

**THE MODULATING EFFECT OF CONJUGATED LINOLEIC ACID (CLA) ON  
CANCER CELL SURVIVAL *IN VITRO***

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A thesis submitted in partial fulfilment of the requirements for the degree of Magister  
Scientiae in the Department of Medical Biosciences, University of the Western Cape.



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# **THE MODULATING EFFECT OF CONJUGATED LINOLEIC ACID (CLA) ON CANCER CELL SURVIVAL *IN VITRO***

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## **KEYWORDS**

Conjugated linoleic acid

Cancer

Lipid peroxidation

Cytotoxicity

Apoptosis

Cell proliferation

Cell cycle

Fatty acid incorporation

Cell membrane

Cell biology

HepG2



## ABSTRACT

### THE MODULATING EFFECT OF CONJUGATED LINOLEIC ACID (CLA) ON CANCER CELL SURVIVAL *IN VITRO*

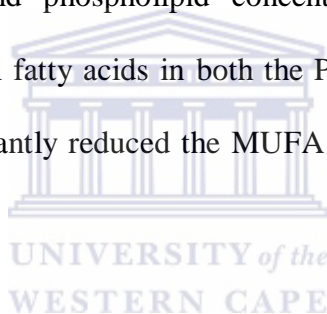
Conjugated linoleic acids (CLA) are geometrical and positional isomers of n-6 octadecadenoic acid (linoleic acid, LA, 18:2n-6), which form part of a family of essential polyunsaturated fatty acids (PUFA). There are 28 identified CLA isomers that mostly found in the meat and milk from ruminant animals. CLA has shown to possess a number of health benefits including; reduction in body fat and increased lean body mass, prevention of atherosclerosis, hypertension, increased immune function and in particular the prevention of cancer. The effects of CLA on cancer cell lines will be evaluated to discover the mechanisms that are employed to achieve this great phenomenon on cell growth.

The aim of this study was to determine the effect of CLA on various parameters that are essential in the development of cancer cell phenotype. The objectives were to evaluate the effect of CLA on iron-induced lipid peroxidation of microsomes isolated from rat liver cells and *in vitro* cytotoxicity, cell proliferation and apoptosis in HepG2 hepatocarcinoma cells. The Fatty acid incorporation in HepG2 cells was also assessed.

Iron-induced lipid peroxidation was measured by the production of malondialdehyde (MDA) as a thiobarbituric acid reactive substance (TBARS), utilising rat liver microsomes. Briefly, microsomes were exposed (30 min) to the following FA's: oleic acid (OA, C18:1n9) and vaccenic acid (VA, C18:1n7), linoleic acid: *c9t11* CLA, *t10c12* CLA,  $\alpha$ -linolenic acid (ALA, C18:3n3), and  $\gamma$ -linolenic acid (GLA, C18:3n6) before lipid peroxidation was measured. Cytotoxicity, cell viability, apoptosis and cell proliferation in HepG2 cells was measured after 48 hour treatment to determine the effect of the CLA isomers, LA and  $\alpha$ -linolenic acid

(ALA) on these parameters. For FA incorporation, cells were supplemented with concentration of FA-BSA complexes for 48 hours. FA were extracted, separated into respective phospholipid fractions (PC and PE) and measured by gas chromatography. Cholesterol and phospholipid concentrations were also quantified by cholesterol enzymes and malachite green methods respectively.

The *t10c12* CLA isomer tended to exhibit weaker protection against lipid peroxidation than the *c9t11* isomer although the difference was not statistically significant. The *c9t11* CLA isomer exhibited greater cytotoxicity and increased apoptosis compared to *t10c12* CLA ( $P < 0.05$ ). Both CLA isomers significantly reduced cell proliferation ( $P < 0.05$ ). CLA did not significantly affect cholesterol and phospholipid concentration. The *c9t11* CLA isomer increased the concentration of all fatty acids in both the PC and PE phospholipid fractions. The *t10c12* CLA isomer significantly reduced the MUFA concentration in both PC and PE fractions.



The *c9t11* CLA isomer markedly increased apoptosis and the overall FA content. MUFA concentrations increased, reducing susceptibility to lipid peroxidation, thereby preventing cellular stress. *c9t11* Increased total FA content of the cell membrane, suggesting increase de novo synthesis of FA required for the formation of new cells. However, it could also be implicated in the formation of apoptotic bodies, which are membranous bodies containing cell fragments that are formed at the end of the apoptosis process. The *t10c12* CLA isomer reduced the activity of delta-9 desaturase due to decline in MUFA composition of the cell membrane. Reduced delta-9 desaturase activity limits FA availability for proliferation of new cells. The anti-cancer mechanisms that are in place await full explanation, therefore further studies in cycle events and gene expression are required to fully uncover the anticancer effect of both *c9t11* and *t10c12* CLA isomers.

**DECLARATION:**

I declare that *The Modulating Effect of Conjugated Linoleic Acid CLA on Cancer Cell Survival in vitro* is my own work, that it has not been submitted for any degree or examination in any other university, and that all the sources I have used or quoted have been indicated and acknowledged by complete references.

Lyle Arendse

November 2014

Signed.....



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## LIST OF COMMON ABBREVIATIONS

CLA	Conjugated linoleic acid
AA	Arachidonic acid
ALA	$\alpha$ -linolenic acid
BrdU	Bromodeoxyuridine
C18	18-carbon fatty acids
<i>c9t11</i>	<i>cis9-trans11</i>
COX	Cyclooxygenase
DHA	DHA docosahexaenoic acid
DPA	Docosapentaenoic acid
EFA	Essential FAs
EPA	Eicosapentaenoic acid
FA	Fatty acid
Fe <sup>2+</sup>	Iron
GLA	$\gamma$ -linolenic acid
LA	Linoleic acid
LT	Leukotrienes
MDA	Malondialdehyde
MUFA	Monounsaturated fatty acids
N3	Omega-3
N6	Omega-6
OA	Oleic acid
PA	Phosphatidic acid
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PG	Prostaglandins
PI	Phosphatidylinositol
PS	Phosphatidylserine
PUFA	Polyunsaturated fatty acids
SATS	Saturated FA
<i>t10c12</i>	<i>trans10-cis12</i>
TBARS	Thiobarbituric acid reactive substance



TVA	Transvaccenic acid
TX	Thromboxanes
VA	Vaccenic acid





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**CHAPTER 1**

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## 1.1. Introduction

Since its discovery, conjugated linoleic acid (CLA) has received a great deal of interest in scientific research as well as the public eye. Limited recent information is available that addresses the cellular effect of CLA in cancer cells. The anticarcinogenic potential of CLA was found by chance when beef hamburger patties reduced tumour growth by more than 50% (Pariza et al., 1979). This sparked the interest in identifying and studying the active element of these beef patties that was responsible for inhibiting tumour cell growth. Later findings showed that the element was in fact a conjugated derivative of linoleic acid (LA) (Nunes et al., 2008). CLA has since been shown to possess activity against the development of many diseases such as atherogenesis, obesity and, for the purpose of this study, most importantly carcinogenesis (Nagao and Yanagita, 2005).

Cancer has for many years been an imperative research focus area. This is due to its ability to develop at any point in the life of an individual. Cancer is described as the uncontrolled growth of cells (WHO, 2009). However, it is now known that many underlying factors exist in the development of tumourigenic cells. These factors have been designated “The Hallmarks of Cancer” (Hanahan and Weinberg, 2011). These characteristics are essential for the development of tumorous cells and are discussed in chapter 2, with emphasis placed on those important in this study. Cancer prevention and therapeutic endeavours have been set out to target at least two cancer hallmarks to be an effective agent. The activity of CLA against the growth of cancer cells has shown to involve apoptosis, cell proliferation, immune response modulation as well as cell cycle events (Crumb, 2011). These studies employed a combination of the two main CLA isomers found, *cis9-trans11* (*c9t11*) CLA and *trans10-cis12* (*t10c12*) CLA. However, the isomers of CLA could possess different mechanisms that



are yet to be defined (Rossi et al., 2012). Therefore CLA remains an effectual treatment possibility due to its easy introduction into the diet of an individual.

The current project evaluated the respective oxidant potential of two CLA isomers, *c9t11* CLA and *t10c12* CLA, in a rat liver microsome modal as well as the modulating effect on HepG2 human hepatocarcinoma cell survival and fatty acid incorporation. These two isomers are found in the greatest proportion of total CLA in meat and milk of ruminant animals. Chapter 2 provides a vital review of literature on cancer, chemotherapy, cell membrane components and important processes involved in tumourigenesis. Also discussed is the history of CLA and current knowledge on its anticarcinogenic properties. Chapter 3 looks at the antioxidant potential of two CLA isomers and compares it to five 18-carbon fatty acids (C18 FA) belonging to monounsaturated and polyunsaturated fatty acid families. Polyunsaturated fatty acids are susceptible to lipid peroxidation, which induces oxidative stress. Cancer cells have high antioxidative nature, therefore increased intracellular oxidative stress can induce apoptosis, a possible anti-tumour mechanism (Verrax et al., 2009). The cytotoxic effect of the two CLA isomers and their effect on apoptosis and cell proliferation are discussed in detail in chapter 4. Chapter 5 presents data regarding the fatty acid incorporation of the two CLA isomers and the modulating effects in the cell membrane fatty acids composition in HepG2 cells. The main findings of the study will be integrated and summarized in Chapter 6, while supportive experimental procedures will be summarized in the Addendum section.

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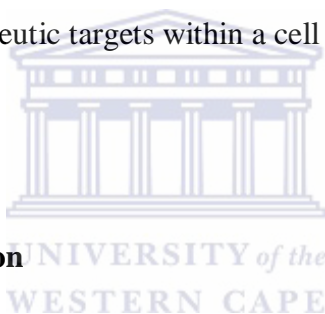
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**CHAPTER 2**

## Literature review

### 2.1. Introduction

It is becoming well recognized that, at least in the long term, future advances in clinical cancer research will come from an emphasis on prevention rather than the treatment of metastatic disease. This endeavor aims to develop compounds that are especially effective against the development and progression of cancer cells and are not harmful or toxic to normal healthy cells. Cancer cells exhibit several hallmarks such as: extensive cell proliferation, hyperactive metabolism, genomic instability and evasion of apoptosis (Table 2.1) (Negrini et al., 2010). These hallmarks provide a dense foundation for cancer research, and have served to identify therapeutic targets within a cell (Hanahan and Weinberg, 2000).



### 2.2. Cancer and chemoprevention

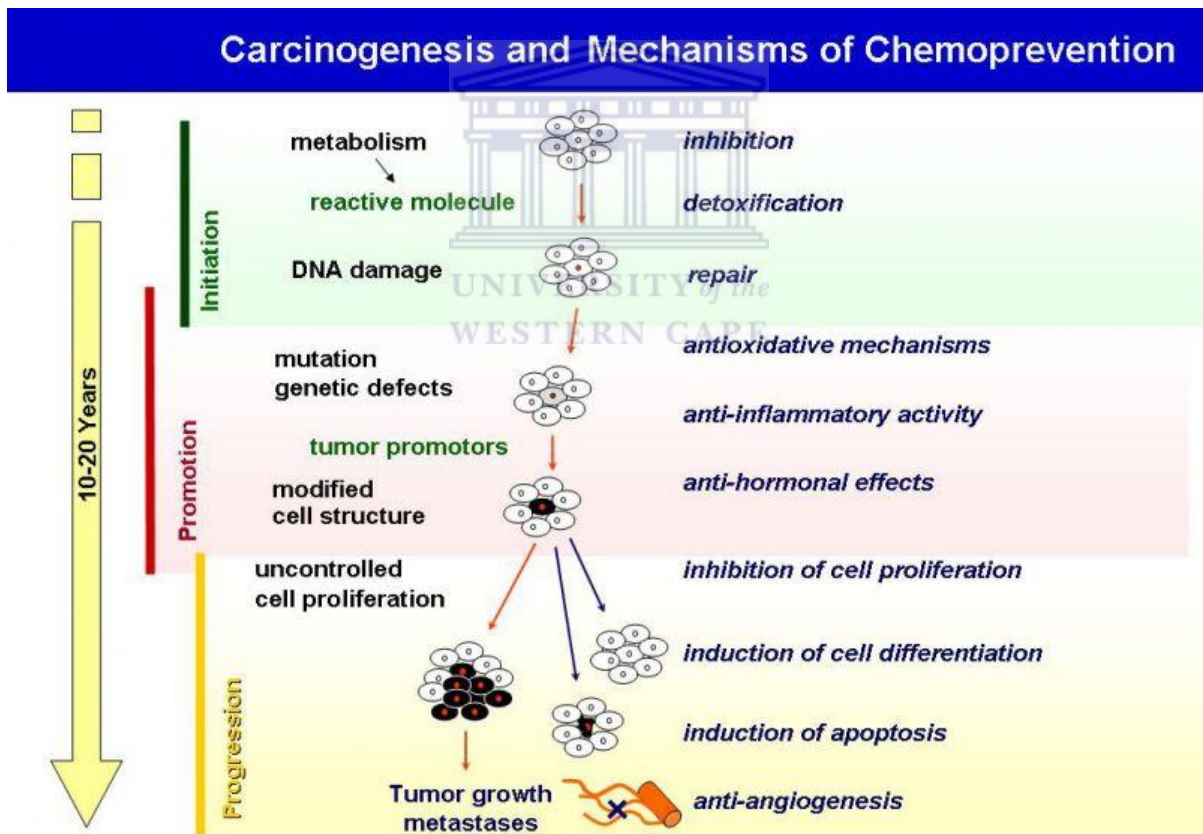
Cancer, tumors or neoplasm's refer to a broad group of diseases, which can affect anyone at any stage in their lives. A tumour begins with genomic instability and uncontrollable, unregulated growth of abnormal cells that may spread and affect other organs. Tumour cells that have spread are metastatic and the leading cause of death worldwide with 7.6 million deaths (around 13% of all deaths from all diseases) in 2008 (Jemal et al., 2011). It is estimated that in 2030 cancer would claim approximately 12 million lives globally (WHO, 2009). Of this number only about 5–10% of all cancer cases have been accredited to hereditary genetic imperfections. The remaining 90–95% of cancer cases was influenced by external risk factors within the environment. These risk factors include; cigarette smoking,

diet, alcohol, sun exposure, environmental pollutants, infections, stress, obesity, and physical inactivity (Anand et al., 2008).

Cancer incidence rises with age, which is an essential factor in tumor development. As the body ages, mechanisms whereby the body repair itself do not operate as efficiently, therefore when a cell is under stress, the DNA repair machinery does not function properly. This will result in mutations in the DNA and the development of a tumor (Gorbunova V. et al., 2007). The type of cancer can be classified by stage, pathology, grade, receptor status, and the presence or absence of genes as determined by DNA testing (Romond E.H., 2005, Sotirou C. and L., 2009). A great deal of research has gone into the development of chemoprevention agents that would actively target and interrupt tumour development *in vivo*; however clinical testing is unethical without sufficient *in vitro* evidence of cellular effects of the compounds.

Cancer is a disease involving dynamic changes in the genomes that result in the abnormal growth of a cancerous lesion. The etiology of cancer encompasses a series of events that may require up to 20 years developing. No less than three gene classes are involved in tumour initiation: these are oncogenes, tumour suppressor genes, and DNA repair genes (Tysnes and Bjerkvig, 2007). During the first stage an oncogene develops within the DNA of somatic cells (Figure 2.1). Oncogenes are mutated genes that are responsible for normal growth of cells (Croce, 2008). The mutated DNA are either not repaired or repaired incorrectly through DNA repair enzymes ultimately leading to atypical proliferation of single cells. Tumour suppressor genes are altered and cannot perform its natural function thus producing excessive cell proliferation that is uncontrolled, leading to the outgrowth of a population tumor cells, establishing a primary tumour site (Fearon and Bommer, 2008). Mutations may occur mainly when an individuals' genetic factors interact with various external agents such as; ultraviolet and ionizing radiation, asbestos, components of tobacco smoke, aflatoxin (a food

contaminant) and arsenic (a drinking water contaminant), infections from certain viruses, bacteria or parasites (WHO, 2009). Chemoprevention, targeting cancer induction, aims to inhibit cell proliferation and stimulate repair of cell machinery to ensure the progression of normal growth phases. During the promotion stage of cancer development, through clonal expansion, cells proliferate excessively without dying forming a tumour growth (Tysnes and Bjerkvig, 2007). After establishment of the primary tumour, cells invade and penetrate the blood vessels and enter the circulatory system. The tumour becomes metastatic and results in a secondary tumor at a distant location in the body. This is the progression and final stage of cancer development (Martini F.H. and Ober, 2005).



**Figure 2.1: The Process of Carcinogenesis and possible targets for Chemoprevention (DKFZ, 2013).** The three stages of carcinogenesis: Initiation, promotion and progression may take between 10 and 20 years to fully develop.

Cell biology is a branch of science that deals with understanding and explaining *in vivo* cellular activities by conducting *in vitro* experiments. The main target for chemotherapy drugs is the induction of apoptosis and the prevention of cellular repair mechanisms. Because of the highly adaptability of cancer cells and development of drug resistant tumours, it has been suggested that targeting at least two hallmark pathways simultaneous may lead to more effective cancer therapies (Hanahan and Weinberg, 2000).

### **2.2.1. Hallmarks of cancer**

A review by Hanahan and Weinberg, 2011 identified six acquired capabilities of cancer cells. These “hallmarks of cancer” (Hanahan and Weinberg, 2000), has provided the basis for approaching new therapies. The authors have since expanded the list to ten features (summarized in Table 2.1) of cancer cells that are essential for developing their distinctive phenotype (Hanahan and Weinberg, 2011).

#### **2.2.1.1. Activated growth signals**

This is arguably the most essential trait of cancer cells involving their ability to sustain chronic proliferation signals. Production and release of growth-promoting signals are highly controlled in normal cells to ensure homeostasis is preserved and thus upkeep of normal tissue architecture and function. These pathways maintain a balance between cell death and cell survival signals (Tzur et al., 2009).



**Table 2.1: The Hallmarks of cancer**

	<b>Hallmarks</b>	<b>Description</b>
1.	Activated growth signals	Most fundamental trait of cancer cells can be achieved through various mechanisms.
2.	Unresponsive to anti-growth signals	Evasion of both intracellular and extracellular growth suppressor signals.
3.	Evading apoptosis	Resisting cell death signals.
4.	Infinite reproductive ability	Reproductive immortality induced by avoiding apoptotic and senescence triggers
5.	Prolonged angiogenesis	Sustain expanding neoplastic growths.
6.	Tissue invasion and metastasis	Tumour cells ‘break off ‘from the original tumour site, enter the blood stream and invade distal tissues.
7.	Genomic instability	Generation of random mutations including chromosomal rearrangement.
8.	Inflammation	Tumour promoting inflammation that helps to acquire hallmark capabilities.
9.	Reprogramming energy metabolism	Fuel cell growth and division.
10.	Evading immune destruction	Longstanding tumours avoid detection by the immune system or have limit the extent of immunological killing.

(Hanahan and Weinberg, 2000, Hanahan and Weinberg, 2011)

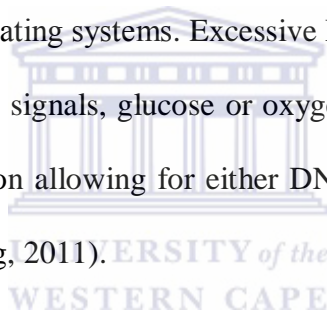
Cancer cells receive growth signals through numerous mechanisms. They produce growth factor ligands, which result in autocrine proliferative stimulation and stimulate surrounding cells to produce various growth factors to sustain the cancer cells. Deregulation of receptor signaling in tumour cells can also occur by increasing the levels of receptor proteins, rendering a hyper-responsive state to a relatively low amount of growth factor ligand. These

adaptations by tumour cells contribute to the development and maintenance of tumour morphology.

### **2.2.1.2. Unresponsive to anti-growth signals**

Two major tumor suppressors key in cell growth control encode the retinoblastoma (Rb) and p53 proteins (Szekely et al., 1993).

The Rb pathway acts as a crucial gatekeeper of cell-cycle progression and any defects or absence permits continual cell proliferation. P53 integrates stress and abnormality sensors within the cell's intracellular operating systems. Excessive DNA damage or suboptimal levels of nucleotides, growth promoting signals, glucose or oxygen increase p53 levels that lead to inhibition of cell-cycle progression allowing for either DNA excision repair or induction of apoptosis (Hanahan and Weinberg, 2011).

