaagcatcag	tgatggcattgtgagacatc	202	XM_006505737.1
CPT1	tcgacaggtggtttgacaag	gaatgtcccactgtagcctg	221	NM_013495
AdipoQ	gctcctgctttggtcctccac	gcccttcagctcctgtcattcc	177	NM_009605.4
FABP4	tttctcacctggaagacagc	cttcaccttctgtcgtctg	287	NM_024406
ACOX1	gataaactcccaagattcaa	aagtcaaaggcatccacaaa	214	NM015729.2
FASN	acacatcctagcatccgag	tttctgaaagtttccgcagc	173	NM_007988.3
CIDEA	tgctcttctgta tcgccagct	gccgtgttaaggaatctgctg	112	NM007702.2

<sup>a</sup>RP13A ribosomal protein large 13A,  $\beta$ -actin beta-actin, HPRT hypoxanthine

ribosyltransferase, SOD3 superoxide dismutase 3, ARK1C1 aldo keto reductase isoform 1c1, CCL5 chemokine 5, MCP-1 monocyte chemoattractin protein 1, caspase-3 cysteine- aspartic protease 3, BAX apoptosis regulator protein, Bcl-2 b-cell lymphoma 2 apoptosis regulator, PPAR $\gamma$  peroxisome proliferator activated receptor gamma, CPT1 carnitine palmitoyl transfer protein 1, AdipoQ adiponectin, FABP4 fatty acid binding protein 4, ACOX1 peroxisomal acyl-coenzyme A oxidase 1, FASN fatty acid synthase, CIDEA cell death activator. *Expression of secreted chemokines.* Cell culture media was adjusted to DMEM with reduced fetal bovine serum at 0.5% on mature adipocytes for 24h or before starting fatty acid treatments to aid in the detection of cytokines. Complete removal of serum severely compromised the 3T3 mature adipocytes survival. Treatments were started in the BSA supplemented media and the cells or cell media was collected in 100ul aliquots and kept at -80C until analysis. Detection of the chemokines was performed by Eve Technologies Inc. (Calgary, AB, Canada). The bead analyzer (Bio-Plex 200) includes a dual-laser system and a flow-cytometry system. One laser activates the fluorescent dye within the beads which identifies the specific analyte. The second laser excites the fluorescent conjugate (streptavidin-phycoerythrin) that has been bound to the beads during the assay. The amount of the conjugate detected by the analyzer is in direct proportion to the amount of the target analyte. The proteins were quantified using a standard curve. A Bio-Plex 200 machine (Eve Technologies) was used to identify fluorophore signatures for each target cytokine in a Mouse Cytokine Array / Chemokine Array 31-Plex Cat #: MD31 and adiponectin. Targets in the array included :CCL-5 (RANTES), CCL-11, CxCl-1 (KC), MCP-1 CCL-11 (Eotaxin), G-CSF, GM-CSF, IFN gamma, IL-1alpha, IL-1beta, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-17A, IP-10, KC, LIF, LIX, MCP-1, M-CSF, MIG, MIP-1alpha, MIP-1beta, MIP-2, RANTES, TNFalpha, VEGF. Protein concentrations were recorded for the serum free media using the bicinchoninic acid (BCA) assay kit (Pierce, Life Technologies Inc., Burlington, ON, Canada).

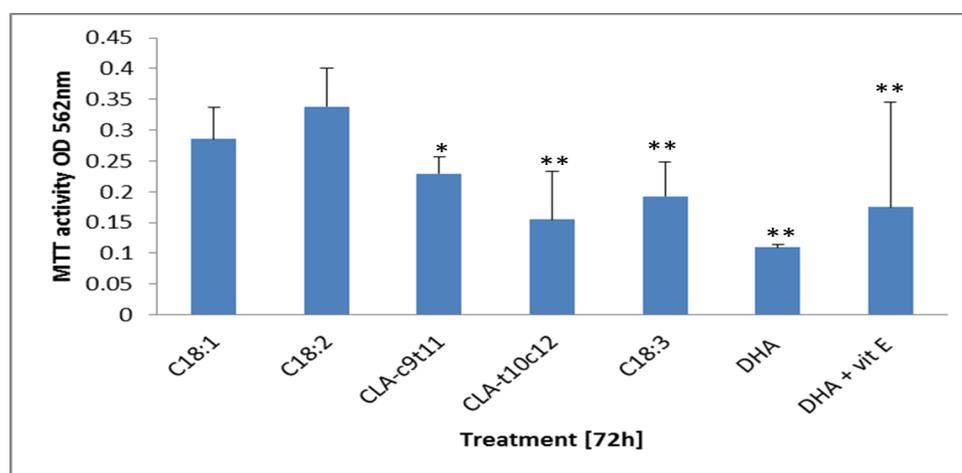
*Lipid Hydroperoxide Assay.* LPO assay kit was obtained from Cayman Chemicals (Ann Arbor , MI, USA). 3T3 mature adipocytes samples were treated in 9 cm<sup>2</sup> cultures for 24h, 48 h and 72h and were harvested in protein assay RIPA buffer [25mM Tris-HCl pH 7.6, 150mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS] and homogenized on ice. The samples [500ul] were then extracted with 500ul methanol and 1ml of chloroform. The methanol/chloroform extract [450ul] was then mixed with 0.25uM ferrous sulfate, 10mM HCl and 0.15% NH<sub>4</sub>SCN and read at 500nm to detect iron thiocyanate. The absorbance was compared using a 13-HpODE (13-hydroperoxy octadecadanoic acid) standard curve.