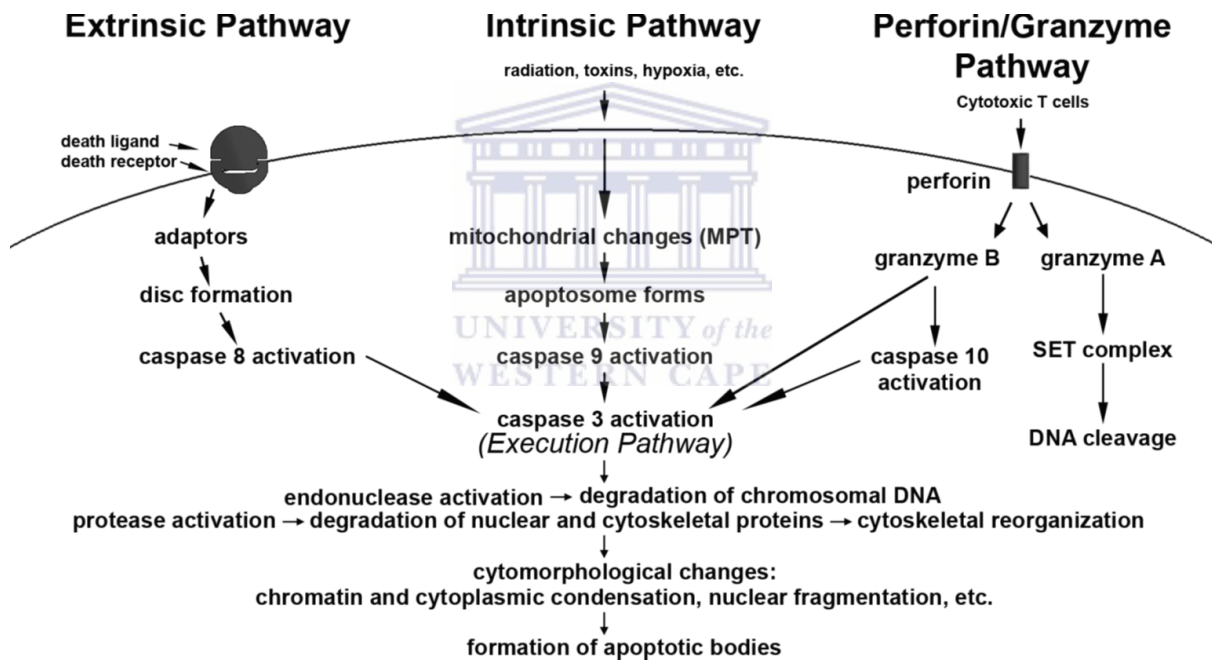


### **2.2.1.3. Evading apoptosis**

Apoptosis is a form of programmed cell death that requires energy to activate biochemical pathways that kill the cell in a neat and orderly manner. (Vaculova A. et al., 2010, O'Brien M.A. and Kirby R., 2008, Sreedhar A. S. and P., 2004).

Evading apoptosis is a key role likened to cancer cell morphology. There are three known pathways leading to apoptosis, as outlined in Figure 2.2. The extrinsic pathway is stimulated via death receptors on the cell membrane. The intrinsic pathway is stimulated by homeostatic changes that result in mitochondrial damage. The third pathway involves immune cytotoxic T

cells, which stimulate perforin incorporation in the cell membrane and release granzyme A and B intracellularly. Activation of caspase 3 via any of the mentioned pathways leads to activation of execution caspases resulting in the formation of apoptotic bodies and the final stage of apoptosis (Elmore, 2007). Apoptosis, like many other biological processes, can be disrupted by mutation in cancer cells (Lowe and Lin, 2000). With the loss of this function, cells containing mutated DNA have no cell death trigger, which result in the multiplication of functionally abnormal cells.



**Figure 2.2: Apoptotic pathways (Elmore, 2007).** Diagram displaying the different pathways leading to programmed cell death. Three distinct pathways namely; extrinsic pathway (death signal is received from outside the cell), intrinsic pathway (death response to intracellular stress signals) and finally granzyme pathway (involving granzyme complexes). All three pathways lead to apoptosis via caspase 3.

#### **2.2.1.4. Enabling and emerging hallmarks**

The above hallmarks of cancer are acquired functional capabilities that allow cancer cell to survive, multiply and spread. Genomic instability in cancer cells creates random mutations that enable the tumorigenic process. while the inflammatory state of premalignant lesions also contribute to tumor progression (Hanahan and Weinberg, 2011).

Furthermore major reprogramming of cellular energy metabolism is effected by cancer cells to maintain their growth, proliferation and metabolic program (Negrini et al., 2010) and evasion of immune destruction by cancer cells enhances tumor development and progression (Kim R, 2007). These hallmarks facilitate the development and progression of many forms of human cancer and can be measured as emerging hallmarks of cancer.



#### **2.3. Cell membrane**

A biological membrane present in all cells that protects its interior from the external environment. The cell membrane regulates substances entering and leaving cells through its selectively permeability to ions and organic molecules. The structure of the cell membrane is a phospholipid bilayer embedded with proteins (Figure 2.3). Cell membranes are involved in a number of cellular processes including cell adhesion, ion conductivity and cell signaling and serve as the attachment surface for several structures like the cell wall, glycocalyx, and intracellular cytoskeleton (Edidin, 2003).

The cell membrane structure is composed of a combination of three classes of lipids with amphipathic properties: phospholipids, glycolipids, and sterols. The individual quantity of each depends upon the cell type, but in most cases phospholipids are most abundant (Alberts

et al., 2002). Lipids are important biological molecules, which include fats, sterols, glycerides, fat- soluble vitamins and phospholipids that functions as energy stores, signaling molecules, and structural components of cellular membranes. These molecules have low water solubility, but are highly soluble in nonpolar solvents, making them hydrophobic (containing nonpolar groups) or amphipathic (both polar and nonpolar groups), possessing properties that are both hydrophilic and lipophilic. (van Meer et al., 2008).

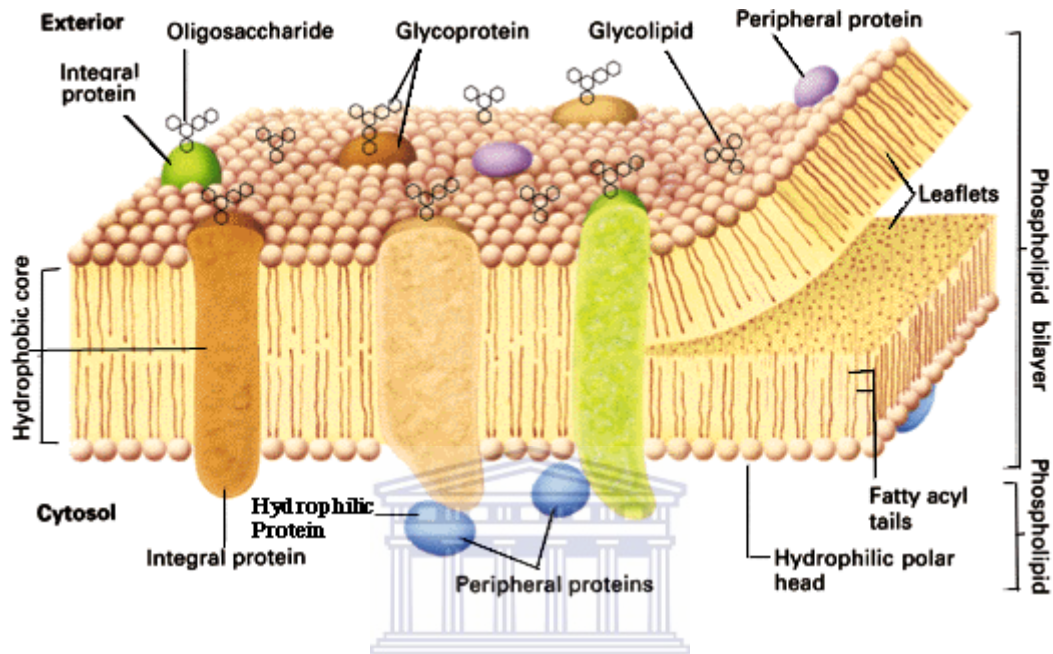
Lipids are vital components of eukaryotic cells. They are responsible for numerous functions in the cell namely; energy storage, cellular matrix formation, protein aggregation and dispersion within the cell membrane and finally act as first and secondary messengers in signal transduction and molecular recognitions processes (van Meer et al., 2008).

Phospholipids, one of the different classes of lipids, are a major element in the lipid bilayer of cellular membranes. Most phospholipids contain a diglyceride, a phosphate group, and a simple organic molecule such as choline. Glycerophospholipids, a glycerol-based phospholipid, are the main type of phospholipids in the biological membranes and include: phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI) and phosphatidic acid (PA) (Zhang and Rock, 2008).

### **2.3.1. Phospholipids**

Phospholipids are a class of lipids that are a major component of all cell membranes as they can form lipid bilayers. Most phospholipids contain a phosphate group, a simple organic molecule such as choline or ethanolamine and two fatty acid (FA) chains. The nonpolar Carbon-Hydrogen bonds found in the hydrocarbon chains are incapable of water interaction, making them hydrophobic in nature. This, together with the naturally hydrophilic nature of

carboxyl groups, gives FAs their amphiphatic properties, which describes the behaviour of phospholipids in biological membranes (van Meer et al., 2008).

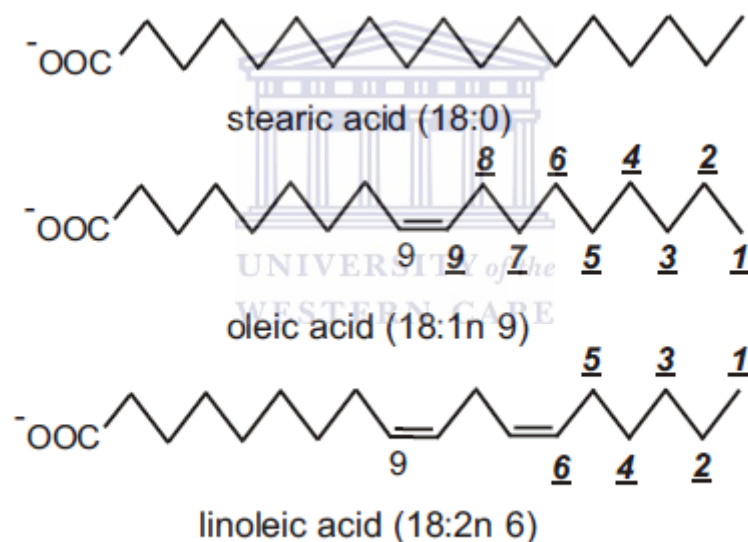


**Figure 2.3 Cell membrane lipid bilayer (Muskopf, 2010).** Diagram show the mammalian cell membrane containing 2 lipid layers embedded with proteins.

### 2.3.1.1. FA chains

The FA chains in phospholipids and glycolipids usually contain an even number of carbon atoms (14 to 24), with the exception of a few marine organisms with odd numbers of carbon atoms. They are found in two forms, saturated and unsaturated (Figure 2.4). Saturated FA (SATS) contain no double bonds and therefore all carbons are saturated by single hydrogen bonds. They are extremely flexible and found in diverse conformations. Because SATS can pack closely together, they can form inflexible arrangements under certain conditions, thereby reducing fluidity of mosaic cellular membranes. Unsaturated FAs are slightly more abundant in nature than SATS. An unsaturated FA has one or more C-C double bonds in its

hydrocarbon. When only one double bond is found, the FA is called monounsaturated fatty acids (MUFA), when two or more double bonds are present, the FAs are called polyunsaturated fatty acids (PUFA). Unsaturated FA are found in either *cis* or *trans* configuration, which create a kink, preventing the FAs from packing together as tightly, thus decreasing the melting temperature and increasing the membrane fluidity. Unsaturated FA are also distinguished according to the position of the first double bond from the methyl end of the hydrocarbon chain. As in in Figure 2.4, oleic acid has 18 carbons in the fatty acid chain and 1 double bond at position 9 from the methyl end hence 18:1n9. Whereas, linoleic acid has 18 carbon, 2 double bond, with the first from the methyl end at position 6, hence 18:2n6.



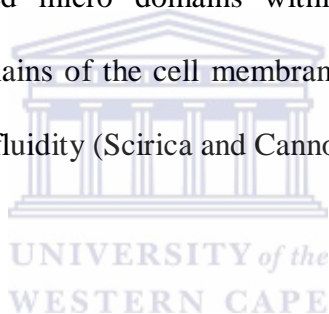
**Figure 2.4: Three 18 carbon (C18) FAs** (Tvrzicka et al., 2011). Saturated FA, steric acid, MUFA, oleic acid, and PUFA, LA (LA).

In its entirety, the cell membrane is supported via non-covalent interaction of hydrophobic tails. Under physiological conditions phospholipid molecules in the cell membrane are in a distinct phase of matter between solid and liquid states called the liquid crystalline state. This

means that the lipid molecules are able to diffuse laterally along their present layer. However, the exchange of phospholipid molecules between intracellular and extracellular leaflets of the bilayer is a very slow process (Muskopf, 2010).

### **2.3.2. Cholesterol**

In animal cells cholesterol is normally found dispersed in varying degrees throughout cell membranes, in the irregular spaces between the hydrophobic tails of the membrane lipids, where it confers stiffening and strengthening effects on the membrane. Lipid rafts are examples of cholesterol-enriched micro domains within the cell membrane. Through interaction with non-polar FA chains of the cell membrane, cholesterol increase membrane packing and decrease membrane fluidity (Scirica and Cannon, 2005).



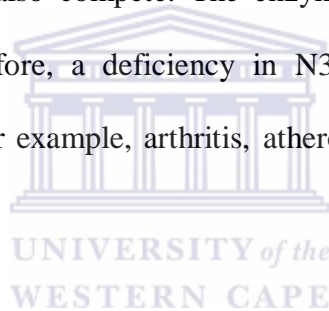
### **2.4.1. FA metabolism**

Essential fatty acids (EFA) are FAs required from the diet because of the inability of humans to synthesize these FA. These include two main EFA; linoleic acid (LA, C18:2n6) and  $\alpha$ -linolenic acid (ALA, C18:3n3). Once in the human system, these C18 FA can be elongated by elongase enzymes and desaturated by desaturase enzymes to produce more biologically active FAs like;  $\gamma$ -linolenic acid (GLA, C18:3n6), arachidonic acid (AA, C20:4n6), docosahexaenoic acid (DHA, C22:6n3) and eicosapentaenoic acid (EPA, C20:5n3) (Tvrzicka et al., 2011) as outlined in Figure 2.5.



Omega-3 (N3) FAs have its first double bond at carbon position 3 from the methyl end of the FA chain, whereas omega-6 (N6) FA is at position 6. Omega-3 (N3) and omega-6 (N6) EFAs are best characterized by their interactions. Arachidonic acid (AA) is a 20-carbon n6 EFA that is the major compound of the AA cascade, involving more than 20 different signalling paths that regulator a number of different biological functions, including functions involving inflammation. LA (LA), an EFA, is acquired from vegetable oils and animal fats, and is the precursor for the majority of AA in the body (Burr et al., 1930).

EPA has been associated with anti-inflammatory effects. It is on the same level of the FA metabolic pathway as AA (Figure 2.4) and they compete for  $\Delta 5$ -desaturase enzyme upstream, but their downstream products also compete. The enzymes in the FA pathway a greater affinity for N3 FA and therefore, a deficiency in N3 FA have been associated with inflammatory related diseases for example, arthritis, atherosclerosis and cancer (Riediger et al., 2009, Williams et al., 2011).

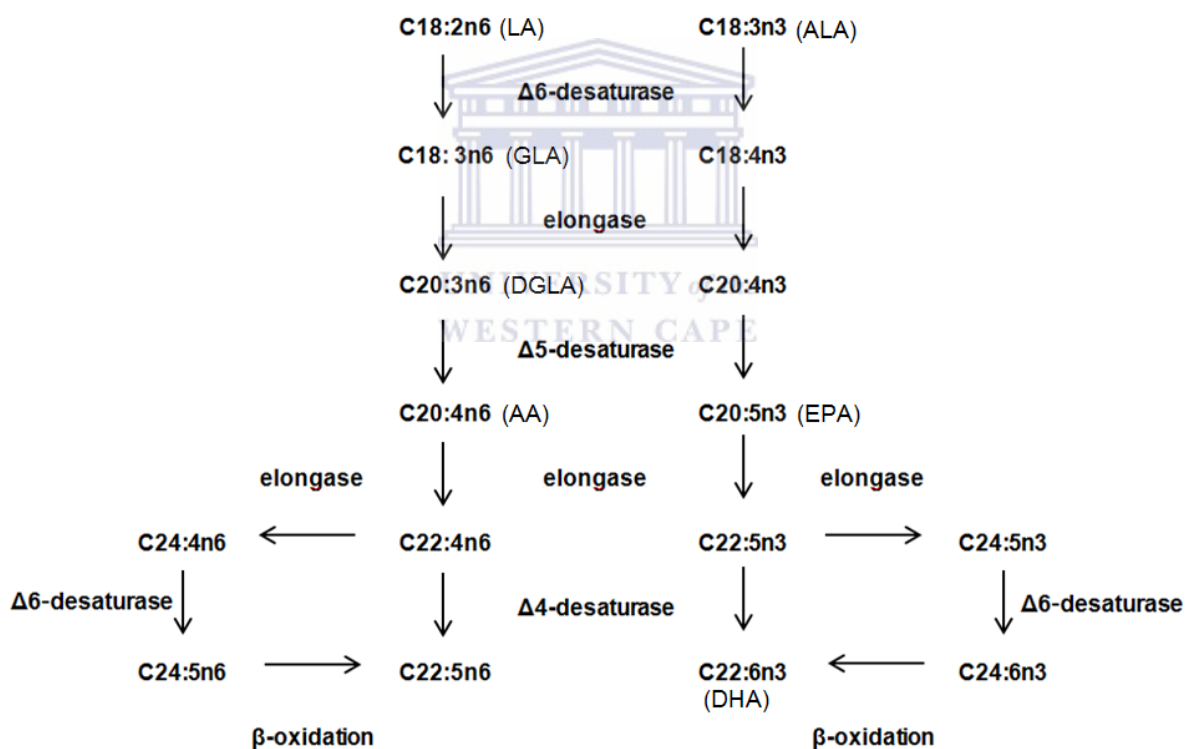


#### **2.4.2. Role of PUFA in disease and cancer**

An extensive body of research discusses the role of PUFA in health and disease (Simopoulos, 1991, Calder, 1997, Calder, 2006). The omega 6/3 (N6/N3) ratio was found to be a major concern to the health of modern man (Simopoulos, 1991). The ratio increased from about 1 to between 10:1 and 25:1, indicating a clear deficiency in N3 FAs in the modern Western diet.

### 2.4.2.1. FAs as precursors of lipid mediators

FAs of the cell membrane are precursors of lipid mediators, with eicosanoids (prostaglandins, PG; thromboxanes, TX; leukotrienes, LT) being one of the most important. Eicosanoids contain 20 carbon atoms, specifically PUFA derived from the sn-2 glycerol carbon (hydroxyl group on the second carbon in the FA chain) of cell membrane phospholipids, such as AA and EPA. Other FA such as DHA, docosapentaenoic acid (DPA; 22:5n-3) and LA are also precursors of various other lipid mediators (Kremmyda et al., 2011, Calviello et al., 2006).



**Figure 2.5: Dietary essential FA pathways (Nakamura and Nara, 2003).** Linoleic acid (LA),  $\gamma$ -linolenic acid (GLA), dihomo- $\gamma$ -linolenic acid (DGLA), arachidonic acid (AA),  $\alpha$ -linolenic acid (ALA), eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA).

Phospholipases assist the release of cell membrane FAs, which are transformed by cyclooxygenase (COX) type 1 and 2 to PG and TX (Berquin et al., 2011). These eicosanoids are basic hormones or regulating molecules that appear in most organisms. Unlike endocrine hormones, which travel in the blood stream, eicosanoids have autocrine or paracrine actions. They alter the activity of the cells from which they were synthesized and of adjoining cells. PGs stimulate inflammation, regulate blood flow to particular organs, control ion transport across membranes, and modulate synaptic transmission. TXs are vasoconstrictors and potent hypertensive agents which also facilitate platelet aggregation (Kremmyda et al., 2011).

The effect of lipid mediators produced is dependent on their FA substrate. Different eicosanoids are produced from AA and EPA, which have different actions. For example, AA is the precursor of 2-series PG and 4-series LT, and EPA is the precursor of 3-series PG and 5-series LT. The AA-derived PG and LT are synthesized in response to injury or cellular stress, whereas the EPA-derived PG and LT appear to control the effects of 2-series PG and 4-series LT produced from AA. EPA-derived PG are formed at a slower rate and weakens the effects AA-derived PG. Sufficient production of these PG appears to defend against heart attack and stroke as well as certain inflammatory diseases such as arthritis, lupus, and asthma (Calder, 2003).

#### **2.4.2.2. FAs in signal transduction**

Signal transduction is the process of activation of a cell surface receptor through extracellular signal molecules, creating an intracellular response. The process occurs in two stages. Firstly, a signaling molecule activates a specific receptor on the surface membrane of the cell. Then a

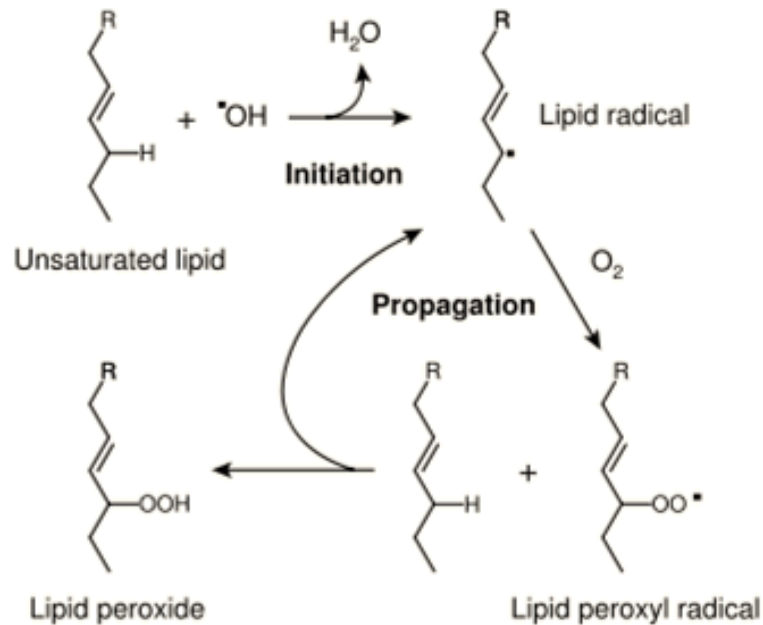
second messenger transmits the signal intracellularly that produces a physiological response (Lodish et al., 2000).

Cell membrane FAs have been identified to play the role of second messenger and modulators of signal transduction. This regulation may be positive or negative, all part of a feedback control mechanism, since phospholipases are also controlled by FA (Sumida et al., 1993). FA act as second messengers in the inositide phospholipid and the cyclic AMP signal transduction pathways. They are also modulators that may intensify, weaken or diverge a signal. FAs modify the activities of many stimulus-response coupling mechanisms such as phospholipases, protein kinases (PKC), G-proteins, adenylate and guanylate cyclases as well as ion channels and other biochemical events involved (Graber et al., 1994). The activation of PKC is dependent on the lipid and FA composition of membrane phospholipids (Nishizuka, 1992).



#### **2.4.2.3. Oxidative stress**

Oxidative stress is defined as a disruption in the equilibrium between the reactive oxygen species (ROS) and antioxidant production in cells. The excessive ROS leads to free radical damage and the induction of lipid peroxidation (Betteridge, 2000). Persistent exposure to oxidative stress has been associated to numerous biological disorders such as cancer. The active role of oxidative stress in cancer has been correlated to activated transcription factors and proto-oncogenes, genomic instability, chemotherapy-resistance, invasion and metastasis (Toyokuni et al., 1995).



**Figure 2.6: Lipid peroxidation of unsaturated FAs (Vickers, 2007).** Diagram showing the process of lipid peroxidation on unsaturated FA forming lipid peroxides.

Unsaturated FAs are highly susceptible to lipid peroxidation. Some studies show that FA can activate oxidative stress and production of prothrombotic markers (Soardo et al., 2011). One study even shows that a saturated FAs enriched diet offers protection against oxidative stress (Lemieux et al., 2011). As outlined in Figure 2.6, exposure of unsaturated FAs to free radicals ( $\cdot\text{OH}$ ) initiates the process of lipid peroxidation and formation of lipid radicals. The lipid radicals are propagated by oxygen ( $\text{O}_2$ ) to form lipid peroxy radicals and ultimately lipid peroxides. Accumulation of lipid peroxides has been implicated in certain diseases and cancer (Yagi, 1987). Cancer cells show a loss in lipid peroxidation early in the tumorigenic process that has been attributed to change in lipid profile of cellular membranes, with a clear decrease in polyunsaturated FAs, the key substrate for lipid peroxidation (Dianzani, 1989). Some studies suggest increasing PUFA content of the cell membrane would increase lipid peroxidation and increase the oxidative stress in cancer cells (Barrera, 2012).

## 2.5. Conjugated Linoleic Acid

Conjugated linoleic acids (CLA) refer to a group of FAs which are geometrical, *cis* or *trans*, and positional, position of double bond, isomers of octadecadenoic acid (LA) (Aydin, 2005). An increasing interest in CLA is attributed to its potential health benefits such as anticarcinogenic, antiatherogenic, antidiabetic and antiadipogenic effects. In the FA chain of CLA there are two conjugated double bonds at different carbon positions. They form part of the omega 6 family of essential PUFAs containing 2 double bonds in its chemical structure (Christie et al., 2007). There are 28 CLA identified isomers, which produced naturally in the rumen and milk udders of ruminant animals (grazing cows). The two major and most extensively studied isomers are *cis9-trans11* CLA (*c9t11* CLA), also called rumenic acid) and *trans10-cis12* CLA (*t10c12* CLA). The *c9t11* CLA isomer is the most prevalent CLA isomer present in milk fat and beef (about 70-90 % of total CLA content), whereas the concentration of the *t10c12* CLA isomer (3-5 %) vary considerably depending of consumption, grazing or feed fed, of these animals (Collomb et al., 2004, Park et al., 2001, Khanal and Dhiman, 2004). Total conjugated dienes (CD) content of milk fat and meat are also season dependent. During spring and summer, grazing months, CD content is higher than during autumn and winter, feed fed months.

Numerous studies exemplify health benefits of CLA in mouse system, such as anticancer, adipogenic, antiatherogenic and increase immune function (Smedman and Vessby, 2001, Aydin, 2005). These benefits raise interest in studying and understanding mechanisms that CLA would protect humans from these disorders. Each isomer however shows different biological activity. For example, *c9t11* CLA isomer has shown to reduce the risk cancer development in mouse (van Meer et al., 2008), whereas *t10c12* CLA isomer plays a more active role in reducing body fat (Muskopf, 2010). It is therefore important to study each

isomer individually and also various combinations to fully understand how they react in the body.

### 2.5.1. History of CLA

The first reported incidence of the presence of conjugated FA in milk fat was by Booth et al. (1935) by demonstrating the occurrences of seasonal differences in FA composition of milk fat. While doing spectrophotometric analyses of milk fat, they observed marked increase in absorption at 230 nm when comparing winter to summer milk fat, when cows were changed on to pasture feed (Booth et al.). This increase absorption at 230 nm was later accredited to the presence of conjugated double bonds by Moore (1939) (Khanal and Dhiman). Hilditch and Jasperson suggested C18 PUFA chains displayed conjugated unsaturation (Ha et al., 1990, Kemp and Lander, 1984, Palmquist et al., 2005). Shorland et al (1957) supported this by showing that PUFAs are hydrogenated, biohydrogenation, in the rumen of cows (Bauman et al., 2000). Bartlett and Chapman proposed conjugated intermediate in reactions leading to the conversion of LA to trans11-vaccenic acid in the rumen. This was after the discovering a constant relationship between trans-C18:1 and conjugated unsaturation determined by differential infrared spectroscopy (Floor et al., 2012). A few years later while studying milk fat FA profiles of 29 creameries across Canada for every month of the year, Riel (1963) found that cows grazing on pasture during summer months yielded twice the amount of conjugated dienes in milk fats as when cows were fed total mixed rations (TMR) during winter (Søreide et al., 2009). These findings confirmed Booths' observations in 1935.

Kepler et al. (1966) investigated the process of biohydrogenation by rumen bacteria *Butyrivibrio fibrisolvens* in the reduction of LA to stearic acid. They confirmed *B.*

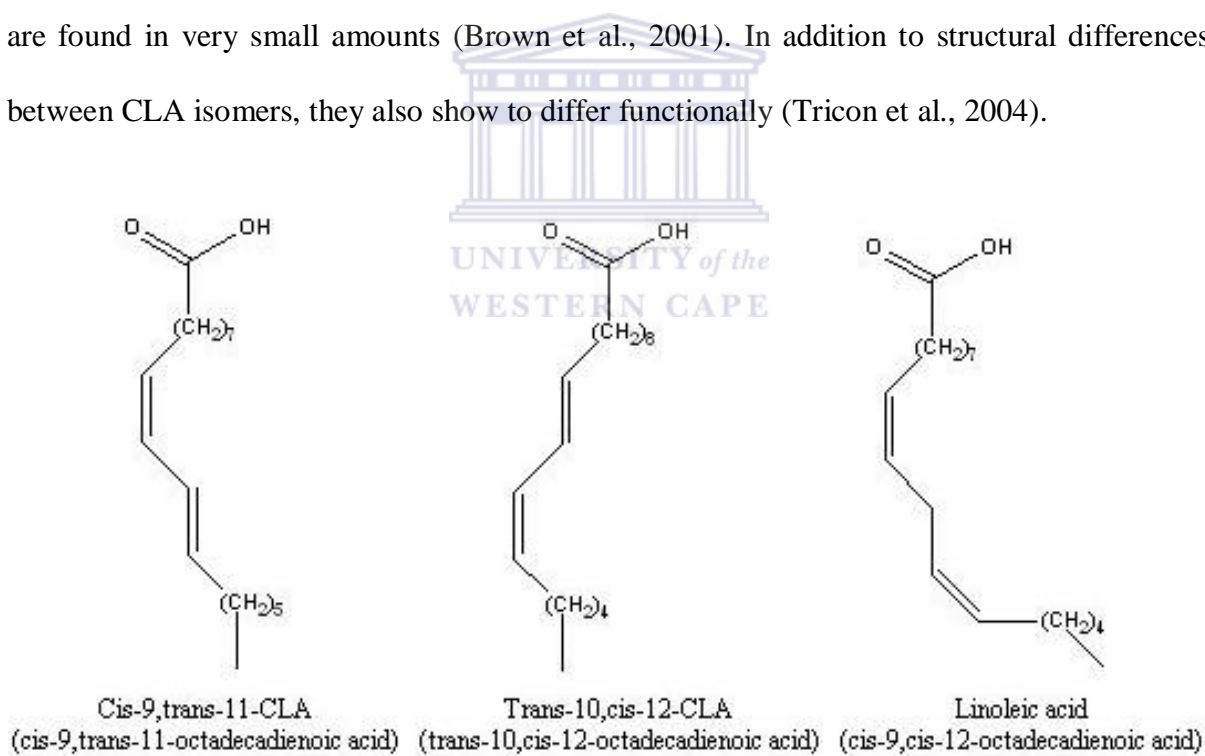
*fibrisolvens* specificity for LA and proposed an intermediate step in the reduction process. In the first step of biohydrogenation, LA isomerization occurred forming a conjugated *cis-trans* octadecadienoic intermediate, which is subsequently hydrogenated and *trans*-monoenoic acids are formed (Milinkovic et al., 2012). Kepler and Tove (1967) later confirmed the *cis-trans* octadecadienoic intermediate formed following isomerization of LA to be *cis9-trans11* octadecanoic acid, which was the first time *c9t11* octadecanoic acid was identified in the rumen (Beckman, 2009). Later, Parodi (1977) was first to establish the presence of *c9t11* octadecanoic acid in milk fat by isolating conjugated *cis, trans*- octadecadienoic acids and studying their FA profiles through gas liquid chromatography (Bissonauth et al., 2006). In 1987, Ha et al. inhibited mutagenesis in bacteria and reduced chemically induced epidermal carcinogenesis in mice using fried ground beef. They said that the responsible compound is an isomeric derivative of LA containing a conjugated double-bond, and called it CLA (CLA) (Nunes et al., 2008). They then induced epidermal cancer in mice and treated them with LA and CLA. When compared to LA, CLA treated mice produced half as many papillomas and exhibited a lower tumor incidence. (Fa et al., 2005). For the first time, CLA was shown to have a potential benefit in reducing cancer development.

### 2.5.2. Structure of CLA

The term CLA has been dedicated to a number of positional and geometrical isomers of an essential PUFA, LA. PUFA, together with saturated and MUFA, are integral constituents of triglycerides, phospholipids and other complex lipid molecules, and therefore form an essential part of cell membranes (Iannone et al., 2009). PUFAs have a common structure of a hydrocarbon chain containing two or more carbon-carbon double bonds. Figure 2.7 illustrates



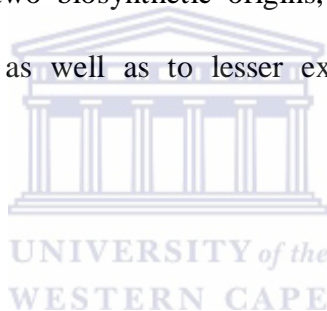
structural differences of the two most extensively studied CLA isomers compared to its parent FA, LA. Like LA, CLA has two carbon double bonds, but these two double bonds are separated by a single hydrogen bond found in either ‘*cis*’ or ‘*trans*’ configuration and are therefore termed ‘conjugated’ (Nunes et al., 2008). Compared to other dienes, the double bonds are either separated by a methylene group or two single hydrogen bonds. The CLA double bonds may occur in the 7,9; 8,10; 9,11; 10,12; or 11,13 positions (Reynolds and Roche, 2010) and geometrically in *cis-cis*, *cis-trans*, *trans-cis* or *trans-trans* configuration. There are 28 identified structurally different isomers of CLA found in various concentrations in beef and dairy products of ruminants (Banni, 2002). The *c9t11* CLA isomer accounts for about 80-90% of total CLA content (Cho et al., 2006), *t10c12* CLA and other CLA isomers are found in very small amounts (Brown et al., 2001). In addition to structural differences between CLA isomers, they also show to differ functionally (Tricon et al., 2004).



**Figure 2.7: Structure of *c9t11* CLA isomer, *t10c12* CLA isomer and LA (Mooney et al., 2012).** Diagram comparing the structure of *c9t11* CLA and *t10c12* CLA to LA. The double bonds of the conjugated FAs was separated by one single bond, whereas the double bonds of LA are separated by 2 single bonds.

### 2.5.3. Biosynthesis of CLA

CLA was first discovered to be present in milk fat and meat of ruminant animals. It was later found to be produced in the rumen as a conjugated intermediate in the process of biohydrogenation of dietary FAs by ruminal bacteria (Kemp and Lander, 1984). Although the yield of CLA was significant, this only accounted for a fraction (10-15%) of CLA present in the meat and milk of these animals (Bauman et al., 2003). Therefore another process had to be involved in the endogenous formation of CLA (Khanal and Dhiman, 2004). CLA was subsequently found to be produced through endogenous conversion of transvaccenic acid (TVA), a later intermediate during biohydrogenation, to CLA in tissues. The formation of CLA is now accepted to have two biosynthetic origins, the rumen and endogenously in tissues, occurring in ruminants as well as to lesser extent non-ruminants (Khanal and Dhiman, 2004).



#### 2.5.3.1. Ruminal synthesis of CLA

Two CLA isomers, *c9t11* and *t10c12* CLA, have previously been shown to be synthesized in the rumen of ruminant animals through a process called biohydrogenation (Crumb, 2011). Biohydrogenation requires free FAs for initiation and is the second foremost conversion of dietary lipids in the rumen following lipid hydrolysis (Palmquist et al., 2005). Pure cultures of ruminal organisms were used to establish biohydrogenation pathways. Two groups of bacteria, classified based on their metabolic pathways, have been identified in their involvement in biohydrogenation, group A and group B bacteria (Table 2.2) (Bauman et al., 2000). Both groups are required for complete biohydrogenation of PUFA in the rumen. But because of the smaller population of group B bacteria, group A products accumulate in the

rumen and can be absorbed by endogenous tissues of the animal (Palmquist et al., 2005). Kemp and Lander, 1984, grouped ruminal bacteria based on their respective metabolic pathways (Table 2.2). This was discovered by incubating pure cultures of ruminal bacteria with dietary FAs of ruminants (Kemp and Lander, 1984). Group A bacteria isomerize, shifting hydrogen bonds (*cis* to *trans*), and hydrogenate, adding hydrogen to remove double bonds, PUFAs. Group B bacteria further hydrogenate unsaturated FAs to form a saturated FA, stearic acid (Bauman et al., 2000).

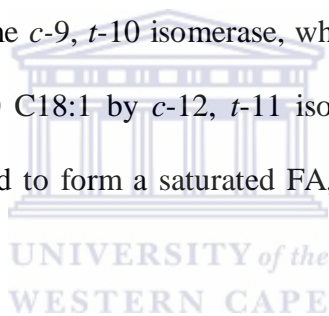
**Table 2.2: Ruminal bacteria groups showing organisms, substrate and major products of group A and group B bacteria.**

Group	Organism	Substrate	Major products
A	F2/6 ( <i>Ruminococcus albus</i> )	18:1; <i>c</i> -9, <i>c</i> -11, <i>t</i> -9, <i>t</i> -11	Not hydrogenated
	S2 ( <i>Butyrivibrio fibrisolvens</i> )	18:2; <i>c</i> -9, <i>c</i> -12 18:3; <i>c</i> -9, <i>c</i> -12, <i>c</i> -15	18 :1; <i>t</i> -11(+ <i>t</i> -10 with F2/6) 18:1; <i>t</i> -11 (+ <i>t</i> -10 and 18:2; <i>t</i> -11, <i>c</i> -15 with F2/6)
B	P2/2 ( <i>Fusocillus babrahamensis</i> )	18:1; <i>c</i> -9, <i>c</i> -11, <i>t</i> -9, <i>t</i> -11	Stearic acid (30% hydroxystearic acid with <i>c</i> -9 and P2/2)
	T344 ( <i>Fusocillus</i> sp.)	18:2; <i>c</i> -9, <i>c</i> -12	Stearic acid
	R8/5 (Gram-negative rod)	18:3; <i>c</i> -9, <i>c</i> -12, <i>c</i> -15	18:1; <i>c</i> -15, <i>t</i> -15 (+ <i>c</i> / <i>t</i> -13, 14 and 16 with P2/2 and T344)

Adapted from (Kemp and Lander, 1984)

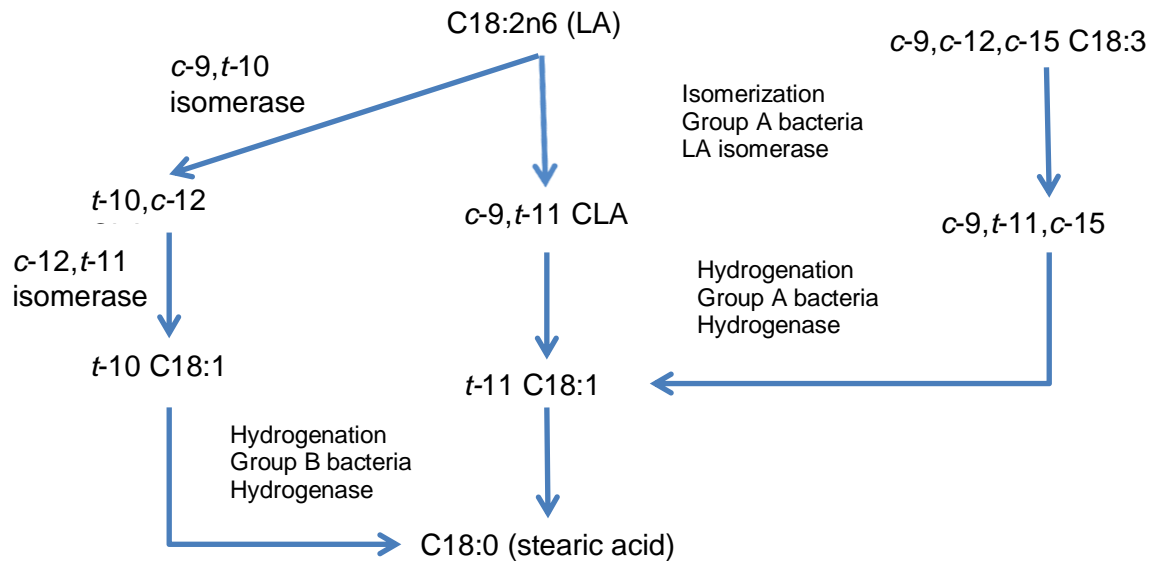
The biohydrogenation of dietary FAs in the rumen and the bacteria and enzymes involved in the process of the biohydrogenation of LA (C18:2), group A bacteria first isomerizes the double bond at *c*-12 to *t*-11, thus forming *c*-9, *t*-11 CLA (Figure 2.8). The double bond at *c*-9 is then rapidly hydrogenated to form TVA (*t*-11 C18:1). The final step utilizes group B bacteria to further hydrogenate the *t*-11 bond, thus converting TVA to stearic acid (C18:0). A

membrane bound bacterial enzyme, LA isomerase, is responsible for the isomerization of *cis*-9, *cis*-12 C18:2 and is highly specific for this substrate (Bauman et al., 2000, Khanal and Dhiman, 2004, Crumb, 2011). The biohydrogenation of linolenic acid (*c*-9, *c*-12, *c*-15, C18:3) occurs through a similar process: isomerization at *c*-12 forming *c*-9, *t*-11, *c*-15 C18:3 and then hydrogenation of the double bonds at *c*-9 and *c*-15 to give transvaccenic acid, which is finally reduced at *t*-11 to give stearic acid. The biohydrogenation of ALA employ the exact same bacteria and enzymes as for LA biohydrogenation (Bauman et al., 2000, Khanal and Dhiman, 2004, Crumb, 2011). Although the processes are similar, no CLA isomer intermediate is formed during the biohydrogenation. The *t*10*c*12 CLA isomer is also synthesized in the rumen as an intermediate during the biohydrogenation of LA. This pathway is initiated by the enzyme *c*-9, *t*-10 isomerase, which forms *t*10*c*12 CLA. The next reaction is the formation of *t*-10 C18:1 by *c*-12, *t*-11 isomerase. The final reaction is the reduction of the *t*-10 double bond to form a saturated FA, stearic acid (figure 2.8) (Khanal and Dhiman, 2004).