*Statistical analysis.* The competitive binding TR-FRET PPAR $\gamma$  assay was analysed using non-linear regression and graphed using the Prism Graph Pad software. The assay was performed over a concentration range of 0.001 uM to 100 uM (0.28 ng/ml to 0.028 mg/ml) with at least 4 replicates. The means on standard deviations on the MTT ad lipid hydroperoxide assays were compared using Student t-test. The LPO assays were measured using a standard curve based on 13-hydroperoxy octadecadienoic acid. The gene expression measures were compared by analysis of treatment means after adjusting expression levels relative to C18:2 values. Means were compared between groups according to ANOVA and

then t-test and Tukey's test using Graph Pad Prism software (La Jolla, CA, USA).

## RESULTS.

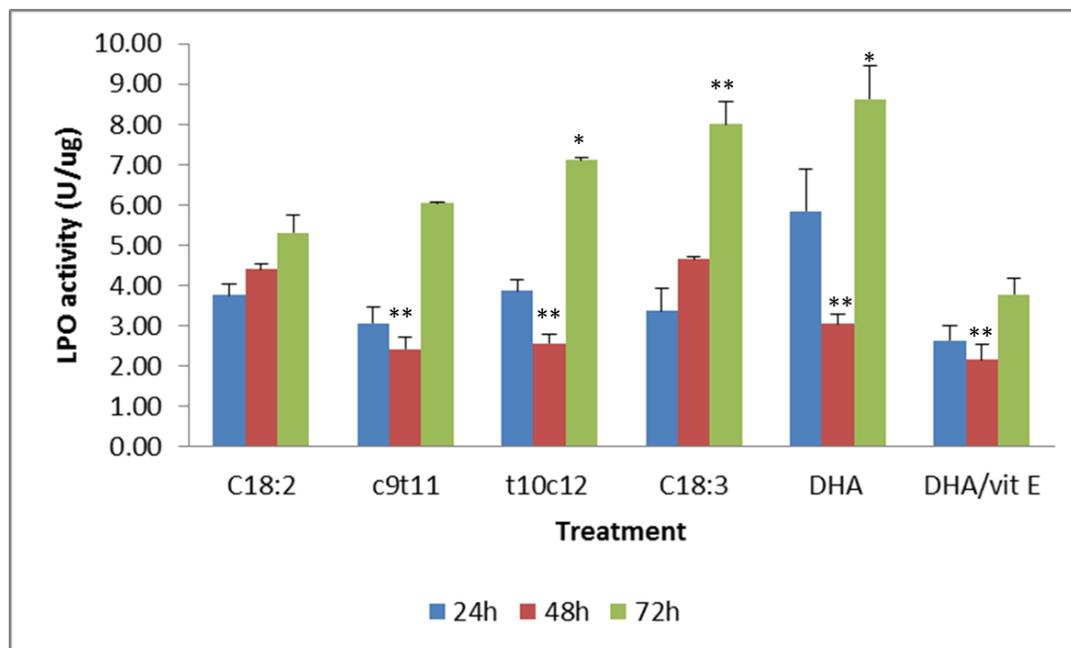
*Cell Viability as determined by MTT assay.* Treatment of the 3T3 mature adipocytes showed a significant inhibitory effect when exposed to 100  $\mu$ M doses of 18:3n-3, CLA-t10c12. [Figure 2] There was too much inhibition of DHA treated cultures at 100 $\mu$ M and it was reduced to 50  $\mu$ M, which still showed a significant degree of 66% inhibition after 72h. The inhibition of cell activity by 50  $\mu$ M DHA was reduced by co-culturing with 100  $\mu$ M alpha-tocopherol to only 51% of the C18:2 activities but not completely to control levels. The CLA-c9t11 treated cultures appeared to experience some inhibition after 72h but this was not significant. In preparation for this experiment, the mature adipocytes experienced too much inhibition according to the MTT activity assay at 250 $\mu$ M treatment after 24h to be a useful model of investigation [data not shown].



**Figure 2. Measure of survival of 3T3 mature adipocytes by MTT assay.** The free fatty acids were loaded at 100  $\mu$ M into the media and incubated for 72h, except 50  $\mu$ M DHA and  $\alpha$ -tocopherol (vit E). Survival of cellular activity is represented by the conversion of MTT into blue formazan to give OD reading of 562nm. Data represent the average from 5 replicates of cell cultures treated. Standard deviations are given as error bars. Significance difference from the C18:2 treated cells is show as (\*\* P < 0.01, \*P < 0.05, # P < 0.1).

*Lipid hydroperoxide activity.* The measurement of lipid peroxides in the treated culture was used to indirectly assess the amount of oxidative stress the cells were undergoing at 24h 48h, and 72h treatment. [Figure 3] The assay directly measured the amount of hydroperoxides which are able to react with ferrous ions to produce ferric ions which are then detected by thiocyanate which acts as a chromophore. The results of the assays, adjusted according the protein in the sample, showed that the culture treatments were not significant at 24h and were generally unchanged by 48h. On day 3 (72h) treatment effect of the free fatty acid were quite pronounced with the C18:3n-3 at 100  $\mu$ M and DHA at 50  $\mu$ M treated culture experiencing the most hydroperoxides in culture. The CLA-t10c12 also caused a significantly increased by day 3 and this was close to the amount in the C18:3n-3 treated cells. CLA-c9t11 also had a