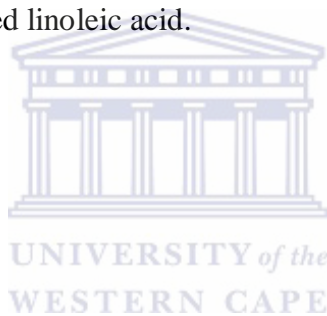
### **2.5.3.2. Endogenous Conversion of CLA**

Evidence shows the production of *c*9*t*11 CLA isomer following the removal of two hydrogen atoms from 11-*trans*-octadecenoate (vaccenic acid) at carbon position 9 and 10 by  $\Delta$ 9-desaturase enzyme, creating a carbon-carbon double bond at that position (Adlof et al., 2000, Miller et al., 2003). This forms a CLA FA as an intermediate in biohydrogenation of LA and ALA. The primarily source of  $\Delta$ 9-desaturase enzyme is in adipose tissue of beef cattle and in the mammary gland of lactating dairy cattle. This biosynthetic process accounts for 60-90% of the total CLA content in ruminant food products (Khanal and Dhiman, 2004).



**Figure 2.8: Biohydrogenation of dietary FAs in the rumen** (Khanal and Dhiman, 2004).

LA, linoleic acid, CLA, conjugated linoleic acid.



#### 2.5.4. Sources of CLA

CLA is found in beef and dairy products, such as milk and cheese and is produced in the rumen and udders of ruminant cows. The CLA content of milk fat and beef is dependent on the type of feed the cow receives (grazing, oil rich feed) and also on the breed of the cattle (Peterson et al., 2002). The concentration of CLA found in milk fat and dairy products was shown to increase in cows fed a diet rich in unsaturated FAs; oleic acid, LA and ALA (Collomb et al., 2006).

### 2.5.5. CLA and cell membranes

Numerous studies show that when cells are treated with CLA isomers, they are incorporated into cell membranous lipids to the same extent as LA and AA (Moya-Camarena et al., 1999). The majority of the incorporated CLA were present in lipid rafts of the membranes. Unlike LA, which mainly occupies sn2-position of phospholipids, CLA incorporated in phospholipid fractions were found to occupy the sn-1 and sn-2 positions. These findings show that the incorporation profiles of CLA isomers differ significantly from that of LA, and have the potential to be incorporated twice as well as LA. This could lead to modifications in membrane function. CLA showed varying effects on desaturation index (18:1/18:0 and 16:1/16:0) and  $\Delta$ 9-desaturase (Subbaiah et al., 2011).



### 2.5.6. Effect of CLA in cancer cells

It is well recorded that CLA plays a potentially beneficial role in the prevention of cancer development. Table 2.3 summarizes the major finding of CLA over the years with regard to carcinogenesis. The first incident recorded that FAs derived from beef reduced chemically induced tumour formation (Ha et al., 1987, Liu and Belury, 1998). Later findings revealed that treatment with CLA increased apoptosis (Evans et al., 2000, Ip et al., 2000), decreased cell proliferation (Cho et al., 2003), altered FA metabolism (Banni et al., 1999, Igarashi and Miyazawa, 2001) and modify components of the cell cycle (Belury, 2002). Furthermore, CLA treatment modulates markers of immunity (O'Shea et al., 2004) and eicosanoid formation (Banni et al., 2004, Belury, 2002). One or more of these pathways are responsible for the inhibitory properties CLA in carcinogenesis, with some tissue specificity and differences in isomer responses.

**Table 2.3: The major findings on CLA and carcinogenesis.**

Reference

↓ Mutagenesis mediated by liver S-9 from normal rats.	(Pariza et al., 1979)
↓ 7,12-dimethylbenzanthracene (DMBA) initiated papilloma in mice.	(Ha et al., 1987)
↓ 12-O-tetradecanoyl-phorbol-13-acetate (TPA)-induced tumor promotion in mouse skin.	(Liu and Belury, 1998)
↓ Mammary cancer incidence by affecting LA metabolites	(Banni et al., 1999)
↑ Apoptosis in 3T3-L1 pre-adipocytes.	(Evans et al., 2000)
↑ Apoptosis and reduced the expression of bcl-2 in premalignant lesions.	(Ip et al., 2000)
↓ Cancer cell growth by affecting FA metabolism.	(Igarashi and Miyazawa, 2001)
↓ DMH-induced colon carcinogenesis in rats.	(Park et al., 2001)
↑ Cytotoxicity of dRLh-84 rat hepatoma cells.	(Yamasaki et al., 2002)
↓ Cell proliferation and ErbB3 signalling in HT-29 human colon cell line.	(Cho et al., 2003)
↑ p53 response that leads to the accumulation of pRb and cell growth arrest.	(Kemp et al., 2003)
↓ Insulin like growth factor-II synthesis and downregulate IGF-IR signalling and the PI3K/Akt and ERK-1/2 pathways.	(Kim et al., 2003)
↓ Membrane invasion by reducing type IV collagenase activities in the serum-free supernatant of SGC-7901.	(Yang et al., 2003)
↓ Proliferation of PC-3 cells via modulation of apoptosis and cell cycle control.	(Ochoa et al., 2004)
↓ 5-lipoxygenase metabolite, 5-HETE, increasing apoptosis and decreasing cell proliferation.	(Kim et al., 2005)
↑ Apoptosis via the mitochondrial pathway in rat hepatoma cells.	(Yamasaki et al., 2005)
↓ Expression of Bcl-2 proteins, increasing apoptosis.	(Beppu et al., 2006)
↓ G1-S Progression in HT-29 Human Colon Cancer Cells.	(Cho et al., 2006)
↑ Apoptosis in MDA-MB-231 breast cancer cells through ERK/MAPK signalling and mitochondrial pathway.	(Miglietta et al., 2006)
↓ breast cancer cell growth and invasion through ERalpha and PI3K/Akt pathways.	(Bocca et al., 2010)

↓ Viability and proliferation of HepG2 cancer cells in relation with cell cycle arrest and induction of apoptosis.	(Melaku et al., 2012)
↑apoptosis by c9, t11-CLA in human endometrial cancer RL 95-2 cells via ERalpha-mediated pathway.	(Wang et al., 2013)

Adapted from (Terk, 2007)

### 2.5.7. CLA and health implications

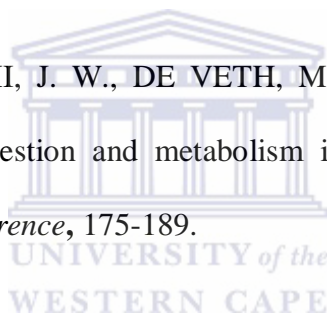
Various studies show that some of the effects attributed to CLA include reduction in body fat and increased lean body mass, prevention of atherosclerosis, hypertension, different types of cancers and also increase immune function (Bhattacharya et al., 2006, McGuire and McGuire, 2000). Not many clinical studies have been conducted using CLA isomers to treat cancer in humans. However reports indicated that CLA may play a protective role against cancer development by inhibiting tumor initiation, promotion and progression. (Smedman and Vessby, 2001). The exact mechanisms on not well understood or well define, but a number of proposed mechanisms have been identified. These include: altering cell cycle events, induction of apoptosis, gene expression modulation via the activation of peroxisome proliferator activated receptors, lipid peroxidation, cell membrane structure and/or functional changes, and growth factor receptor signaling interference (Amarù et al., 2010).



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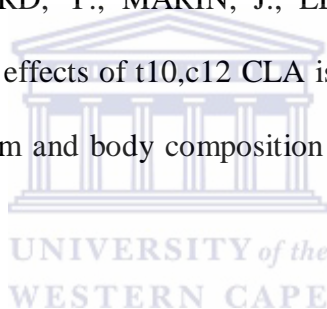


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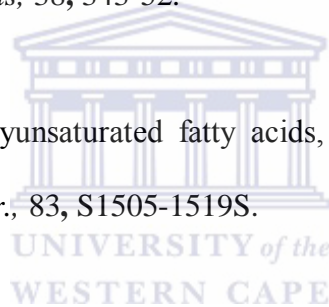
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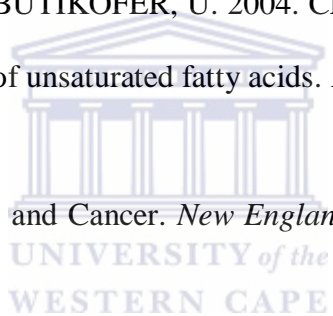
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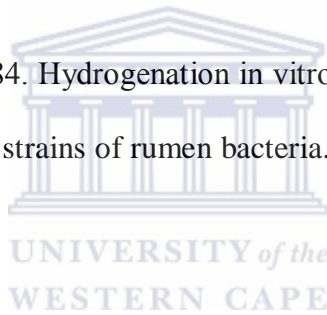
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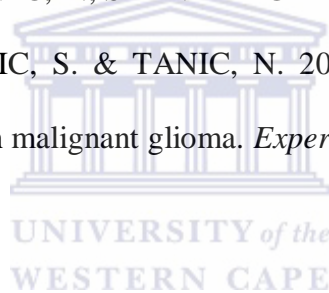
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**CHAPTER 3**

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# THE ANTIOXIDATIVE POTENTIAL OF *CIS9-TRANS11* AND *TRANS10-CIS12* CONJUGATED LINOLEIC ACID IN A RAT LIVER MICROSOMAL MODEL

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## 3.1. Abstract

Intracellular accumulation of peroxides and reactive oxygen species are established targets for cancer therapy intervention as they result in oxidative stress, which cause damage to cellular components such as lipids, proteins and DNA. Unsaturated fatty acids (FA's), found at high concentrations in mammalian cell membranes, are highly susceptible to oxidative stress via the formation of lipid peroxides, hydroxyl radicals and reactive aldehydes. Conjugated linoleic acids (CLA, C18:2) are conjugated dienoic isomers of linoleic acid (LA, C18:2n6) found naturally in the meat and milk of ruminant animals, that are speculated to protect against oxidative stress. This study was aimed to determine the potential anti-oxidative properties of two CLA isomers, *cis9-trans11* (*c9t11*) CLA and *trans10-cis12* (*t10c12*), CLA against iron (Fe<sup>2+</sup>)-induced microsomal lipid peroxidation compared to unconjugated 18-carbon (C18) FA's, including mono-unsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA); containing two and three carbon-carbon double bonds. Iron-induced lipid peroxidation was measured by the production of malondialdehyde (MDA) as a thiobarbituric acid reactive substance (TBARS), utilising rat liver microsomes. Microsomes were pre-exposed (30 min) to the following FA's: oleic acid (OA, C18:1n9) and

vaccenic acid (VA, C18:1n7), linoleic acid; *c9t11* CLA, *t10c12* CLA,  $\alpha$ -linolenic acid (ALA, C18:3n3), and  $\gamma$ -linolenic acid (GLA, C18:3n6). Results, expressed as the 50% inhibitory concentration (IC<sub>50</sub>), indicated that vaccenic acid exhibited the highest (P<0.05) protection against lipid peroxidation followed by OA>LA>*c9t11*>*t10c12*>ALA with GLA exhibiting the weakest protection. The two CLA isomers did not differ from LA or ALA although the *t10c12* CLA isomer tended to exhibit weaker protection than the *c9t11* isomer. The number of double bonds plays a determining role of the FA against lipid peroxidation with GLA being to be significantly (P<0.05) more susceptible than LA. Apart from the number of double bonds, the position of the double bonds from the methyl end also seems to play a role in the susceptibility as VA exhibited a higher protection than OA, ALA more protective than GLA whilst *c9t11* CLA exhibited a higher protection when compared to *t10c12* CLA.

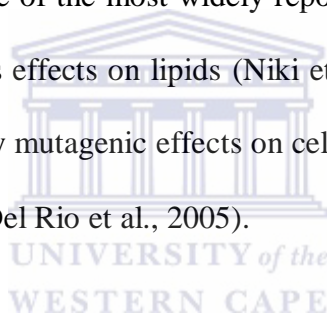


### 3.2. Introduction

Lipid peroxidation is a major mechanism of cellular damage in many biological systems. It involves a process whereby unsaturated FA's react with cellular free radicals, are oxidized and form additional radical species and toxic by-products that can be harmful to the organism. Peroxidation reactions are accompanied by disturbances in cellular membrane structure and function (Esterbauer et al., 1982). Intracellular accumulations of lipid peroxides and products of lipid peroxidation are associated with cellular stress and linked to a variety of diseases, most notably heart disease and cancer. (Nakamura et al., 2012, Del Rio et al., 2005). The effects of lipid peroxides are 100-1000 times more potent than hydrogen peroxide and have shown to be strong inducers of cell death by inactivating protein tyrosine phosphatases, thus regulating the cellular communication of receptor tyrosine kinases (Conrad et al., 2010). Lipid peroxides are themselves unstable, and undergo additional decomposition to form a

complex series of compounds including highly reactive carbonyl compounds (Kulkarni et al., 2013).

Polyunsaturated fatty acids in the cell membrane are especially susceptible to lipid peroxidation when exposed to oxidizing agents, resulting in the formation of lipid peroxides (Song et al., 2000). This gains its importance from the ability of free radicals to initiate oxidative degradation of PUFA's in cell membranes, resulting in membrane damage or production of lipid peroxides through a series of events or cascade (Palacios et al., 2003). PUFA peroxides are broken down resulting in the formation of malondialdehyde (MDA) and 4-hydroxynonenal (HNE), which are found in most biological samples as products of lipid peroxidation, and has become one of the most widely reported components for the detection and estimation of oxidative stress effects on lipids (Niki et al., 2005). These aldehydes have been described to have potentially mutagenic effects on cells through interaction with nucleic acid bases of DNA and protein (Del Rio et al., 2005).



Conjugated linoleic acids (CLA) are conjugated dienoic isomers of linoleic acid (LA) found mostly in the beef and dairy products of ruminant animals. This naturally occurring conjugated polyunsaturated fatty acid (PUFA) has been shown to possess anti-cancer, anti-atherogenesis, anti-hypertensive and anti-obesity properties beneficial to human health (Bhattacharya et al., 2006). Extensive research has been conducted to elucidate the exact cancer protective mechanisms exhibited by CLA, one of which is the potential antioxidative role (McGuire and McGuire, 1999).

Rat liver microsomes are commonly used as substrate to study lipid peroxidation in determining the pro- or anti-oxidant potential of compounds (Esterbauer et al., 1982). In this study the antioxidant potential of CLA isomers in a lipophilic rat liver microsomal model are

compared to other 18 carbon FA, representing the N9, N3 and N6 FA families, differing in number and position of double bonds.

### **3.3. Materials and Methods**

#### **3.3.1. Chemicals**

Sepharose 2B (Sigma-Aldrich CAS# 9050946), 0.01 M potassium phosphate buffer ( $K_2HPO_4$  and  $KH_2PO_4$ ) pH 7.4 supplemented with 1.15% KCl made up with distilled water ( $dH_2O$ ), SDS solution (2% sodium dodecyl sulfate, 20 mM  $NaHCO_3$ , 2 mM EDTA), bicinchoninic acid (BCA) protein assay reagent A (Pierce #23223), reagent B (4%  $CuSO_4 \cdot 5H_2O$ ), bovine serum albumin (BSA) standard 0.1 mg, trichloroacetic acid (TCA) reagent (containing 10% TCA, 1 mM EDTA and 125  $\mu$ l BHT), 0.67% thiobarbituric acid (TBA), 2.5 mM  $FeSO_4$  (Sigma-Aldrich CAS#7782630). All FA were acquired from Sigma-Aldrich: Linoleic acid (CAS# 60333), *c9t11* CLA (CAS# 2540569), *t10c12* CLA (CAS# 2420566), vaccenic acid (CAS# 506172), oleic acid (CAS# 112801),  $\gamma$ -linolenic acid (CAS# 506363),  $\alpha$ -linolenic acid (CAS# 463-40-1), 99% ethanol.

#### **3.3.2. Preparation of rat liver microsomes**

Ethical approval for the use of rodents for standard biochemical techniques was obtained from the MRC Ethics Committee for Research on Animals (ECRA) (Addendum 1). Rat liver homogenate fractions (S9 supernatant fraction) were prepared in 0.01M potassium phosphate buffer containing 1.15M KCl (pH = 7.4) from male Fischer rats with a body weight of approximately 200 g, according to a modified method described previously (Maron and

Ames, 1983). S9 fractions were aliquoted into sterile 50 ml falcon tubes and stored at -80 °C. All procedures were carried out on ice (4°C).

Microsomes were prepared from the S9 fraction according to the method described previously (Gelderblom et al., 1984) by column (2.5 x 40 cm) chromatography using Sepharose 2B with potassium phosphate buffer (pH 7.4) as eluent at 4 °C. Microsomes, eluted in the void volume, were collected in 15 ml falcon tubes and stored at -80 °C.

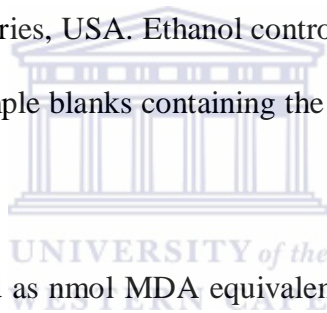
### **3.3.3. Protein determination**

The microsomal protein concentration was determined by the Bicinchoninic Acid (BCA) protein assay (Kaushal and Barnes, 1986). Samples were diluted in SDS solution to denature proteins. A BSA standard was prepared ranging from 1 µg to 10 µg proteins to construct a standard curve. Absorbance readings were determined spectrophotometrically at 562 nm and the unknown protein concentration determined from the standard curve (Addendum 2).

### **3.3.4. Determination of lipid peroxidation**

Antioxidative activity of two CLA isomers, *c9t11* CLA and *t10c12* CLA and other C18 FA; oleic acid (OA, C18:1n9), vaccenic acid (VA, C18:1n7), linoleic acid (LA, C18:2n6),  $\alpha$ -linolenic acid (ALA, C18:3n3) and  $\gamma$ -linolenic acid (GLA, C18:3n6) was monitored against iron (Fe<sup>2+</sup>)-induced lipid peroxidation utilizing rat liver microsomes. The TBARS assay was performed by monitoring the formation of malondialdehyde (MDA) (Esterbauer and Cheeseman, 1990) (Addendum 3). Microsomes were dounced ten times using a tight glass dounce on ice and diluted to contain 1 mg protein per sample in each reaction tube. Stock

solutions of each FA were prepared in ethanol and serially diluted to the required concentration range (mM range; 0.2, 0.4, 0.6, 0.8 and 1 mM). The individual FA's were incubated with microsomes at 37 °C for 30 minutes in a shaking water bath, Fe<sup>2+</sup> (2.5 mM in distilled water) was added to induce lipid peroxidation (MDA formation). The samples were incubated at 37 °C for 1 hour in a shaking water bath after which, 2 ml TCA reagent (composition above) was added to each tube to stop the lipid peroxidation reaction. The samples were mixed by vortexing, centrifuged at 2000 rpm for 15 minutes. A volume of 2 ml of the supernatant was combined and mixed with 2 ml TBA solution in a clean reaction tube. The reaction tubes were tightly capped and incubated in a water-bath at 90 °C for 20 minutes and cooled to room temperature. Absorbance (optical density) was measured at 532 nm on a spectrophotometer, Jenway 67 Series, USA. Ethanol controls samples were included for each FA dilution in addition to the sample blanks containing the working phosphate buffer without the microsomes.



Lipid peroxidation was expressed as nmol MDA equivalents per mg protein using the molar extinction coefficient of  $1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$  at 532 nm for MDA (Esterbauer and Cheeseman, 1990). The percentage reduction of total lipid peroxidation by each FA was obtained from a untreated microsomal Fe<sup>2+</sup> control sample and the 50% inhibitory concentration (IC<sub>50</sub>) were calculated using GraphPad Prism version 5.04 for Windows, GraphPad Software, (La Jolla, California, USA, [www.graphpad.com](http://www.graphpad.com)).