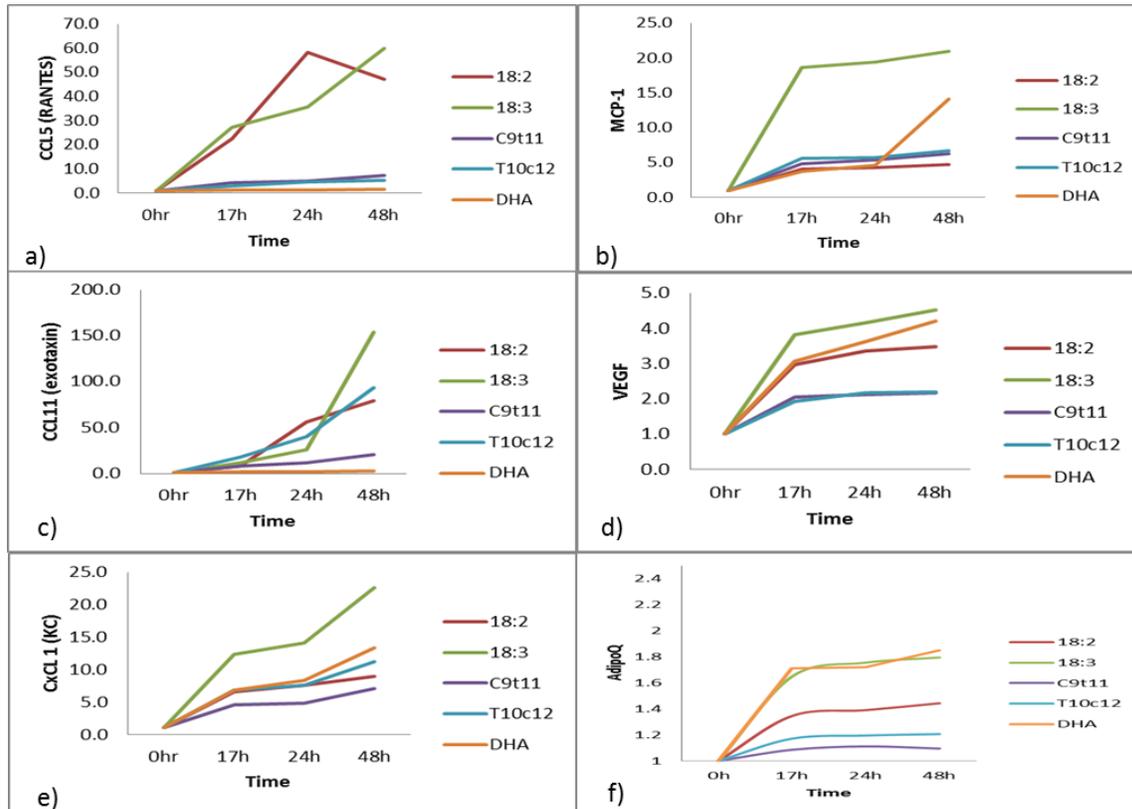
showed an increase in the lipid hydroperoxides in day 2 but by day 3 but this was not significant relative to C18:2. The co-treatment of DHA with the vitamin E showed that the peroxides were not being generated at the same levels in the 24h and 48h treatment but this protection was becoming less evident after 72h co-treatment with DHA and vitamin E.



**Figure 3. Lipid hydroperoxide activity in mature 3T3 adipocytes treated for 24h, 48h, 72h with free fatty acids.** Lipid hydroperoxides were measured by the reduction of ferrous sulfate ions which react with ammonium thiocyanate in chloroform and methanol. Data shown is the cells treated for up to 72h with 100  $\mu$ M of fatty acids except the 50  $\mu$ M DHA and DHA + vitamin E. Bars represent treatment mean  $\pm$  SE. Significantly different from the C18:2 at equivalent time point. \*\*  $P < 0.01$ , \*  $P < 0.05$ .

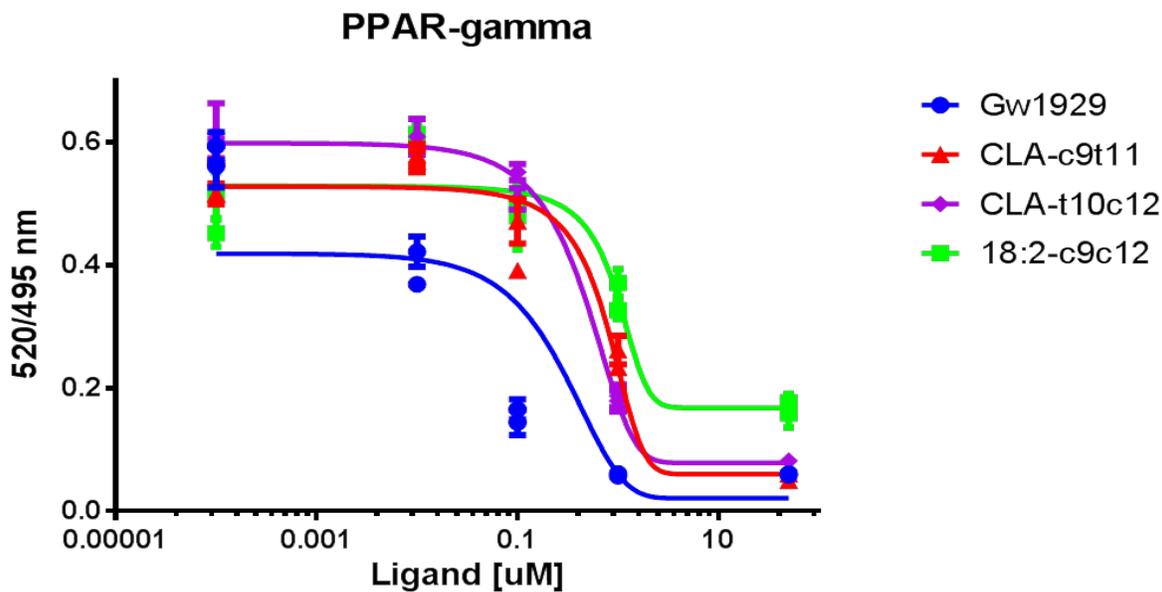
*Expression of secreted chemokines.* The 31 secreted chemokine array panel showed an increase of RANTES (CCL5) up 60X, MCP-1 up 20X, Exotaxin (CCL11) up 150X, KC (CxCL1) up 20X, VEGF up 4X, and AdipoQ up 1.8X, after the 48h treatment with C18:3(n-3), relative to time 0 [Figure 4]. DHA treatment stimulated the VEGF up 4X, CxC1 up 15X and AdipoQ up 1.8X after 48h relative to time 0. The CCL5, CCL11, CxC11, MCP-1, VEGF and CCL-5 also known as RANTES are chemokine attractant normally expressed in T-cells and inflammation. It has been reported that these cytokines are activated by long term exposure of 3T3 mature adipocytes to CLA t10c12 [40] but relative to the C18:2 treatment, only the CxC1 and CC11 secreted protein were stimulated after 48h [figure 4c,4e]. MCP1 (monocyte chemo-attractant protein 1) was stimulated by C18:3n-3 and DHA relative to C18:2 treatments. CCL11 also known as eosinophil chemotactic protein and CxC11 (chemokine (C-X-C motif) ligand 1) are associated with inflammation and their expression were stimulated significantly ( $p < 0.05$ ) C18:3(n-3) and CLA-t10c12 insignificantly ( $p < 0.1$ ) in the 48h cultures. The c9t11 treated cultures were not stimulated to secrete cytokines

relative to the C18:2 treated cells. Vascular endothelia growth factor (VEGF) is secreted as a chemoattractant and is also expressed in hypoxic conditions and was particularly stimulated by C18:3n-3 and DHA treatment. Secreted Adiponectin protein did show an increase in the DHA and C18:3(n-3) treated 3T3 culture media and was significantly stimulated ( $p < 0.05$ ) according to the qPCR results [figure 6]. The CLA-c9t11 showed no increase in AdipoQ protein secretion into the media but the qPCR results suggested that the AdipoQ transcript was stimulated by CLA-c9t11 treatment [figure 6].



**Figure 4. Cytokines secreted into the media of 3T3 mature adipocytes.** The increase of the cytokines ) CCL5 (RANTES), b) MCP-1, c) CCL11, d) VEGF, e)CxC11 (KC), f) AdipoQ proteins were measured for 48h in the presence of 100uM C18:2, C18:3, CLA–c9t11, CLA-t10c12, or 50uM DHA, in DMEM media with reduced serum at 0.5%. The data represent the fold increase in the level of the cytokine divided by the estimated level at t=0.

*TR-FRET Assay of PPAR $\gamma$  binding.* According to the TR-FRET PPAR-gamma receptor competitive binding assay, the CLA-c9t11 bound at 5.4 uM which was less than either the CLA-t10,c12 at 1.3 uM or the linoleic acid (18:2-c9,c12) with an IC50 of 1.2 uM. This was in comparison to the strong synthetic PPAR-gamma agonist GW1929, which bound at IC50 of 0.9 uM. [Figure 5] These values are in agreement with other studies who have looked at the free fatty acids binding PPAR $\gamma$  independently but the ability of the PPAR to form complexes with other transcription factors such as retinoic acid receptor (RXR) complicates the in vitro study of ligands such as DHA (Gani and Sylte 2008).