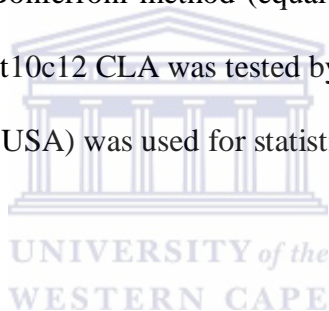
### **3.3.5. Statistical analyses**

Normality among the groups for the 2 parameters was investigated using the Kolmogorov Smirnov Test. An ANOVA with two main effects (Concentration & FA group) and an



interaction effect was used to analyze each parameter. An interaction effect was included to test for a difference in slope (concentrations) for the FA's. A significant interaction effect would indicate that the different concentrations reacted differently for all the FA's. Least Squares Means were calculated, as well as the LSMeans differences and their 95% CI's. Non-parametric Kruskal-Wallis test group differences and Non-parametric Tukey-type test whether the groups differ at 5% level. Statistical analyses were performed with SAS v9.2 and statistical significance was considered at 5% ( $P < 0.05$ ). Inhibitory concentrations at 50% (IC<sub>50</sub>) were compared by ANOVA analyses.

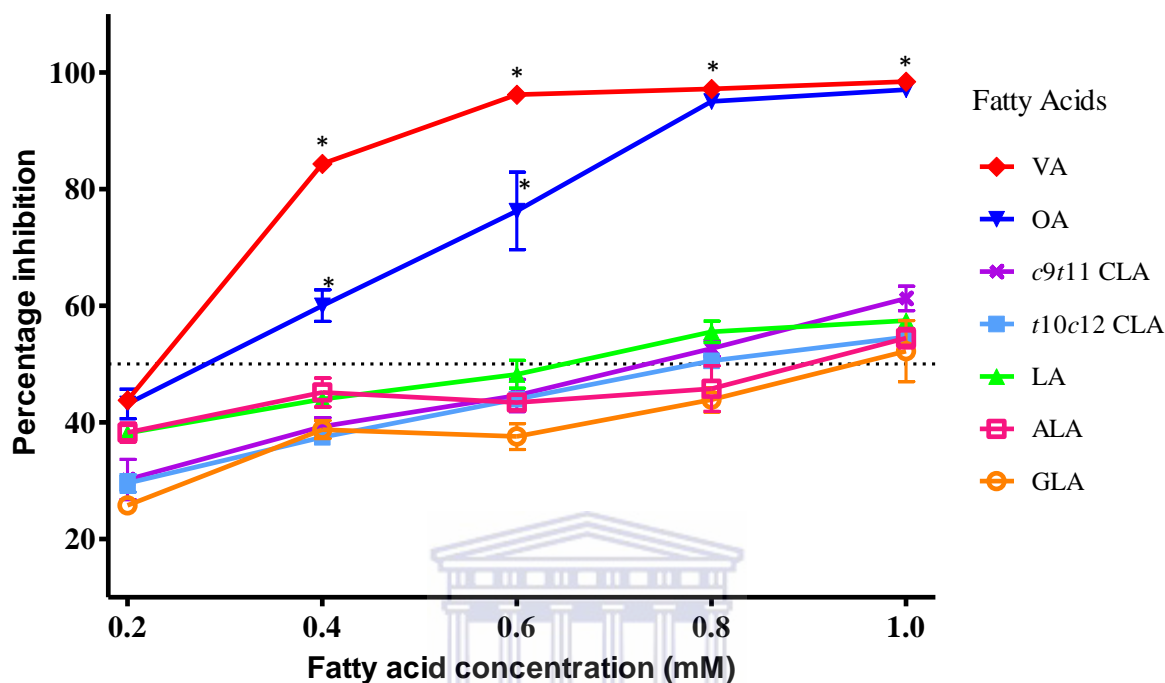
The TBARS IC<sub>50</sub> data was analysed within a one-way ANOVA analysis, with multiple comparison of means using the Bonferroni method (equal variances assumed). Significance between the IC<sub>50</sub> data of LA and *t*10*c*12 CLA was tested by a two-sample t-test. The Stata 11 software (College Station, Texas, USA) was used for statistical analysis.



### 3.4. Results

TBARS assay was used to determine the antioxidant potential of the two major CLA isomers, *c*9*t*11 CLA and *t*10*c*12 CLA, and 5 other C18 unsaturated FA's, representing FA's with 1 (VA and OA), 2 (LA) and 3 (ALA and GLA) double bonds. The dose response curve in Figure 3.1 illustrates the protection against lipid peroxidation presented by each FA at different concentrations. Interaction between percentage reduction and FA concentration revealed typical dose response effects which differ between the different FA's ( $P < 0.05$ ), while IC<sub>50</sub> of the Fe<sup>2+</sup>-induced lipid peroxidation was obtained for all the FA's. The MUFA's, VA and OA, differed significantly ( $P < 0.05$ ) from the other FA's and provided the greatest protection against microsomal membrane lipid peroxidation. The PUFA's showed

comparable protection against lipid peroxidation but showed significantly ( $P<0.05$ ) lower antioxidant potential compared to the MUFA's.



**Figure 3.1: Dose response curves of FA susceptibility to  $Fe^{2+}$ -induced lipid peroxidation.** The dotted line represents 50% reduction in lipid peroxidation compared to untreated control. Percentage inhibition refers to the percentage of lipid peroxidation that had been reduced compared to the lipid peroxidation observed when microsomes were treated with a vector control (ethanol) in the presence of  $Fe^{2+}$ . VA (vaccenic acid), OA (oleic acid), LA (linoleic acid), c9t11 CLA (cis9-trans11 conjugated linoleic acid), t10c12 CLA (trans10-cis12 conjugated linoleic acid), ALA ( $\alpha$ -linolenic acid), GLA ( $\gamma$ -linolenic acid).

The  $IC_{50}$  obtained for each FA are presented in Table 3.1. The FA's indicated in the significant difference column differed at the 5 % level ( $P<0.05$ ). VA and OA exhibited the greatest reduction of lipid peroxidation ( $IC_{50}$ ; 0.11 and 0.19 mM, respectively) which differed significantly ( $P<0.05$ ) from each other and all tested FA's. LA and c9t10 CLA isomer produced a similar  $IC_{50}$  of 0.57 and 0.66 mM respectively, that significantly ( $P<0.05$ ) differs

from VA, OA and GLA. The *t10c12* CLA isomer and ALA produced a similar IC<sub>50</sub> of 0.72 and 0.80 mM respectively, with the CLA isomer differed significantly (P<0.05) from VA, OA, GLA and LA. GLA provided the least inhibition of lipid peroxidation, IC<sub>50</sub>; 0.99 μM, which was significantly (P<0.05) different when compared to VA, OA, both CLA isomers and LA.

**Table 3.1: IC<sub>50</sub> values reflecting the inhibition of Fe<sup>2+</sup>-induced lipid peroxidation of the individual fatty acids.**

Fatty Acid	IC <sub>50</sub> (mM) ± SD	Significant difference
Vaccenic acid (VA, C18:1n7)	0.11 ± 0.04	LA, <i>c9t11</i> , <i>t10c12</i> , ALA, GLA
Oleic acid (OA, C18:1n9)	0.19 ± 0.05	LA, <i>c9t11</i> , <i>t10c12</i> , ALA, GLA
Linoleic acid (LA, C18:2n6)	0.57 ± 0.06	VA, OA, GLA, ALA
<i>cis9-trans11</i> CLA ( <i>c9t11</i> , C18:2)	0.65 ± 0.13	VA, OA, GLA
<i>trans10-cis12</i> CLA ( <i>t10c12</i> , C18:2)	0.72 ± 0.03	VA, OA, GLA, LA
α-Linolenic acid (ALA, C18:3n3)	0.81 ± 0.13	VA, OA, LA
γ-Linolenic acid (GLA, C18:3n6)	0.99 ± 0.07	VA, OA, LA, <i>c9t11</i> , <i>t10c12</i>

IC<sub>50</sub> values are the means ± SD of two repetitions. FA's are presented with common name, abbreviation and chemical notation. Significant difference is indicated when P<0.05. VA (vaccenic acid), OA (oleic acid), LA (linoleic acid), *c9t11* CLA (cis9-trans11 conjugated linoleic acid), *t10c12* CLA (trans10-cis12 conjugated linoleic acid), ALA (α-linolenic acid), GLA (γ-linolenic acid).

The FA's with two double bonds showed interesting responses. Linoleic acid showed a comparable protection than *c9t11* CLA while a significant higher protection was noticed when compared to the *t10c12* isomer. Alpha linolenic acid, containing 3 double bonds,

showed a comparable protection against lipid peroxidation with *t10c12* CLA but differed significantly from *t10c12* CLA, LA and the MUFAs. Except for ALA, GLA differed significantly from all the other FA's and provided the least protection against microsomal lipid peroxidation.

### 3.5. Discussion

The microsomal model used provides a biological system to study the direct effect of the FA on  $\text{Fe}^{2+}$ -induced lipid peroxidation (Hochstein and Ernster, 1963). As a membranous structure lipid model, the microsomes can be subjected to lipid peroxidation when treated with  $\text{Fe}^{2+}$  to investigate the stimulating or protecting effect of unsaturated FA (Wilhelm, 1990). The oxidative potential is given by the ability of FA to be oxidized by free radicals and subsequently form lipid peroxides. It would therefore be of interest to study the interactions of different FA in the microsomal model regarding their possible antioxidant or pro-oxidant potencies. The purpose of this study was to investigate the protective and therefore antioxidant effect of two CLA isomers, *c9t11* CLA and *t10c12* CLA, against  $\text{Fe}^{2+}$ -induced lipid peroxidation. Their protective effect was subsequently compared to selected C18 FA's containing 1 (VA and OA), 2 (LA) and 3 (ALA and GLA) double bonds representing monounsaturated fatty acids (MUFA's) and PUFA's.

Microsomes, pre-treated for 30 minutes with different concentrations of OA, VA, LA, *c9t11* CLA, *t10c12* CLA, ALA and GLA, revealed distinguishable variability in the antioxidant potential against  $\text{Fe}^{2+}$ -induced lipid peroxidation. The greatest protection was obtained by the MUFA's i.e. VA and OA which is in accordance with previous studies and known properties of OA. It was indicated that these MUFA's, are less susceptible to oxidation and

have lower pro-inflammatory activity when exposed to conditions resulting in oxidative stress (Lee et al., 1998). Although MUFA's showed the highest protection against lipid peroxidation than the PUFA's in this experiment, a slight difference in the protective activity, depending on the concentration used, exists between VA and OA. At low concentrations VA appears to be more protective than OA, hence the slight lower IC<sub>50</sub> value obtained. A type of plateau effect is reached at higher concentrations which could point towards a pro-oxidative effect above where no additional protection is obtained.

PUFA's are known to be more vulnerable to lipid peroxidation or rancidity than MUFA's, as they contain two or more double bonds, which are separated by methylene bridges (-CH<sub>2</sub>-) that hold an especially reactive hydrogen atom (Song et al., 2000). To explain the relationship between MUFA and PUFA activity against lipid peroxidation, the lipid peroxidation can be appraised, the initial step is the subtraction of a hydrogen atom from the double bond by an hydroxyl or hydroperoxyl radical to produce lipid radicals (Halliwell and Chirico, 1993). Thus, with MUFA only one free hydrogen atom exists for interaction, therefore they are less susceptible to lipid peroxidation as compared to PUFA's and therefore may act as scavengers of free radicals, exhibiting antioxidant potential.

The current study showed that PUFA is more susceptible to oxidation and therefore less protection was achieved against lipid peroxidation. Of the PUFA containing 2 double bonds the CLA isomers and LA exhibited a higher protection than ALA and GLA (each containing 3 double bonds). Linoleic acid showed the highest protection against lipid peroxidation of the PUFA's containing 2 double bonds. The difference between the PUFA's with 2 double bonds is the position of the double bond relative to the methyl end. LA has the double bond at carbon position 6 (C18:2n6), followed by *c9t11* CLA and *t10c12* CLA isomers, with the double bonds at carbon position 11 and 12, respectively. ALA and GLA, containing 3 double bonds

produced higher IC<sub>50</sub> values and therefore provide less protection and lower antioxidative potential against Fe<sup>2+</sup>-induced lipid peroxidation than the C18:2 FAs. These FA contain 3 double bonds with the first double bond at carbon position 3 and 6, respectively with ALA showing a slight greater susceptibility to lipid peroxidation than GLA. This indicates an apparent relationship between the position of the first double bond closest to the methyl end of the FA chain and the protection against lipid peroxidation.

Both the CLA isomers tend to exhibit a weaker response regarding the protection against lipid peroxidation when compared to LA. The *c9t11* isomer exhibited a similar but weaker protection against lipid peroxidation as compare to LA while the *t10c12* isomer exhibited significant weaker response with a similar activity to ALA (Table 3.1). Confounding evidence on the antioxidant potential of CLA exist in literature. CLA (isolated isomers and mixtures) showed an antioxidative effect when treated with a stable DPPH free radical (2, 2-diphenyl-1-picrylhydrazyl), by a direct free radical scavenging (Ali et al., 2012.). In another study, dietary CLA (0.75% and 1.5% CLA mixtures diet) was reported not to directly affect the oxidative status in Broiler chickens, although hepatic catalase activity increased which may affect hepatic antioxidant defence system and lipid metabolism by increasing hepatic peroxisomal activity (Ko et al., 2004). To the contrary, *c9t11* CLA was reported to increase the susceptibility of breast cancer cells to undergo lipid peroxidation (Devery et al., 2001). The levels tend to increase the vulnerability of the cells above the normal oxidative stress levels and exhibiting a cytotoxic effect that would contribute to the anti-tumour effects of CLA. These studies suggest that CLA may possibly scavenge free radicals present in a healthy system, but due to its polyunsaturated nature, are more susceptible to lipid peroxidation, which may positively affect the anti-tumour mechanism of CLA (Stachowska et al., 2008). Although no significant difference between *c9t11* CLA and *t10c12* CLA isomers was found, they did however show minor difference in the protection against lipid

peroxidation when compared to LA and ALA i.e. less protective than LA and more protective than ALA. The least reduction of Fe<sup>2+</sup>-induced lipid peroxidation was found in GLA treated samples. This indicates that GLA offers little protection against lipid peroxidation. Many studies highlight the lipid peroxidation promoting ability of GLA in cancer cells and, similar to the *t10c12* CLA isomer, this mechanism plays a role in GLA cytotoxicity (Ramanathan et al., 1994 , Ge et al., 2009).

The current study investigated, for the first time, the comparative antioxidant properties of different classes of mono- and polyunsaturated fatty acids. Each individual unsaturated FA reacts differently in response when exposed to free radicals and therefore should be tested separately with respect to dietary recommendations and should not be generally categorized solely as MUFA's and PUFA's, but to extend them to their individual effect related to oxidative stress (Di Nunzio et al., 2011). It became evident that a relationship exists between the number of double bonds, the position of the first double bond closest to the methyl group and susceptibility to lipid peroxidation. It is evident that the more double bonds present, the more susceptible to lipid peroxidation. And the closer the first double bond is to the methyl end of the FA, the less susceptible to lipid peroxidation. These two factors clearly influence the degree of reduction in lipid peroxidation.

In the present study, MUFA exhibited the highest protection against lipid peroxidation, while depending on the CLA isomer; it showed similar antioxidant potential to LA and ALA. Therefore, CLA membrane incorporation and influence of cellular events, such as cell proliferation and apoptosis, need to be evaluated to fully assess the mechanism of chemoprevention of CLA.

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**CHAPTER 4**

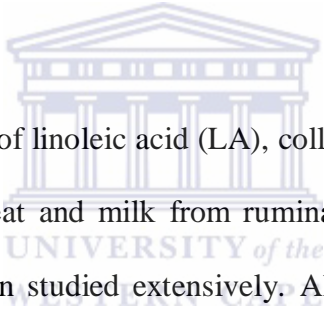
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# THE EFFECTS OF *TRANS10-CIS12* AND *CIS9-TRANS11* CONJUGATED LINOLEIC ACID ISOMERS ON GROWTH PARAMETERS IN HepG2 LIVER CANCER CELLS

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## 4.1. Abstract



Positional and geometric isomers of linoleic acid (LA), collectively called conjugated linoleic acid (CLA), are found in the meat and milk from ruminant animals. There are anticancer properties of CLA that have been studied extensively. Although the exact mechanisms of action are not yet known, CLA has shown to affect apoptosis and cell proliferation, with some isomer and tissue differences. This study aimed to determine the effect of two CLA isomers, *cis9-trans11* (*c9t11*) and *trans10-cis12* (*t10c12*), on the growth and survival indices of HepG2 human hepatocarcinoma cells. Cytotoxicity, cell viability, apoptosis and cell proliferation was measured after 48 hour treatment to determine the effect of the CLA isomers, LA and  $\alpha$ -linolenic acid (ALA) on these parameters. The *c9t11* CLA isomer showed greater cytotoxicity and an increase in apoptosis compared to *t10c12* CLA (all significant,  $P < 0.05$ ). Both CLA isomers significantly reduced cell proliferation ( $P < 0.05$ ). The activity of *c9t11* was comparable to the omega 3 fatty acid, ALA, for all tested parameters, and exhibited a significantly higher activity than LA. Differences existed in anti-proliferative properties of between the two CLA isomers; however both are effective in reducing cell

proliferation a key effect in chemoprevention. The activity of *c9t11* is linked to apoptosis and cytotoxicity, illustrated in this study, but *t10c12* possess a different mechanism that involved cell cycle arrest and senescence.

## 4.2. Introduction


Conjugated linoleic acid (CLA) was originally described as an anticarcinogen isolated from fried ground beef (Ha et al., 1987) and was later discovered to have additional health benefits such as: protecting against obesity (Miner et al., 2001, Kennedy et al., 2010), atherosclerosis (Mooney et al., 2012) and diabetes (Noto et al., 2006, Moloney et al., 2004). To date, *in vivo* animal studies as well as *in vitro* tissue culture studies have demonstrated CLA to have beneficial effects as a chemo-preventative measure, though extensive research is required before it can be used as such. CLA has shown to be an effective agent for reducing the growth and development of a variety of cancer cell types, which are substantiated by extensive research. High CLA levels (*c9t11* and *t10c12* CLA isomers) reduced cell growth, induced apoptosis and also modulated FA metabolism of mammary cells (Keating et al., 2008) with similar responses seen in colon cancer cells (Cho et al., 2003), prostate cancer cells (Ochoa et al., 2004) and liver cancer cells (Melaku et al., 2012). However, the isomer and tissue specific anticancer mechanisms of CLA are yet to be fully defined as some studies have shown differences in activity between the two CLA isomers (Melaku et al., 2012). Phosphoinositide 3-kinase/Protein kinase B (PI3K/Akt) cascade, which promotes cell proliferation, glucose metabolism and survival, is down regulated in mammary cells by addition of CLA – 1:1 *c9t11*: *t10c12* CLA mixture (Bocca et al., 2010). The same mechanism was found to prevail in colon cancer cells (HT29), but included down regulation of ErbB3 signalling (Cho et al., 2003), decrease in insulin-like growth factor (IGF) II synthesis and

down-regulation of extracellular signal-regulated kinase-1/2 pathway and IGF-I receptor signalling (Kim et al., 2003). Moreover, CLA has shown to inhibit the growth of cancer cells through induction of p21 (CIP1/WAF1), a cyclin-dependent kinase inhibitor, thereby regulating the cell cycle progression at G<sub>1</sub> and S phase (Lim et al., 2005).

The aim of this study was to determine the effect of the CLA isomers, *c9t11* CLA and *t10c12* CLA, ability to prevent the survival and growth of human liver HepG2 cancer cell line as compared to LA and ALA by measuring cytotoxicity, cell viability, cell proliferation and apoptotic indices.

### 4.3. Materials and Methods

#### 4.3.1. Chemicals



Promega (South Africa) assay kits: CellTiter-Glo® Luminescent Cell Viability assay (Cat # G7571), Caspase-Glo® 3/7 assay (Cat# ZZG8092), Roche Cell Proliferation ELISA, BrdU (Cat# 11669915001). All FA's were acquired from Sigma-Aldrich (South Africa): linoleic acid (Cat# 60333), conjugated linoleic acids *c9t11* (Cat# 2540569), *t10c12* (Cat# 2420566),  $\alpha$ -linolenic acid (Cat# 463401), 99 % Ethanol (Sigma-Aldrich Cat# 64175), Dulbecco's phosphate buffered saline (DPBS; pH = 7.0 - 7.2, Cat# 14190250). Culture media: Eagle's Minimum Essential Medium (Lonza Cat# BE12-125F) supplemented with 10 % fetal bovine serum (FBS), 1 % non-essential amino acids, 1 % pyruvate and 1 % L-glutamate. Treatment media: Eagle's Minimum Essential Medium supplemented with 5 % FBS, 1 % non-essential amino acids, 1 % pyruvate and 1 % L-glutamate. Trypsin/EDTA (Lonza Cat# CC5012), Hanks' Balanced Salt Solution (HBSS) (Lonza Cat# 10-508F), Phenolphthalein (Analar 37188), 0.1M NaOH, Fatty acid free bovine serum albumin (BSA, Roche Cat# 70335128)

#### **4.3.2. Maintenance of Hep G2 cell culture**

HepG2 cells were obtained from ATCC and cultured in 75 cm<sup>3</sup> flasks with culture medium (above) and sub-cultured at 80% confluency. Culture medium was replaced when necessary.