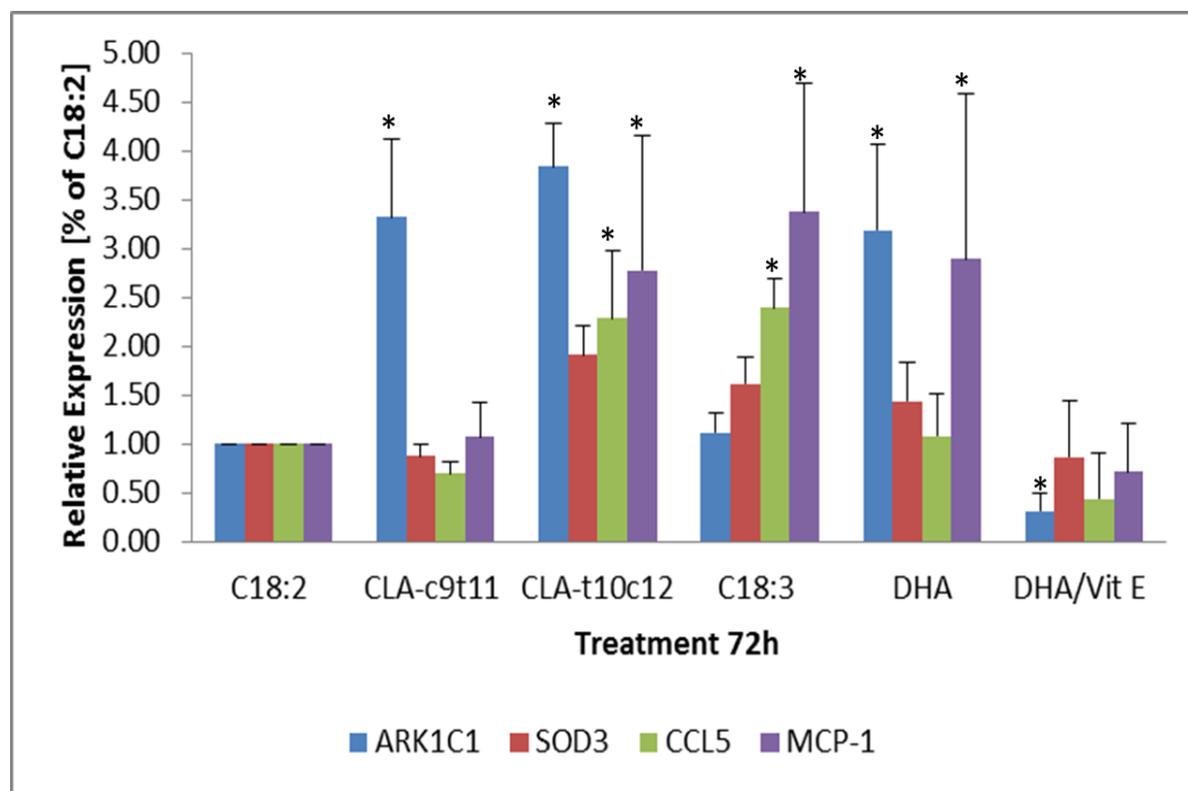


**Figure 5. Binding of CLA-t10c12 and CLA-c9t11 to PPAR $\gamma$ .** The binding of CLA were measured in a TR-FRET assay to recombinant PPAR $\gamma$ . The control substance GW1929 was bound to the PPAR $\gamma$  at competitive IC<sub>50</sub> of 0.9 uM.

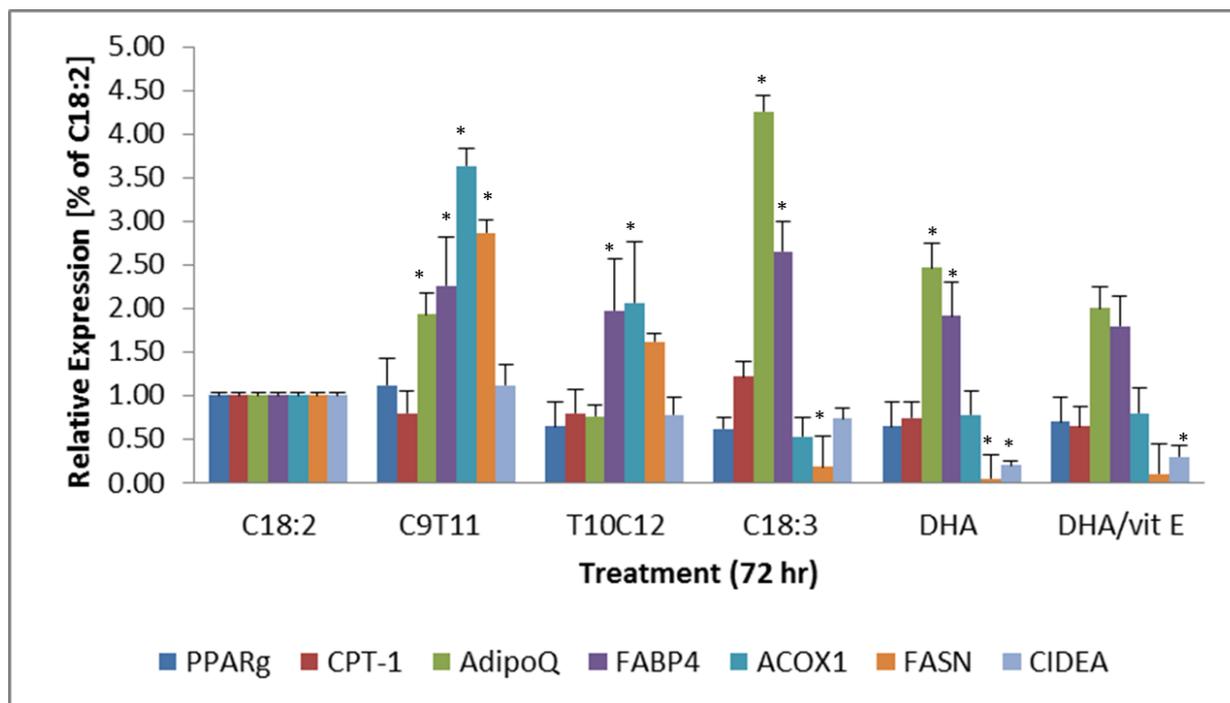
*Expression of lipid metabolic markers.* The expression of the fat metabolism genes, ACOX1, FASN, and FABP4 were generally upregulated in the c9t12 treated cells from the C18:2 treated cells and the trend was also seen in the t10c12 treated cells but the amount was distinctly less  $P < 0.05$ . [Figure 6] The expression of FASN and ACOX1 was significantly inhibited by C18:3n-3 and DHA treatment but the AdipoQ and FABP4 transcripts were stimulated relative the C18:2. In the DHA and vitamin E treated samples, the AdipoQ, and FABP4 expression was also stimulated but the FASN was inhibited. AdipoQ(aka. adiponectin, Acrp30) is secreted in the blood by adipose tissue and is generally associated an increased sensitivity to insulin and promotes beta-oxidation of fats. AdipoQ is also associated increased by dietary EPA and DHA [22]. The transcripts of PPAR $\gamma$ , CPT-1 and CIDEA a biomarker of lipid droplet size, were all not significantly increased by the high dose treatments but the C18:3n-3 and DHA suppressed expression of FASN and CIDEA indicating a reduction in new fatty acid accumulation. Vitamin E appeared to alleviate some of the negative effects of DHA treatment on FASN expression but the CIDEA expression was still inhibited. The most distinguishing factor is that AdipoQ is stimulated by omega-3 fatty acids, C18:3 n-3 and DHA and not by CLA-t10c12, which agrees with other studies [23].