#### **4.3.3. Preparation of fatty acids for treatment**

Stocks (10 mM) of the individual FAs were coupled with FA-free BSA for treatment of cell cultures, according to a previously described method (Ellsworth et al., 1986). Briefly, the required FAs were measured relative to the amount needed to make up the final concentration and mixed with 1 ml pure ethanol and 1 mg phenolphthalein. A 0.1M NaOH solution was used to titrate the mixture and solvent was evaporated on a 37°C heating block under N<sub>2</sub> gas. The FA salt was then dissolved in DPBS, half the volume required to make the 10 mM solution. A BSA solution (5 mM) was prepared in DPBS, and added to the respective FA salt solution in equal volume to make up a 10 mM FA stock solution required for treatment of the cells.

#### **4.3.4. Fatty acid treatment of Hep G2 cells**

The respective 10 mM FA stock solutions prepared as described above, were diluted with treatment media containing 5% FBS (above) to the required concentrations ranging between 0.01 mM and 0.8 mM. Hep G2 cells were seeded at 20000 cells per well in a 96 well microtitre plate and allowed to attach for 24 hours in culture media (above). Thereafter, the culture media was removed and replaced with 200 µl treatment media containing the FA BSA solution mentioned above at the required concentration between 0.01 mM and 0.8 mM.

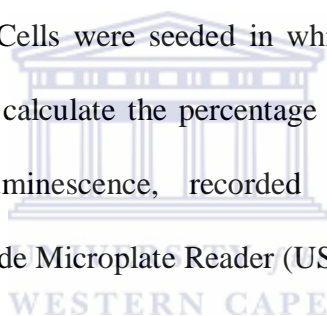


Cells were then incubated for 48 hours and different growth parameters monitored using the appropriate assay kits.

#### **4.3.5. Cell culture assays**

##### **4.3.5.1. Cytotoxicity**

Cytotoxicity was determined by measuring lactate dehydrogenase (LDH) release following 48 hour treatment with FA at concentrations 0.05, 0.1, 0.2, 0.4 and 0.8 mM. LDH was measured using the Promega CytoTox 96® Non-Radioactive Cytotoxicity Assay according to the manufacturer's instructions. Cells were seeded in white 96 well plates; untreated cells were used as positive control to calculate the percentage of viable cells in the experiment. LDH was determined by luminescence, recorded using a Biotek Synergy Mx Monochromator-Based Multi-Mode Microplate Reader (USA).



##### **4.3.5.2. Cell viability**

Cell viability was determined by monitoring ATP production using the Promega CellTiter-Glo® Luminescent Cell Viability assay. Cells were seeded in white 96 well plates and treated with FA at 0.01, 0.05, 0.1, 0.2, 0.4 and 0.8 mM for 48 hours. Untreated cells were used as positive control to calculate the percentage of viable cells in the experiment. ATP was determined according to the manufacturers' kit instruction. Luminescence was recorded using a Biotek Synergy Mx Monochromator-Based Multi-Mode Microplate Reader (USA).

#### **4.3.5.3. Cell proliferation**

Cell proliferation was determined by DNA incorporation of BrdU using the Roche Cell Proliferation ELISA, BrdU. After 48 hour treatment with FA at 0.01, 0.05 and 0.2 mM concentrations, 10 µl BrdU labelling solution was added to each well and incubated for 2 hours at 37°C. BrdU was determined according to the manufacturers' kit instructions by luminescence using a Biotek Synergy Mx Monochromator-Based Multi-Mode Microplate Reader (USA).

#### **4.3.5.4. Apoptosis**

Caspase 3, as an indicator of apoptosis, was measured using the Promega Caspase-Glo<sup>®</sup> 3/7 assay. Cells were seeded in 96 well plated and treated with FA at 0.1, 0.2, 0.4 and 0.8 mM concentrations for 48 hours. Caspase 3 was determined according to the manufacturers' kit instruction. Luminescence was recorded using a Biotek Synergy Mx Monochromator-Based Multi-Mode Microplate Reader (USA).

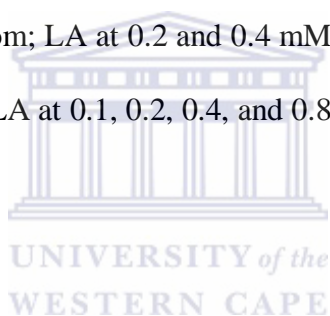
#### **4.3.6. Statistical analyses**

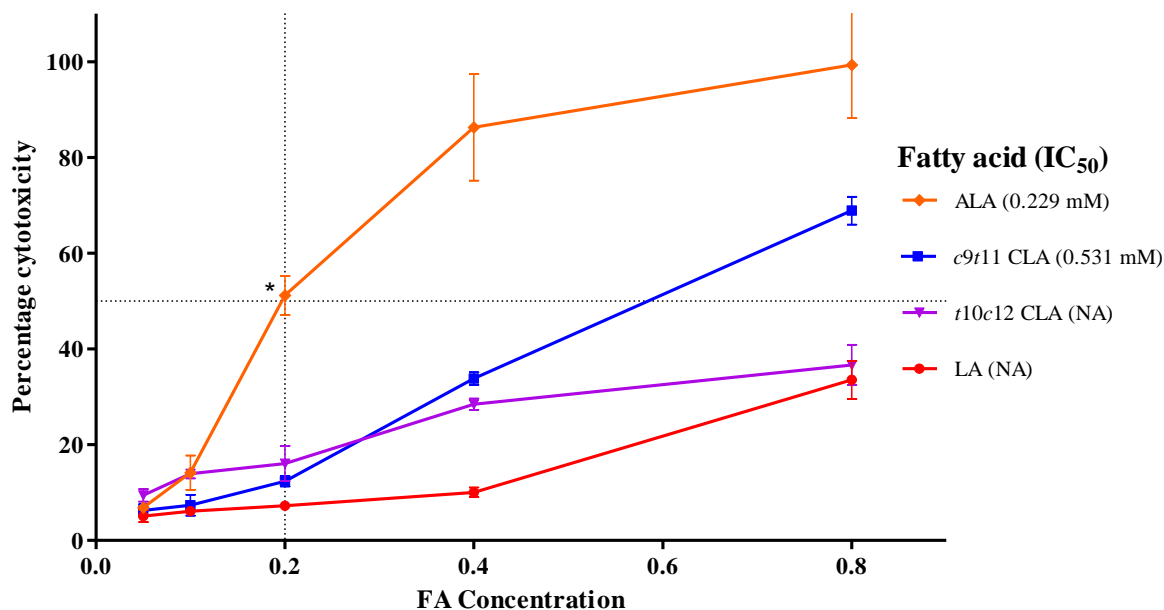
All experiments were carried out in quadruplicate and repeated for consistency. The statistical significance between groups was assessed by analysis of variance (ANOVA). All statistical analyses were performed with SPSS and the IC<sub>50</sub> for each assay determined using GraphPad Prism version 5.04 for Windows, GraphPad Software, (La Jolla, California, USA, [www.graphpad.com](http://www.graphpad.com)).

## 4.4. Results

### 4.4.1. Cytotoxicity

Percentage cytotoxicity, as determined by LDH release, of HepG2 cells, following treatment with different concentrations of *c9t11* CLA, *t10c12* CLA, LA and ALA for 48 hours (Figure 4.1). ALA showed the greatest cytotoxicity. CLA isomers and LA were comparable below 0.2 mM however, *c9t11* CLA showed greater cytotoxicity than *t10c12* CLA and LA at higher concentrations. Descriptive statistics comparing differences between concentrations showed that the *c9t11* CLA differed significantly ( $P < 0.05$ ) from; the *t10c12* CLA isomer at 0.8 mM, LA at 0.4 mM and 0.8 mM, and ALA at 0.2, 0.4 and 0.8 mM. The *t10c12* CLA isomer differed significantly ( $P < 0.05$ ) from; LA at 0.2 and 0.4 mM and ALA at 0.2, 0.4 and 0.8 mM. LA differed significantly from ALA at 0.1, 0.2, 0.4, and 0.8 mM.



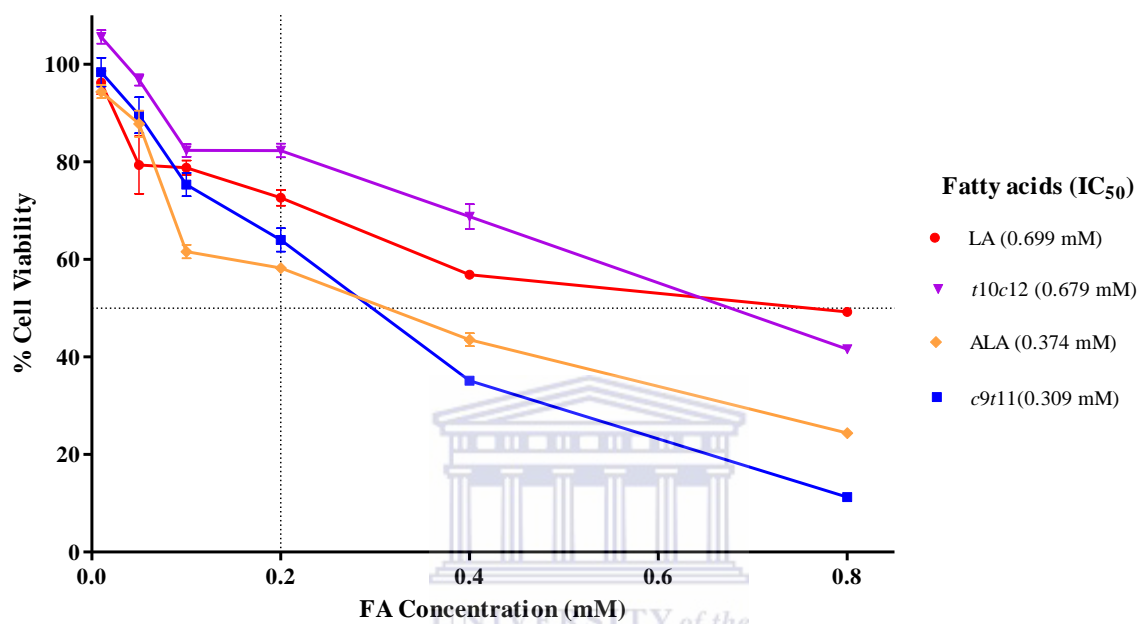


**Figure 4.1: Percentage cytotoxicity as determined by LDH release on Hep G2 cancer cells following treatment with *c9t11* and *t10c12* CLA isomers, LA and ALA for 48 hours.** Percentage cytotoxicity is presented as a mean (n=4) percent of untreated controls (with standard error bars). The broken line represents 50 % reduction in cell proliferation. The \* denotes significantly different (p<0.05) activity to other FAs at the same concentration (0.2 mM). LDH, lactase dehydrogenase; FA, fatty acid; LA, linoleic acid; ALA, alpha-linolenic acid; CLA, conjugated linoleic acid; NA, not available, IC<sub>50</sub> was not reached within the tested concentration range for LA and *t10c12* CLA.

#### 4.4.2. Cell Viability

Percentage viability of HepG2 cells following treatment with different concentrations of *c9t11* CLA, *t10c12* CLA, LA and ALA for 48 hours is illustrated in Figure 4.2. Cell viability decreased in a dose-dependent manner in all FA treatment groups. Comparing the slopes for each treatment group, all FA produced statistically significant (P<0.05) slope differences response, indicating that all treatment FAs responded differently. The median inhibitory concentrations (IC<sub>50</sub> ± SD) for *c9t11* CLA, *t10c12* CLA, LA and ALA were calculated to be 0.309 ± 0.063, 0.679 ± 0.035, 0.69 ± 0.064, and 0.374 ± 0.035 mM respectively. Comparing

the  $IC_{50}$ <sup>s</sup> revealed no significant difference between treatment with *c9t11* CLA and ALA and between treatment with *t10c12* and LA. These two groups (*c9t11* CLA; ALA and *t10c12* CLA; LA) differed significantly ( $P < 0.05$ ), indicating that *c9t11* CLA produced a significantly greater cytotoxic effect than *t10c12* CLA.

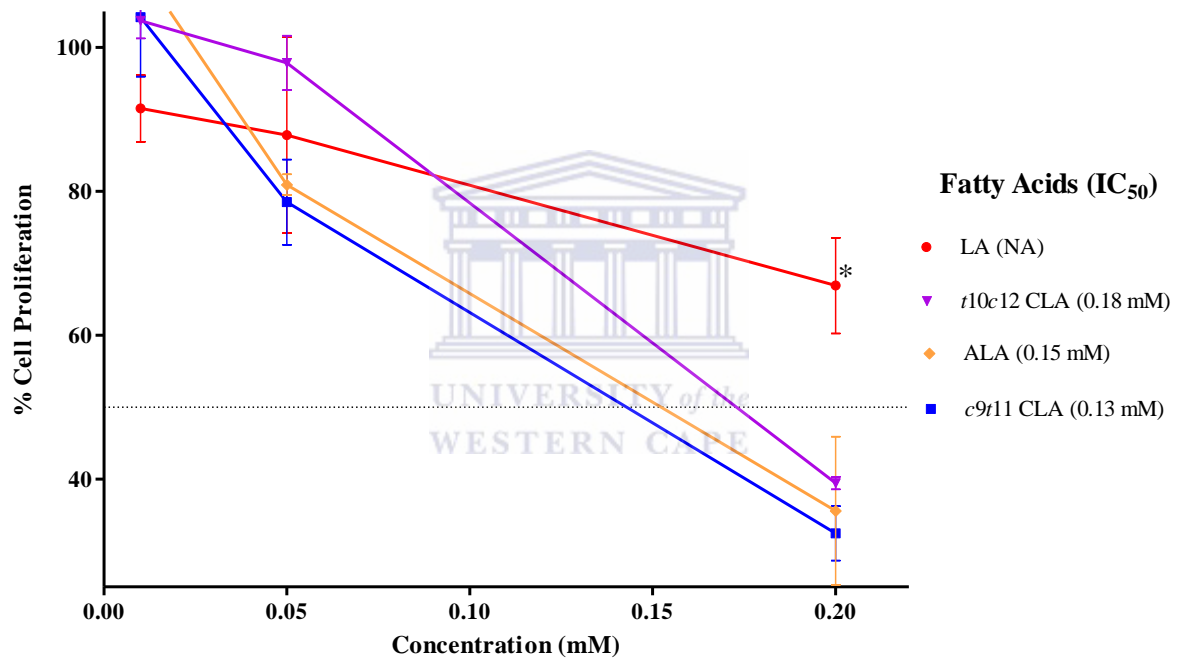


**Figure 4.2: Viability (ATP) of Hep G2 cancer cells following treatment with *c9t11* and *t10c12* CLA isomers, LA and ALA for 48 hours.** Percentage viability is expressed as mean ( $n=4$ ), with SEM error, percentage of treated against untreated controls. The horizontal dashed line denotes 50% reduction in cell viability. The vertical dashed line represents 0.2 mM, the highest tested concentration at which all FA are below the  $IC_{50}$ , and common concentration for all tested parameters. FA, fatty acid; LA, linoleic acid; ALA, alpha-linolenic acid; CLA, conjugated linoleic acid

#### 4.4.2. Cell proliferation

Cell proliferation was measured as a percentage of DNA incorporated BrdU present following treatment with the respective FA concentrations for 48 hours (Figure 4.3). The treatment concentrations were selected on the basis below the cell viability  $IC_{50}$ , where cells are still

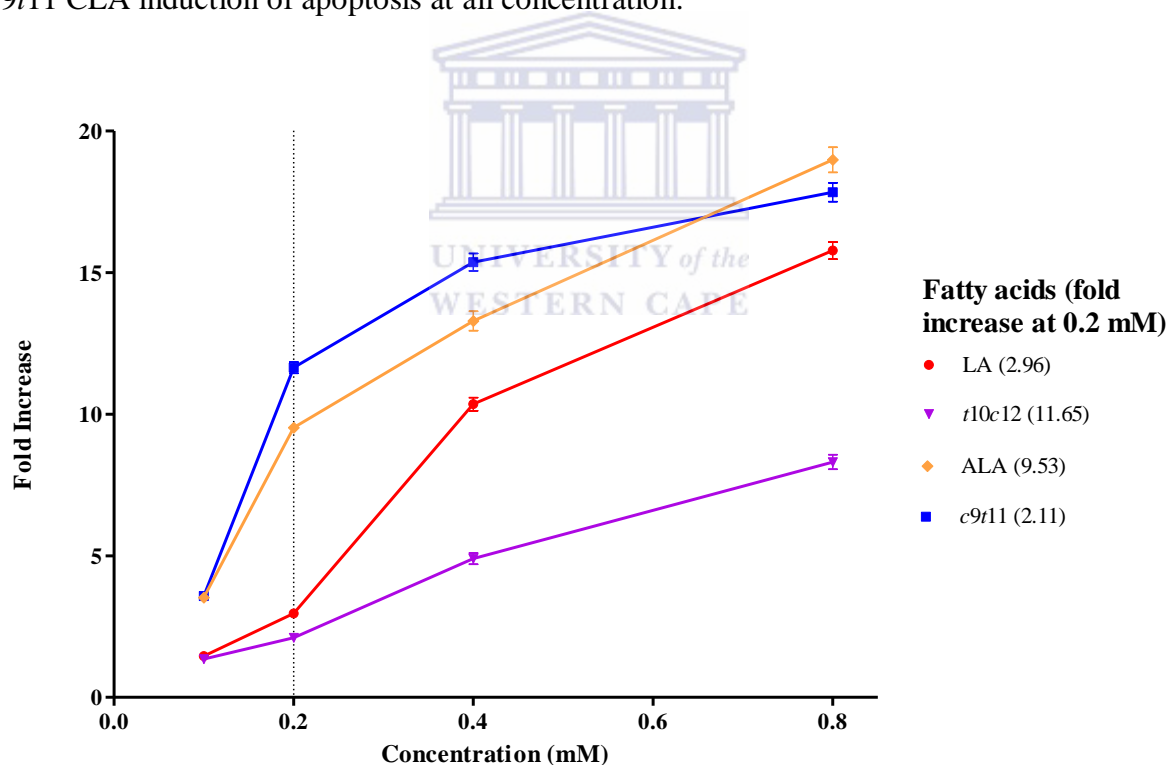
actively producing ATP. Overall, a dose dependent reduction in cell proliferation was observed with all FA treatments, with *c9t11* CLA and ALA showing the highest and LA the least response. When comparing the means, a significant difference was found between concentrations ( $P<0.05$ ) but not between the various FAs, although LA produced a weaker response. Comparing the slopes of Figure 4.3, LA was significantly different ( $P<0.05$ ) from *c9t11* CLA, *t10c12* CLA and ALA. No significant difference was found between *t10c12* CLA and *c9t11* CLA.



**Figure 4.3: Percentage cell proliferation on Hep G2 cancer cells following treatment with *c9t11* and *t10c12* CLA isomers, LA and ALA for 48 hours.** Percentage cell proliferation is presented as a mean ( $n=4$ ) percent of untreated controls (with standard error bars). The broken line represents 50 % reduction in cell proliferation. The \* denotes significantly different ( $p<0.05$ ) activity to other FAs at the same concentration (0.2 mM). FA, fatty acid; LA, linoleic acid; ALA, alpha-linolenic acid; CLA, conjugated linoleic acid; NA, not available, IC50 was not reached within the tested concentration range.

### 4.3. Apoptosis

Apoptosis was measured by the fold increase in caspase 3 levels of Hep G2 cells following treatment with the two CLA isomers, LA and ALA when compared to untreated control after 48 hours (Figure 4.4). A dose-dependent fold increase in caspase 3 was found for all FA groups. Pairwise differences between the means of each FA treatment were significant ( $P < 0.05$ ). Comparing the slopes revealed that *t10c12* CLA treatment differed significantly ( $P < 0.05$ ) from *c9t11* CLA, LA and ALA. At low concentrations (0.1 and 0.2 mM) *t10c12* CLA and LA had a similar effect on apoptosis but at higher concentrations (0.4 and 0.8 mM) LA response was greater. There is statistical significance ( $P < 0.05$ ) between *t10c12* CLA and *c9t11* CLA induction of apoptosis at all concentration.



**Figure 4.4: Apoptotic effect of *c9t11* and *t10c12* CLA isomers, LA and ALA on Hep G2 cancer cell line.** Fold increase refers to the increase in caspase 3 following 48 hour treatment compared to untreated controls. Plotted are the means ( $n=4$ ) with standard error. The broken line indicates 0.2 mM, which is below the apoptosis  $IC_{50}$  for all treatments. FA, fatty acid; LA, linoleic acid; ALA, alpha-linolenic acid; CLA, conjugated linoleic acid

## 4.5. Discussion

The purpose of this study was to evaluate the effect of two CLA isomers, *c9t11* CLA and *t10c12* CLA, in comparison to LA and ALA on HepG2 liver cancer cell survival. These FAs are 18 carbons in length and differ only in the position of their double bond and geometric configuration, with the exception of ALA which is an omega 3 FA with three double bonds. The measuring of cell growth parameters, including cytotoxicity, viability, cell proliferation and apoptosis, are essential in the development of cancer. These factors are amongst the most common known targets for assisting in developing an effective chemo-preventive or chemotherapeutic agent (Hanahan and Weinberg, 2011).

Cytotoxicity was measured by the release of LDH by dead cells. The analysis showed that ALA was the most cytotoxic of the tested FA, with an  $IC_{50}$  of 0.23 mM, followed by the *c9t11* CLA isomer,  $IC_{50}$  0.53 mM. The *t10c12* CLA isomer and LA were less cytotoxic and their respective  $IC_{50}$  were outside the tested FA concentration range (i.e.  $IC_{50} > 0.8$  mM).

Cell viability decreased dose-dependently for all FA; however two groupings could be distinguished. The *c9t11* CLA isomer and ALA showed the greatest decrease in cell viability with the lowest  $IC_{50}$  ( $0.309 \pm 0.063$  and  $0.374 \pm 0.035$  mM, respectively), whereas *t10c12* CLA isomer and LA showed a weaker response with concomitant higher  $IC_{50}$  ( $0.679 \pm 0.035$  and  $0.69 \pm 0.064$  mM, respectively).

Various studies have demonstrated that the cytotoxic effect of the two major CLA isomers, *c9t11* CLA and *t10c12* CLA, are different and tissue specific. The *c9t11* CLA was more cytotoxic than *t10c12* CLA in rat hepatic stellate cells (HSC-T6) (Yun et al., 2008) whereas in mammary cells (MCF-7) the effects was greater for *t10c12* CLA compared to *c9t11* CLA (Amarù and Field, 2009). One of the proposed mechanism of CLA against the growth of



cancer cells are the modulation of cell cycle events such as inhibition of cell proliferation and induction of apoptosis (Kelley et al., 2007, Melaku et al., 2012).

The *c9t11* CLA isomer and ALA displayed the greatest inhibition of cell proliferation and a consequential increase in apoptosis dose dependently. These findings are consistent with previous studies on ALA and omega-3 FAs as being cytotoxic, inhibiting cell proliferation and inducers of apoptosis on SP 2/0 mouse myeloma cells at concentrations of 5 µg/ml (Sravan Kumar and Das, 1997). ALA has shown to positively affect apoptosis by inhibiting the PI3K (phosphoinositide-3-kinase) pathway and Bcl-2 (B-cell lymphoma 2) expression in BCR-ABL positive leukemic cells (Beaulieu et al., 2011). Bcl-2 protein family are a major tumour suppressor gene family. It is well known that an increase in Bax/Bcl-2 ratio, both of which belong to the Bcl-2 family of genes, promotes apoptosis of cancer cells (Salakou et al., 2007). Regarding nuclear morphology and flow cytometric analysis, *c9t11*CLA inhibited viability and triggered apoptosis through increased expression of caspase-3 as well as the ratio of Bax/Bcl-2 genes (Wang et al., 2013).

The *t10c12* CLA isomer and LA were similar regarding their effect on cytotoxicity and cell viability, while at concentrations below 0.2 mM, cell proliferation and apoptosis were similar as well. However, LA increased apoptosis whereas *t10c12* CLA reduced cell proliferation at concentrations greater than 0.2 mM. The collective effects observed at 0.2 mM are shown in Table 4.1. This concentration (0.2 mM) was selected to show how the two CLA isomers differ from each other as well as their comparative effect to LA and ALA. The concentration of 0.2 mM was selected because it is below the IC<sub>50</sub> for all FA. At concentrations greater than the cell viability IC<sub>50</sub>, cytotoxicity is a confounding factor. Above the IC<sub>50</sub>, necrosis would interfere with apoptosis and cell proliferation analyses; there would be an insufficient amount of viable cells in the assay to acquire an accurate

measurement of these parameters. At 0.2 mM the *c9t11* CLA isomer had comparable effects to ALA for all tested parameters i.e. reduced cell viability, inhibition of cell proliferation and increased apoptosis. The *t10c12* CLA isomer was comparable to LA with regard to cell viability and apoptosis, but showed greater inhibition of cell proliferation similar to that of *c9t11* CLA and ALA.

**Table 4.1:** Comparing growth parameters of Hep G2 cells following treatment with two CLA isomers, LA and ALA for 72 hours at 0.2mM concentration.

Cell parameters	LA	<i>t10c12</i>	<i>c9t11</i>	ALA
Cytotoxicity (%)	7.26±0.42 <sup>a</sup>	16.08±3.65 <sup>b</sup>	12.36±1.09 <sup>ab</sup>	51.18±4.11 <sup>c</sup>
Cell Viability (%)	72.61±3.19 <sup>a</sup>	82.32±2.81 <sup>b</sup>	63.99±4.76 <sup>c</sup>	58.21±1.28 <sup>c</sup>
Cell proliferation (%)	72.61±8.4 <sup>a</sup>	39.44±1.73 <sup>b</sup>	32.46±7.56 <sup>b</sup>	35.58±20.59 <sup>b</sup>
Apoptosis (fold increase)	2.97±0.15 <sup>a</sup>	2.11±0.12 <sup>b</sup>	11.65±0.39 <sup>c</sup>	9.53±0.23 <sup>d</sup>

Means with SD are compared within tested parameter. Differing superscript letters indicate significant difference (P<0.05). LA, linoleic acid; ALA, alpha-linolenic acid; CLA, conjugated linoleic acid.

Therefore, at a concentration of 0.2 mM distinguishable differences were observed between *c9t11* CLA and *t10c12* CLA (Table 4.1). The *c9t11* CLA isomer reduced cell viability and increased apoptosis more effectively than *t10c12* CLA even though there is no difference in the inhibition of cell proliferation. These findings suggest that *t10c12* CLA may possess anticancer properties that to a lesser extent involve apoptosis and more actively involve effects on cell growth involving cell cycle arrest and senescence. Of interest is that, *t10c12* CLA was shown to be a greater inhibitor of the genes regulating cell cycle and growth than *c9t11* CLA (Kelley et al., 2007).

Initially, it was thought that the anti-tumour properties of CLA were reduction of cell viability and inhibition cell proliferation, and increasing apoptosis (Khanal, 2004). In the present study, clear differences existed in the activity between the two CLA isomers, *c9t11* CLA and *t10c12* CLA were shown, which are substantiated in previous findings (Kelley et al., 2007, Khanal, 2004). A study showed that different anti-tumour mechanisms exist for these two isomers when CLA decreased prostate cancer cell proliferation. The *t10c12* CLA isomer increased apoptosis by decreasing bcl-2 gene expression and increasing p21<sup>WAF1/Cip1</sup> mRNA, whereas *c9t11* CLA increased apoptosis and decreased cell proliferation by increasing 5-lipoxygenase (5-LOX) expression and cyclooxygenase-2 (COX-2) protein level, which are involved in formation of arachidonic acid-derived eicosanoids (Ochoa et al., 2004).

Loss of cellular growth control and cellular senescence are two hallmarks of cancer described in previous studies (Hanahan and Weinberg, 2011). These factors are critical for cancer development. Cellular senescence has been defined by the loss of proliferative potential, losing the cells ability to proliferate. When a growth signal is reintroduced it typically stimulates cellular hypertrophy and not proliferation (hyperplasia). Therefore cells increase in cell size and not cell number. This should be distinguished from cell cycle arrest, which is avoided by cancer cells to continue proliferation despite unfavourable conditions (Blagosklonny, 2011). Some tumour suppressors (Rb and p16) cause cell cycle arrest while others (PTEN and TSC1/2) inhibit the growth promoting mTOR pathway involved in senescence (Blagosklonny, 2011). The p53 protein is the only identified tumour suppressor that both causes cell cycle arrest and inhibit senescence (Li et al., 2012). The *t10c12* CLA has been shown to inhibit cell proliferation via the p53 pathway (Kemp et al., 2003). Further studies should study the effect of CLA isomers on tumour suppressor genes, cyclin dependent kinases and checkpoint proteins to fully understand the different anticancer properties of CLA isomers.

It is not known at present what the interaction of the different CLA isomers are with the different fatty acid classes including saturated (SATS), monounsaturated (MUFA) and the polyunsaturated fatty acids (PUFAs) within the cell. As many of these FA plays an important role in cell biology the modulating effect of the different CLA isomers are therefore on interest.



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**CHAPTER 5**

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# THE EFFECT OF *CIS9-TRANS11* AND *TRANS10-CIS12* CONJUGATED LINOLEIC ACID ON THE LIPID PROFILE OF HEPG2 CELLS

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## 5.1. Abstract

Although the biological effects of conjugated linoleic acids (CLA) have been studied extensively, their mechanisms of action remain unclear. They have shown to reduce the growth and progression of cancer cells amongst other health benefits including anti-atherogenic and anti-adipogenic activity. The aim of this study is to determine the effect of *c9t11* CLA and *t10c12* CLA isomers on the lipid profile of HepG2 cells and how they compare to linoleic acid (LA) and  $\alpha$ -linolenic acid (ALA). Briefly, cells were supplemented with concentration of FA-BSA complexes for 48 hours. FA were extracted, separated into respective phospholipid fractions (PC and PE) and measured by gas chromatography. Cholesterol and phospholipid concentrations were also quantified by cholesterol enzymes and malachite green methods respectively. No significant effect was seen on cholesterol and phospholipid concentration in all treated samples. The *c9t11* CLA isomer increased the concentration of all fatty acids in both the PC and PE phospholipid fractions. The *t10c12* CLA isomer significantly reduced the MUFA concentration in both PC and PE fractions. LA and ALA expectedly increased N6 and N3 FA concentrations, respectively. The results show that *t10c12* CLA and *c9t11* CLA have varying effects on HepG2 cancer cell membrane composition. This may partially explain their effect on cancer cell growth and development.

Altering the membrane FA composition results in modification of a number of downstream events including; prostaglandin production, inflammation and lipid peroxidation or cellular stress events. Future studies including cycle events and gene expression are required to fully uncover the anticancer effect of CLA.

## **5.2. Introduction**

Conjugated linoleic acid (CLA) is a group name for the positional isomers of linoleic acid, which are characterized by conjugated double bonds. They are intermediate products in the biohydrogenation of unsaturated fatty acids (FA) by microorganisms in the rumen. CLA can also be produced endogenously in tissues, such as the mammary gland of ruminants (Adlof et al., 2000). Several positive physiological effects are reported for CLA, like anti-carcinogenic, anti-atherogenic and immuno-modulatory properties (Bhattacharya et al., 2006). Supplementations with FA can influence the function of immune cells and epithelial cells in the liver and colon through different mechanisms, i.e. change the FA profile of the cellular membrane and subsequently affecting signal transduction pathways and lipid mediators like prostaglandin E2 (Calder, 2013, Serhan and Chiang, 2002). For example CLA supplementation led to decreased lymphocyte activation of healthy men (Tricon et al., 2004) and declined proliferative response in rat splenocytes (Renner et al., 2012). The CLA isomers incorporation profiles in the cell membrane differ greatly from that of LA; this may well lead to membrane function alterations. Dietary FA intake has shown to alter the lipid profile of cellular membranes. Changing the lipid composition on the cell membrane ultimately modifies cellular functions (Gill and Clark, 1980).

Polyunsaturated fatty acids (PUFAs) can induce growth inhibition and cytotoxicity of tumour cells *in vitro* via mechanisms involving free radical generation (Das et al., 1987) resulting in lipid peroxidation (Hawkins et al., 1998) and cell cycle arrest (Palakurthi et al., 2000). CLA isomers showed comparable results to iron induced lipid peroxidation to LA, which was not as effective at protecting against lipid peroxidation as mono-unsaturated FA (MUFA). The CLA isomers also showed a marked effect on cell proliferation and apoptosis, by increasing apoptosis and decreasing cell proliferation in varying degrees.

The purpose of this study was to determine the effect of the two main CLA isomers; *cis*9-*trans*11 (*c9t11*) and *trans*10-*cis*12 (*t10c12*) CLA, as well as two essential fatty acids; linoleic acid (LA) and alpha-linolenic acid (ALA) on FA incorporation, phospholipids and cellular cholesterol.



### 5.3. Materials and Methods

#### 5.3.1. Chemicals

All FA's were acquired from Sigma-Aldrich (South Africa): linoleic acid (Cat # 60333), conjugated linoleic acids *c9t11* (Cat# 2540569), *t10c12* (Cat# 2420566),  $\alpha$ -linolenic acid (Cat # 463401), 99 % ethanol (Sigma-Aldrich Cat # 64175), Dulbecco's phosphate buffered saline (DPBS; pH = 7.0 - 7.2, Cat# 14190250). Culture media: Eagle's Minimum Essential Medium (Lonza Cat # BE12-125F) supplemented with 10 % fetal bovine serum (FBS), 1 % non-essential amino acids, 1 % pyruvate and 1 % L-glutamate. Treatment media: Eagle's Minimum Essential Medium supplemented with 5 % FBS, 1 % non-essential amino acids, 1 % pyruvate and 1 % L-glutamate. Trypsin/EDTA (Lonza Cat # CC5012), Hanks' Balanced Salt Solution (HBSS) (Lonza Cat# 10-508F), phenolphthalein (Analar 37188), 0.1M NaOH,

Fatty acid free bovine serum albumin (BSA, Roche Cat # 70335128), chloroform, methanol, butylated hydroxytoluene (BHT), thin layer chromatography (TLC) solvent: chloroform:methanol:petroleum benzene:acetic acid:boric acid (40:20:30:10:1.8; v/v/v/v/w), 2,5-bis-(5'-tert-butylbenz-oxazolyl-2')thiophene (BBOT, CAS# 7128-64-5), saline, CMS (chlorophorm:methanol:saline, 86:14:1), Malachite green phosphate detection kit from R&D Systems (Cat # DY996), 6 N KOH, perchloric acid, BCA Protein Assay Reagent A (Pierce #23223), Reagent B (4 % CuSO<sub>4</sub>.5H<sub>2</sub>O), 0.1 mg BSA standard, glacial acetic acid, anhydrous sodium sulphate, Triton X-100, SDS solution (2% sodium dodecyl sulphate, 20 mM NaHCO<sub>3</sub>, 2 mM EDTA)

### 5.3.2. Maintenance of HepG2 cell culture

HepG2 cells (ATCC HB-8065) were cultured in 75 cm<sup>3</sup> flasks with culture mediums described above and sub-cultured at 80% confluency. Culture medium was replaced when necessary.

### 5.3.3. Preparation of fatty acids

Stocks (10 mM) of the individual FAs (*c9t11*, *t10c12*, ALA, LA) were complexed with FA-free BSA for treatment of cell cultures, according to a previously described method (Ellsworth et al., 1986). Briefly, the required FAs were measured relative to the amount needed to make up the final concentration and mixed with 1 ml pure ethanol and 1 mg phenolphthalein. A 0.1M NaOH solution was used to titrate the mixture and solvent was evaporated on a 37°C heating block under N<sub>2</sub> gas. The FA salt was then dissolved in DPBS,

half the volume required to make the 10 mM solution. A BSA solution (5 mM) was prepared in DPBS and added to the respective FA salt solution in equal volume to make up a 10 mM FA stock solution required for treatment of the cells.

#### **5.3.4. Fatty acid treatment of HepG2 cells**

The respective 10 mM FA stock solutions prepared as described above, were diluted with treatment media containing 5% FBS (above) to the required concentrations ranging between 0.01 mM and 0.8 mM.

HepG2 cells were trypsinated, spun down and seeded at  $4 \times 10^6$  cells per 10 cm petri dish in 7 ml culture medium and allowed to attach for 24 hours. Thereafter, the culture medium was removed and replaced with 8 ml treatment medium containing the FA-BSA solution prepared above. Cells were then incubated for 48 hours and collected for determining FA, phospholipid and cholesterol content.

#### **5.3.5. Lipid extraction**

After the 48 hour incubation period, cells from 3 petri dishes were scraped off, combined and lipids were extracted with chloroform/methanol (CM; 2:1; v/v) containing 0.01% BHT. Briefly, the method adapted from Folch et al. (1957): the HepG2 cells were resuspended in 0.5 ml saline in a 40ml Teflon-capped glass tube and 0.1 ml was removed for protein determination. CM (12 ml) was added to the 0.4 ml resuspended cells and shaken for 20 minutes. Saline saturated with CMS (4 ml) was added, mixed, centrifuged for 15 minutes and 500 rpm and the lower chloroform-rich phase was transferred to a round bottom flask. The

cell suspension was re-extracted with 10 ml CMS, as above, and the bottom phase were combined with the previous in the round bottom flask. This procedure was repeated once more. The combined lower phases were evaporated on a rota-vapor and the dried lipid extract was washed over into a 12 ml Teflon-capped glass tube with 5 X 2 ml CMS. Thereafter, 1 ml saline saturated with CMS was added, the contents mixed, centrifuged at 500 rpm for 15 min and the top aqueous phase was removed. The CMS was evaporated under N<sub>2</sub> gas in a 37°C water bath till dry, rinsed with 3 X 1 ml CMS while drying and then the tubes containing the dried lipid extract were placed on ice for phospholipid fractionation by TLC.

#### **5.3.6. Thin layer chromatography (TLC)**

The dried lipid extracts, from above, were dissolved in ice cold CMS (100ul) and 2x20 µl was removed for cholesterol determination in a separate glass tube. A further 60 µl was removed for phospholipid fractionation on silica TLC glass plates and applied on 2 separate 10 x 20 mm silica TLC plates for FA (30 µl) and phospholipids (30 µl) analyses. The plates were developed using the TLC solvent (above) containing the fluorescent agent, for 90 minutes. The plates were dried under N<sub>2</sub> gas and BBOT solution was used to visualize the phospholipid fractions under a UV light. The lipid fractions phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were identified against a phospholipid standard containing different phospholipid fractions.



### 5.3.7. Fatty acid analyses

For FA analyses the phospholipid fractions, PC) and (PE, were scraped off the TLC plates into separate Teflon-capped glass tubes. For FAME preparation by base catalysis, it was necessary to first extract the phospholipids from the silica (Christie et al., 2007): 2 ml chlorophorm: methanol: water (C:M:W; 5:5:1) was added to the silica samples, mixed and centrifuged for 10 minutes at 1000 rpm . The supernatant was transferred to a clean Teflon-caped glass tube. The silica was re-extracted with a further 2 ml CMW and the upper solvent phase was combined with the previous extract. The CMW was then evaporated under N<sub>2</sub> gas in a 37°C water bath until dry. Thereafter, FAME were prepared from the dried extract as follows (Christie et al., 2007): 12 µg C17:0 (internal standard) was added together with 1 ml hexane and mixed to dissolve the dried extract. To this mixture, 40 µl sodium methoxide was added and 40 µl methylacetate, the tube mixed, sealed with N<sub>2</sub> gas and placed on a 50°C heating block for 15 minutes. Thereafter, glacial acetic acid (100 µl) was added to neutralize the methylation reaction as well as anhydrous sodium sulphate (1 mg). The solution was mixed and centrifuged at 100 rpm for 10 minutes. The upper solvent phase containing the FAME was transferred to a new Teflon-capped glass tube, evaporated under N<sub>2</sub> gas and dissolved in 10 µl hexane before injecting into a GC (Varian 3300 gas chromatograph) equipped with 30 m fused silica BPX-70 capillary columns (SGE, USA) of 0.32 mm internal diameter.

### 5.3.8. Phospholipid determination

The phospholipid fractions PC and PE were determined colorimetrically using the Malachite green phosphate detection kit from R&D Systems. Briefly, PC and PE fractions were

fractionated by TLC and collected into glass tubes as described above. The dry silica samples were digested in perchloric acid (i.e. PC: 400  $\mu$ l; PE: 300  $\mu$ l) on a 170°C heating block for two hours. After digestion, 6 N KOH (i.e. PC: 775  $\mu$ l; PE: 581  $\mu$ l) and distilled water was added to the samples at a ratio of 5: 1. Samples were mixed and centrifuged at 1000 rpm for 15 minutes and 80  $\mu$ l of the samples were transferred to 96 well microtitre plates in duplicate. The assay was carried out as per the manufacturers' instructions and the colour development was measured spectrophotometrically at 620 nm and referenced against a phosphate standard curve.

#### **5.3.9. Cholesterol determination**

The cholesterol content of HepG2 cells was determined by an enzymatic iodide method using cholesterol-oxidase and cholesterol-esterase (Richmond, 1973). Briefly, 20  $\mu$ l of the lipid extract was used per unknown sample; the solvent was removed under N<sub>2</sub> gas in a 37°C water bath. The dried samples were dissolved in 20  $\mu$ l CM (2:1) and placed on a 50°C heating block for 30 minutes. Thereafter, 50  $\mu$ l Triton X-100 was added mixed and the sample tube centrifuged for 1 minute at 1000 rpm. A 20  $\mu$ l aliquot was transferred to a 96 well microtitre plate, in duplicate. Cholesterol reagent B (20  $\mu$ l) (Addendum 6) was added to sample solution, vortexed and left to incubate for 15 minutes. The absorbance was measured spectrophotometrically at 365 nm with samples being referenced against cholesterol blank. Cholesterol content was quantified against a cholesterol standard curve.