*Expression of lipid oxidative factors.* The activity observed in the lipid hydroperoxide activity assay prompted a more in-depth investigation into oxidative enzymes biomarkers. The initial screening of the oxidative biomarkers was first attempted with Qiagen oxidative macroarray PAMM-065Z comparing CLA-c9t11 against CLA-t10c12 72h treated cells. A panel of 90 genes were investigated and suggested that some were differentially expressed

more strongly in the CLA-t10c12 treated cells. These were the solute carrier protein 38C (SLC38c), dual oxidase (Duox1), cytoglobulin (cygb), superoxidase 3 (SOD3), glutathione peroxidase 3 (Gpx3), and chemokine 5 (CCL5) also known as RANTES [data not shown]. A more detailed investigation into the expression of these genes and also keto reductase 1c1 (ark1c1), which had been noted [33] to be stimulated by lipid oxidation, were performed by qPCR. The expression of ark1c1 was significantly upregulated by both CLA c9t11 and t10c12 treatment after 72h. [Figure 7] CLA-t10c12 also stimulated the expression of CCL5 and SOD3 genes in the 72h treated cells. DHA and C18:3 (n-3) treatments showed a trend ( $P < 0.05$ ) towards stimulation of CCL5 and SOD3 after 72h. This was reduced by the co-treatment of DHA with 100uM alpha-tocopherol (vitamin E) which also reduced the expression of ark1c1, especially in comparison with DHA. MCP-1 was unique as being significantly up regulated by C18:3, DHA and CLA-t10c12 but not by CLA-c9t11. MCP-1 protein secretion into the media was only observed in the omega-3 treated cultures

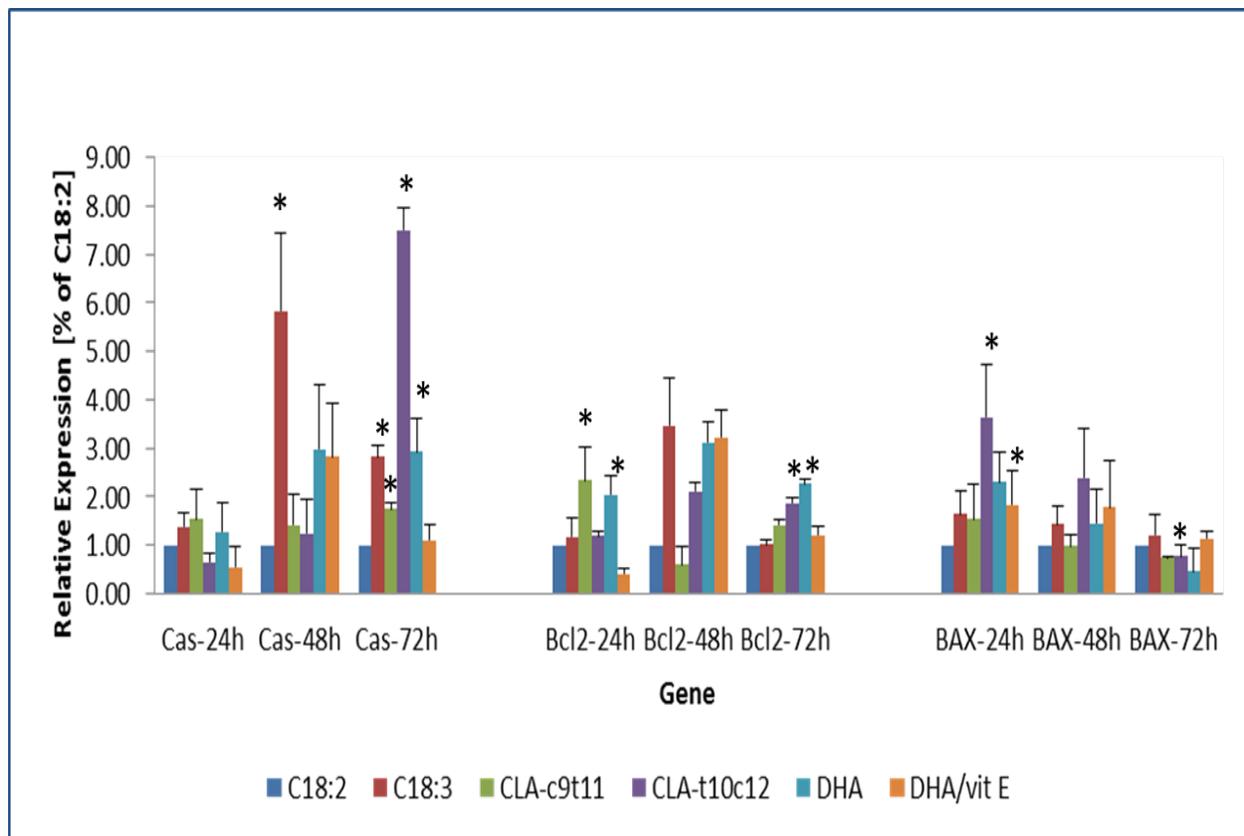


**Figure 6. The expression of lipid metabolism are differentially expressed between CLA and omega-3 fatty acid treated 3T3 mature adipocytes.** The cells were treated for 72h with the fatty acids at 100uM except for the DHA which was treated at 50uM. The transcripts were analyzed via qPCR. Means +/- standard error (n= 5) were compared with C18:2 expressions. Significantly different from the control C18:2 are represented by \*  $P < 0.05$ .



**Figure 7. The expression of lipid oxidation genes in response to omega-3 or CLA fatty acid treated 3T3 mature adipocytes.** 3T3 mature adipocytes were treated for 72 h and the transcripts of the gene *ark1c1*, *SOD3*, *CCL5* and *MCP-1* were evaluated. The transcripts were analyzed via qPCR. Means  $\pm$  standard error (n= 5) were normalized against with C18:2 expressions. Significantly different from C18:2 control are represented by \*  $P < 0.05$

*Expression of apoptosis biomarkers.* The expression of genes involved in oxidative detoxification and xenobiotics and the accumulation of lipid hydroperoxides suggested that the cells were being stressed and that the environment was inducing lipid toxicity. According to the MTT assays, DHA was tolerated at 50uM in the mature adipocytes and the C18:3(n-3) and CLA-t10c12 at 100uM but still the cultures were showing considerable inhibition, when compared to CLA-c9t11. The expression of caspase 3, Bcl2, and BAX were selected as indicators of cell apoptosis but not senescence. Apoptosis is difficult to properly assess in the fibroblast 3T3 cell line but after they have undergone terminal differentiation into a mature adipocyte phenotype, the signalling cascade becomes more repeatable. [Figure 8] Apoptosis signals generated from the qPCR measurements were still highly variable. All three of the apoptosis biomarkers, caspase 3, Bcl2, and BAX were up significantly in the culture treated with 50uM DHA at 24h but after 72h, the BAX signal showed a significant decline relative to the C18:2. C18:3(n-3) showed an 3x- 6x ( $P < 0.05$ ) increase in caspase 3 by 48h and 72h treatment and a 4x increase in the 48h Bcl2 signal. CLA-t10c12 treatment significantly increased the caspase 3 in the 72h treatment, the BAX signal in the 24h and 48h samples and just the 48h Bcl2 signal. The CLA-c9t11 only increased the 24h BCL-2 expression signal slightly and not the caspase 3 or BAX signals.



**Figure 8. The expression of apoptosis biomarker genes in response to omega-3 or CLA fatty acid treated 3T3 mature adipocytes.** 3T3 mature adipocytes were treated for 72 h and the transcripts of the genes caspase 3, Bcl-2 and BAX were evaluated. The transcripts were analyzed via qPCR. Means +/- standard error (n= 3) were normalized against with C18:2 expressions. Significantly different from the control C18:2 are represented by, \*\* P < 0.01, \* P < 0.05.

## DISCUSSION

The subtle differences between CLA-c9t11 and CLA-t10c12 were compared to omega 3 fatty acids C18:3 and DHA to examine if they caused similar stress mechanism in mature 3T3 adipocytes. The results suggest that the CLA-t10c12 caused extra lipid hydroperoxide damage compared to CLA-c9t11, which activated phase 2 detoxification genes. If the cells were exposed for 72h, apoptosis genes were activated in CLA-t10c12, which mirrored the 50uM (~ 164 ng/ml). DHA treatments. Some of the toxicity of DHA could be alleviated by the addition of 100uM alpha-tocopherol (vitamin E) but not enough, as the cells still expressed apoptosis markers at a reduced level after 72h. Changes in lipid metabolism and lipid oxidation and were all observed in the CLA-c9t11 and CLA-t10c12 at 100uM (~ 280ng/ml) but only the CLA-t10c12 was consistent for expression of apoptosis biomarkers after 72h.

CLA-c9t11 occurs much more commonly than CLA-t10c12 in natural system. In typical beef fat, the concentration of CLA-t10c12 is between 0.05% to 0.12 % of total fat, whereas the more common CLA-c9t11 is typically around 0.10% to 0.5% of total fat [24]. In the

milk, the CLA-t10c12 level is usually around 20 mg /100g of milk fat [1] but in extreme cases, CLA-t10c12 concentrations can reach 60 mg/100g of milk fat, which is caused by a high concentrate/low forage diet leading to milk fat depression [3]. In normal milk the concentration of CLA-c9c11 is usually 400 mg to 1070 mg /100g of milk fat. Even if the two isomers of CLA are supplied in equal concentrations, the CLA-c9t11 is preferred. Humans supplemented with 500 mg per day of equal amounts CLA-c9t11 and CLA-t10c12 for 6 months, had blood CLA-c9t11 levels reaching approximately 0.2 % of plasma fat (equivalent to a maximum of ~ 6.5 ug/ml of plasma) but CLA-t10c12 only reached 0.03%. (~0.1 ug/mL of plasma ~0.36 uM) [25].

Lipid hydroperoxides are generated when the double bonds in PUFAs react to oxidation, mainly through reactive oxidation species (ROS) but also by enzyme processes such as P450 mediated xenobiotic metabolism. In systems that are experiencing a lot of oxidation or the concentration of poly unsaturated fatty acids (PUFA) are too high, cellular activity suffers and the newly generated lipid peroxides will react with other targets. The addition of 50 uM DHA to cells generated lipid peroxide levels of 8.4 +/-1.7 unit in the cells after 72h exposure. This was expected since there are 6 double bonds in DHA (all-cis-docosa-4,7,10,13,16,19-hexaenoic acid). CLA-t10c12 was the next highest producer of lipid peroxides at 7.1 +/- 0.11 after 72h, followed closely by the C18:3 and then by the CLA-c9t11 at 6.7+/-2.22 and 6.0+/-0.3 after 72h. CLA-t10c12 has only 2 double bonds but there are other targets such as mitochondrial disruption, DNA adducts and oxidized protein that can act as ROS and these may have contributed to the overall oxidative effect [26]. The effect is pronounced however, as shown by the MTT assays of the mature adipocytes which show the loss of cytosol NAD(P)H-dependent oxidoreductase enzymes activity. MTT results generally correlated with the lipid hydroperoxide assay results, for the selected fatty acid treatments. The DHA at 50uM showed the most inhibition, followed by the CLA-t10c12, the C18:3 and then the CLA-c9t11. The DHA mixed with 100uM alpha-tocopherol was also inhibited relative to the C18:2, which suggest that the cell activity was damaged beyond the ROS interaction with lipids or that a higher concentration of alpha-tocopherol was needed [16]. Brefeldin, a lactone antibiotic which interrupts protein transport, has been shown to block inflammation in 3T3 cultures caused by 50uM CLA-t10c12 [8] and pre-treatment with 300 uM oleic acid (C18:1) has also been shown to partially prevent CLA-t10c12 induced cellular inflammation [11]. It would be interesting to see if Brefeldin and oleic acid could have a similar effect on DHA treatments, although in cattle fed vitamin E with trans fatty acids, it has been difficult to see an effect [27].

The oxidative stress the mature adipocytes were experiencing was also indicated by the expression of chemokines in the cell media. Relative to the control treatment of 100 uM C18:2, the C18:3n-3 appeared to increase the expression of CCL-11, CCL-5 CxCl-1, VEGF, MCP-1 and adipoQ release into the growth media. DHA and the CLA-t10c12 both increased the CCL11 and CxCl1 signal after 48h but only the DHA additionally increased the MCP-1, VEGF and AdipoQ signals. The unique stress of CLA-t10c12 treatment to activate CCL11 and CxCl-1 expression will have to be investigated further; it could be that the 50uM DHA treatment was immediately too lipotoxic for the cells at time 0.

Investigation of cellular apoptosis markers: caspase 3, BAX and Bcl2 showed a similar effect of stress in the DHA, CLA-t10c12 and C18:3(n-3) treated cells. The expression was significantly stimulated for the caspase 3 gene after 48h, and 72h treatment with DHA, CLA-t10c12 and C18:3. This coincided with the increased lipid hydroperoxide production and the reduced cell viability as indicated by the MTT assay. Bcl2 and BAX expression is reported as a ratio, with increased BAX expression being more indicative of apoptosis and Bcl2 indicative of signaling traffic [28]. BAX was up-regulated in the DHA and CLA-t10c12 treatments after 48h but were generally reduced by 72h. Bcl2 was also upregulated in the DHA and C18:3n-3 48h treatments but after 72h of treatment, only the DHA treated cells still showed significant stimulation. The relationship between ROS and apoptosis is commonly found among many system studying the effect of novel drugs including cholesterol statins and omega-3 fatty acids [13]. In these trials, these fatty acids also showed a significant effect on expression but the amount of expression was quite variable, even after 4 separate trials.

Genes which are activated by ROS and other cellular stresses were modified depending on the fatty acid treatment. The CLA-t10c12, CLA-c9t12 and DHA treatment significantly induced the *akr1c1* transcript after 72h. The aldo-keto reductases (*akr1c1*) are a group of enzymes which oxidize/reduce aldehydes or ketones to their secondary alcohols [29]. AKR1C1 is involved in the conversion and detoxification of 4-hydroxy-2-nonenol (HNE), which are the oxidation products of fatty acids [30]. The t10c12 and the omega-3 fatty acids C18:3 and DHA significantly induced the MCP-1 (monocyte chemoattractin protein 1 ~ CCL2) which is involved in signaling the macrophages along with CCL5. CCL5 (chemoattractin 5, RANTES) is also a macrophage attractant and has been shown to be stimulated by CLA-10c12 [23] and we also noted stimulation in the microarray panel and in the cultures treated with C18:3. SOD3 was up regulated but not significantly by t10c12 treatment and was increased in the C18:3(n-3) and DHA treated cells. From the lipid oxidative microarray panel only SOD3 was confirmed to be stimulated by t10c12 over the c9t11, the other oxidation marker targets Duox1, GPX and cog did not show significant stimulation by the treatments by qPCR but these factors warrant further investigation.

Genes involved primarily with lipid metabolism and expression of FABP4 and adipoQ, showed similar effects of significant stimulation by C18:3(n-3) and DHA [31] but only FASN and ACOX1 were significant up regulated by CLA-c9t11 and CLA-t10c12 treatment after 72h. However, CLA-t10c12 did not stimulate adipoQ expression. It is unknown how these same genes respond to apoptosis factors but the evidence suggested that although C18:3 and DHA may be inducing an oxidative stress on the cells; the stress caused by t10c12 is more than just unsaturated bonds and maybe linked to the lack of adipoQ expression in CLA-t10c12 but not CLA-c9t11 [32]. It might be unique to the position of trans bond at position 10 in CLA-t10c12. In contrast, the trans 9 bond in monounsaturated fat C18:1-trans 9, has no effect on FASN and delta-9 desaturation expression [33, 34]. CLA-t10c12 has been noted as causing an increase in oxidative stress in the mitochondria and this coincides with a disruption in mitochondrial transport and increased carnitine palmitoyltransferase (CPT-1) expression [10]. No significant change was observed in CPT1 expression in this trial but the apoptosis markers BAX and Bcl-2 are often associated with changes in the mitochondrial environment

and reflect the oxidative stress.

## CONCLUSIONS

The unique mechanism induced by CLA-t10c12 as compared to CLA-c9t11 appears to be the result of excessive lipid oxidation and ROS. Evidence of this increase in ROS is shown by the increased lipid hydroperoxides, which were also seen in the omega-3 fatty acids treatments of C18:3(n-3) and DHA. However, there are some differences in the cellular response to CLA vs omega-3 DHA, the foremost being the lack of adipoQ expression but an increase in CCL5 and caspase-3 with CLA-t10c12. This difference could be due to the trans-10 bond in the CLA-t10c12 causing it to be poorly absorbed and metabolized [35]. This provides additional warning to food processors on the inadvertent enrichment of extra trans-fat even in the form of CLA-t10c12, in the preparation of milk, butter [36] and meat products.

## Authors Contributions and Acknowledgments.

Dr Meadus designed, generated and interpreted the data presented in the manuscript. Dr Vahmani assisted in the design of experiments and in the qPCR data. Pascale Duff was the main technician responsible for the molecular data. Dr Zantinge assisted in the molecular data generation and sequencing confirmation on the qPCR products. Dr Turner assisted in the preparation of 3T3 cells and the initial preparation of fatty acids. Dr Dugan is the principal investigator for lipid research at Agri-Food & Agriculture Canada in Lacombe.

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## Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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