### 5.3.10. Protein determination

The sample cellular protein concentration was determined by the Bicinchoninic Acid (BCA) protein assay (Kaushal and Barnes, 1986). Samples were diluted in SDS solution to denature proteins. A BSA standard was prepared ranging from 1  $\mu\text{g}$  to 10  $\mu\text{g}$  protein to construct a standard curve. Absorbance readings were determined spectrophotometrically at 562 nm and the unknown protein concentration determined from the standard curve (Addendum 2).

### 5.3.11. Statistical analyses

All experiments were carried out in quadruplicate and repeated for consistency. The statistical significance between groups was assessed by analysis of variance (ANOVA). An interaction effect was included to test for a difference in slope for the FA. A significant interaction effect would indicate that the different concentrations reacted differently for all the FA. Statistical significance was considered at 5% ( $P < 0.05$ ).

## 5.4. Results

### 5.4.2. Phospholipids

The PC fraction (Table 5.1) phospholipid concentration (per mg protein) was not affected by *c9t11* CLA, LA and *t10c12* CLA, but was significantly lower ( $P < 0.05$ ) in ALA treated samples compared to untreated control.

In PE (Table 5.2), the *t10c12* CLA isomer showed a dose dependent phospholipid reduction ( $P < 0.05$ ) between highest and lowest concentration (0.5 and 0.075 mM respectively)

resulting in the highest concentration significantly lower ( $P < 0.05$ ) than the untreated control. PE concentration was significantly lower ( $P < 0.05$ ) in LA and ALA treated samples compared to control, while no significant effect was observed in *c9t11* CLA treated samples although values tended to be lower than control.

The PC/PE phospholipid ratio (Table 5.3) was not significantly affected by the treatments compared to controls, although the ratio tended to be lower in the ALA-treated group.

### 5.4.3. Cholesterol

Cholesterol was not significantly affected for all tested FA at all concentrations (data not shown).



### 5.4.4. Fatty acids

Treatment FAs, *c9t11*, *t10c12*, LA and ALA were incorporated into the cell membrane (PC and PE fractions) in a dose dependent manner ( $P < 0.05$ ) compared to untreated control, while *t10c12* was incorporated ( $P < 0.05$ ) but did not show a dose response.

### 5.4.4. Phosphatidylcholine (PC) (Table 5.1)

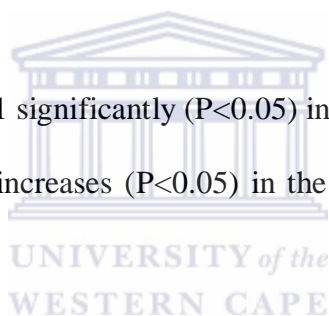
#### 5.4.4.1. Saturated fatty acids (SATS; C14:0, C16:0, C18:0 and C22:0)

**t10c12 CLA:** Compared to control, *t10c12* CLA treatment did not significantly affect the Total SATS, although the lower concentration (0.75mM) resulted in a significantly higher

level of C16:0 and C18:0 as well as with a dose of 0.15mM for C18:0. Overall, *t10c12* tended to increase the SATS at the lower dose resulting in a decrease in FA levels with increase in dose to control levels. However within the treatments, there was a significant ( $P<0.05$ ) interaction resulting in a significant ( $P<0.05$ ) dose difference (decrease) between 0.075 mM and 0.5 mM in Total SATS, was also reflected in C16:0 and C18:0 ( $P<0.05$ ). C14:0 tended to show a decrease as *t10c12* increased dose wise.

**LA**: Compared to control, LA significantly increased the Total SATS ( $P<0.05$ ) at 0.15 mM which drops significantly ( $P<0.05$ ) at 0.3 and 0.6 mM, also reflected by C16:0 and C18:0. Within treatments, C14:0, C16:0 and C18:0 were significantly ( $P<0.05$ ) dose-dependently decreased.

**c9t11 CLA**: Treatment with *c9t11* significantly ( $P<0.05$ ) increased the Total SATS compared to the control, also reflected by increases ( $P<0.05$ ) in the individual SATS FAs in a *c9t11* dose-dependent manner.



**ALA**: Treatment with ALA did not affect any SATS parameters compared to control or between concentrations.

#### **5.4.4.2. Monounsaturated fatty acids (C16:1, C18:1n9, C18:1n7 and C20:1n9)**

**t10c12 CLA**: Compared to control, *t10c12* CLA significantly ( $P<0.05$ ) lowered Total MUFA, reflected by decreases in C16:1n-7, C18:1n-9 and C18:1n-7 ( $P<0.05$ ). Within the treatments, a significant ( $P<0.05$ ) interaction resulting in a dose dependent decrease in Total MUFA, also reflected in C18:1n9 ( $P<0.05$ ).

**LA:** Total MUFA was significantly ( $P<0.05$ ) lowered by LA compared to control in a dose-dependent manner ( $P<0.05$ ), reflected by reduction in C16:1, C18:1n-9 and C18:1n-7 ( $P<0.05$ ). The lower dose of 0.15 mM had a lesser effect on MUFA content than 0.3 and 0.6 mM

**c9t11 CLA:** Compared to control, C9T11 CLA significantly increased ( $P<0.05$ ) Total MUFA, also reflected by increases ( $P<0.05$ ) in the individual MUFAs. Within the treatments, only C20:1n-9 increased dose-dependently ( $P<0.05$ ) from the 0.05 mM to 0.1 mM dose.

**ALA:** Compared to control, ALA significantly ( $P<0.05$ ) lowered Total MUFA, reflected by significantly ( $P<0.05$ ) reduced individual MUFAs, except for C20:1n-9. Between the doses there was a downward trend as ALA concentration increased, although the change was not statistically significant.



#### **5.4.4.3. N6 PUFA (C18:2, C18:3, C20:2, C20:3, C20:4 and C22:5)**

**t10c12 CLA:** Compared to control, t10c12 CLA did not significantly affect Total N6 PUFA, although a significantly ( $P<0.05$ ) lower C20:3n-6 and dose-dependent reduction in C20:4 ( $P<0.05$ ) was observed.

**LA:** Compared to control, LA significantly ( $P<0.05$ ) increased Total N6 PUFAs in a dose-dependent manner ( $P<0.05$ ), reflected in all identified N6 PUFAs.

**c9t11 CLA:** Compared to control, C9T11 CLA significantly ( $P<0.05$ ) increased Total N6 PUFAs dose-dependently ( $P<0.05$ ), reflected by dose-dependent ( $P<0.05$ ) increase of C18:2n-6 and C20:4n-6.

**ALA**: Treatment with ALA increased Total N6 PUFAs slightly compared to the control, which was attributed to a significant increase in C18:2n-6 as well as a dose-dependent ( $P<0.05$ ) increase in C18:2n-6.

#### **5.4.4.4. N3 PUFA (C18:3, C20:3, C20:5, C22:5 and C22:6)**

**t10c12 CLA**: Compared to control, *t10c12* significantly lowered ( $P<0.05$ ) Total N3 PUFA, a dose-dependent ( $P<0.05$ ) between highest and lowest concentration, 0.5 and 0.075 mM respectively) decrease in Total N3 PUFA was observed, as reflected in C22:6.

**LA**: Treatment with LA significantly ( $P<0.05$ ) increased Total N3 PUFA compared to control, reflected by significant ( $P<0.05$ ) increase in C22:5 and C22:6, although at higher doses LA lowered ( $P<0.05$ ) the level within the treatment groups.

**c9t11 CLA**: The *c9t11* CLA isomer significantly ( $P<0.05$ ) increased Total N3 PUFA compared to control which was reflected by significant ( $P<0.05$ ) increases in C22:5, C22:6 and C20:5.

**ALA**: Compared to control, ALA significantly ( $P<0.05$ ) increased Total N3 PUFA in a dose-dependent ( $P<0.05$ ) manner, reflected by significant increases in C20:5, C22:5 and dose-dependent increase in C18:3 and C20:3. ALA also showed a dose-dependent ( $P<0.05$ ) reduction in C22:6 that was not significant when compared to the controls.

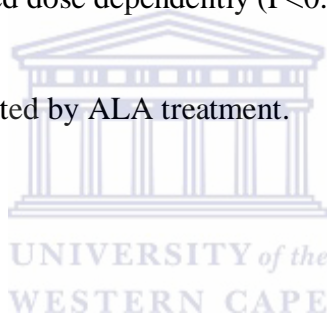
#### 5.4.4.5. PUFA and LCPUFA

**t10c12 CLA:** Treatment with *t10c12* demonstrated no effect on PUFA compared to control, while LCPUFA was significantly ( $P<0.05$ ) lower at the highest dose. Within treatments, there appeared to be a dose dependent decrease.

**LA:** Treatment with LA resulted in a significant increase ( $P<0.05$ ) for both PUFA and LCPUFA compared to control. However within treatments, PUFA significantly ( $P<0.05$ ) increased as per dose but the LCPUFA decreased ( $P<0.05$ ).

**c9t11 CLA:** The *c9t11* CLA isomer significantly increased ( $P<0.05$ ) PUFA and LCPUFA, although only the PUFA responded dose dependently ( $P<0.05$ ).

**ALA:** This same pattern was elicited by ALA treatment.



#### 5.4.5. Phosphatidylethanolamine (PE) Table 5.2

##### 5.4.5.1. Saturated fatty acids (C14:0, 16:0 and 18:0)

**t10c12 CLA:** Compared to control, *t10c12* CLA treatment did not significantly affect the Total SATS. However within the treatments, the Total SATS demonstrated a tendency to decrease dose dependent while there was a significant ( $P<0.05$ ) decrease in C18:0 between 0.075 mM and 0.5 mM.

**LA:** Compared to control, LA significantly ( $P<0.05$ ) increased Total SATS, reflected by significant ( $P<0.05$ ) increase in all individual identified SATS. Within the treatments, C14:0 increased ( $P<0.05$ ) dose dependent in contrast to a dose-dependent decrease ( $P<0.05$ ) C18:0.

**c9t11 CLA:** Treatment with *c9t11* CLA significant ( $P<0.05$ ) increased Total SATS compared



to control in a dose dependent ( $P < 0.05$ ) manner, which was reflected by significant ( $P < 0.05$ ) dose-dependent increase in the entire individual identified SATS.

**ALA**: Compared to control, ALA did not affect Total SATS significantly. No dose dependent treatment effects were observed.

#### **5.4.5.2. Monounsaturated fatty acids (C16:1, C18:1n-9, C18:1n-7 and C20:1n-9)**

**t10c12 CLA**: Compared to control, t10c12 CLA significant ( $P < 0.05$ ) reduced the Total MUFA in a dose dependent manner ( $P < 0.05$ ).

**LA**: Treatment with LA showed a significant ( $P < 0.05$ ) dose-dependent increase in Total MUFA at the lowest dose compared to the control, which was reflected by C18:1n-7 and C20:1n-9. However, this increase was negated by a tendency to decrease dose dependently with LA treatment, particularly with C16:1n-7 and C18:1n-9 ( $P < 0.05$ ). Overall, an increase in Total MUFA is evident, which decreases as the LA concentration increases.

**c9t11 CLA**: The c9t11 CLA isomer significantly increased Total MUFA ( $P < 0.05$ ) compared to control, reflected by significant ( $P < 0.05$ ) increases of all individual identified MUFAs, with C20:1n-9 increasing dose dependently ( $P < 0.05$ ).

**ALA**: Treatment with ALA significantly ( $P < 0.05$ ) reduced Total MUFA compared to control, that is reflected by reduction of all identified MUFAs at 0.3 mM ALA concentration.

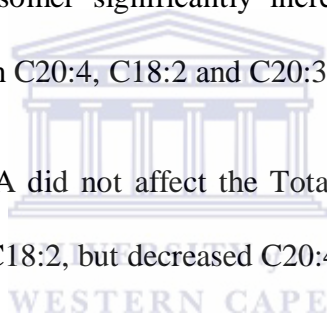
#### 5.4.5.3. N6 PUFA (C18:2, C18:3, C20:2, C20:3, C20:4, C22:4 and C22:5)

**t10c12 CLA:** The *t10c12* CLA isomer appeared to significantly decrease ( $P<0.05$ ) the Total N6 PUFA, only significant ( $P<0.05$ ) at the lowest dose, reflected by significant ( $P<0.05$ ) reduction of all identified N6 PUFA.

**LA:** Compared to control, LA significantly increased Total N6 PUFA ( $P<0.05$ ) as well as in a dose-dependent ( $P<0.05$ ) manner, reflected by significant dose-dependent increases ( $P<0.05$ ) in C18:2, C18:3, C20:2, C22:4. However, C20:3, C20:4 and C22:5 decreased ( $P<0.05$ ) dose dependently.

**c9t11 CLA:** The *c9t11* CLA isomer significantly increased Total N6 PUFA ( $P<0.05$ ), reflected by significant increase in C20:4, C18:2 and C20:3.

**ALA:** Compared to control, ALA did not affect the Total N6 significantly, however ALA significantly ( $P<0.05$ ) increased C18:2, but decreased C20:4.



#### 5.4.5.4. N3 PUFA (C18:3, C20:3, C20:5, C22:5 and C22:6)

**t10c12 CLA:** Compared to control, *t10c12* CLA significantly ( $P<0.05$ ) decreased Total N3 PUFA, reflected by significant decrease in C20:5 and C22:6.

**LA:** Treatment with LA did not significantly affect Total N3 PUFA, however a dose reduction is evident, reflected in the significant ( $P<0.05$ ) dose reduction in C22:6.

**c9t11 CLA:** Compared to control, *c9t11* CLA significantly ( $P<0.05$ ) increased Total N3 PUFA, reflected by significant ( $P<0.05$ ) increase in C22:6 and dose-dependent ( $P<0.05$ ) increase in C20:5.

**ALA:** Treatment with ALA significantly ( $P < 0.05$ ) increased Total N3 PUFA in a dose-dependent ( $P < 0.05$ ) manner compared to control, reflected by significant ( $P < 0.05$ ) dose dependent increases in C18:3 and C20:3.

#### 5.4.5.5. PUFA and LCPUFA

Overall, *t10c12* significantly ( $P < 0.05$ ) lowered PUFA and LCPUFA levels, whereas *c9t11* significantly ( $P < 0.05$ ) increased these two parameters. These two parameters were similarly increased ( $P < 0.05$ ) by LA and ALA.

#### 5.4.6. FA ratios Table 5.3



##### 5.4.6.1. N6/N3

(PC) *c9t11* and ALA significantly decreased ( $P < 0.05$ ) the N6/N3 fatty acid ratio, but the effect of ALA was greater as the concentration increase and the effect of *c9t11* was inversely proportional. LA Significantly increased ( $P < 0.05$ ) the N6/N3 fatty acid ratio in a dose dependent manner ( $P < 0.05$ ), while T10C12 was not significant.

(PE) *c9t11* CLA and ALA significantly decrease ( $P < 0.05$ ) the N6/N3 fatty acid ratio, but the effect of ALA was greater as the concentration increase whereas the effect of *c9t11* was inversely proportional. LA Significantly increased the N6/N3 fatty acid ratio in a dose dependent manner ( $P < 0.05$ ), while *t10c12* was not significant, but did show significant dose increase ( $P < 0.05$ ) in the ratio.

#### **5.4.6.2. P/S**

(PC) The P/S ratio was significantly increased by all fatty acids ( $P < 0.05$ ) except *t10c12*, which did not alter the ratio at all.

(PE) The P/S ratio was significantly increased ( $P < 0.05$ ) by LA and ALA, decreased by *c9t11* CLA, and unaffected by *t10c12* CLA, which did not alter the ratio at all.

#### **5.4.6.3. AA/EPA ratio (arachidonic acid/eisocapentanoic acid, C20:4n6/C20:5n3).**

(PC) Compared to control, *c9t11*CLA and ALA significantly increased ( $P < 0.05$ ) the AA/EPA as a result of increasing EPA to within a detectable level.

(PE) Compared to control, *t10c12* CLA significantly increased ( $P < 0.05$ ) the AA/EPA ratio at 0.15 and 0.5 mM, with a significant difference ( $P < 0.05$ ) seen between 0.075 and 0.5 mM concentrations. In contrast, *c9t11* CLA and ALA significantly reduced ( $P < 0.05$ ) the AA/EPA ratio.

#### **5.4.6.4. Desaturation indices (C16:1n-7/C16:0, C18:1n9/C18:0 and C18:1n7/C16:0)**

(PC) All FA at all concentrations significantly decreased ( $P < 0.05$ ) the desaturation index within the PC phospholipid fraction.

(PE) The *t10c12* CLA isomer and LA significantly decreased ( $P < 0.05$ ) the desaturation index, whereas *c9t11* and ALA had no significant effect on the desaturation index of the PE phospholipid fraction.

#### 5.4.6.4 Delta 6 desaturase substrate and product (Delta 6-S and Delta 6-P)

##### 5.4.6.4.1. PC

**t10c12 CLA:** Compared to the control, t10c12 CLA treatment significantly increased Delta 6 desaturase substrate and product at 0.5 mM. Concentration differences were seen where 0.05 mM showed a significantly higher ( $P < 0.05$ ) effect than 0.075 and 0.15 mM.

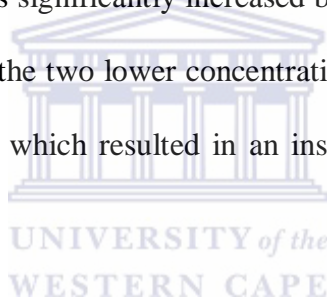
**LA:** Treatment with LA significantly increased ( $P < 0.05$ ) Delta 6 S/P ratio. A significant ( $< 0.05$ ) dose increase was seen between the highest concentration and the lowest concentration.

**c9t11 CLA:** Delta 6 substrate was significantly increased by c9t11 CLA treatment, with dose difference between 0.2 mM and the two lower concentrations. Compared to control, Delta 6 product showed similar increase, which resulted in an insignificant response in the Delta 6 S/P ratio.

**ALA:** Compared to control, ALA treatment significantly increased the Delta 6 substrate, with dose differences seen between 0.3 mM and the two lower concentrations. Slight reduction in Delta 6 product can be seen, which is dose significant ( $P < 0.05$ ). This effect significantly increased ( $P < 0.05$ ) the Delta S/P ratio in a dose dependent manner ( $P < 0.05$ ).

##### 5.4.6.4.2. PE

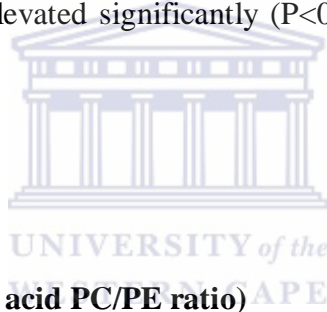
**t10c12 CLA:** Compared to the control, t10c12 CLA treatment significantly decreased delta 6 desaturase substrate at 0.075 and 0.15 mM. The Delta 6 S/P ratio was significantly increased ( $P < 0.05$ ) at 0.5 mM.



**LA:** Treatment with LA significantly increased ( $P<0.05$ ) Delta 6 S in a dose dependent manner ( $P<0.05$ ). Delta 6 P was also significantly increased, but dose difference seen between the higher concentrations and the lowest concentration. The Delta 6 S/P was not significantly affected.

**c9t11 CLA:** Delta 6 substrate was significantly increased ( $P<0.05$ ) by c9t11 CLA treatment, and a slight increase in Delta 6 P is evident, therefore the effect on the ratio did not differ significantly from the control.

**ALA:** Treatment with ALA significantly increased ( $P<0.05$ ) Delta 6 S. A dose increase was notable between 0.3 mM and the two lower concentrations. No effect on Delta 6 P was seen, therefore the Delta 6 S/P ratio elevated significantly ( $P<0.05$ ) in a dose dependent manner ( $P<0.05$ ).



#### **5.4.6.5. AA PC/PE (arachidonic acid PC/PE ratio)**

Treatment with t10c12 CLA was significantly higher than the control at 0.075 mM, which decreased as the concentration increased. LA, c9t11 CLA and ALA significantly increased ( $P<0.05$ ) the AA PC/PE ratio compared to control, but no differences between FA concentrations were seen.

**Table 5.1: Fatty acid analysis ( $\mu\text{g}$  fatty acid/mg protein) of the phosphatidylcholine (PC) phospholipid fraction in HepG2 cells**

Dose (mM)	T10C12 CLA			LA			C9T11 CLA			ALA			Control
	0.5	0.15	0.075	0.6	0.3	0.15	0.2	0.1	0.05	0.3	0.15	0.075	0
<b>SATS</b>													
C14:0	0.25±0.11	0.39±0.26	0.60±0.23	0.44±0.05 <sup>b</sup>	0.37±0.07 <sup>b</sup>	1.14±0.12 <sup>*a</sup>	1.47±0.34 <sup>*b</sup>	0.94±0.26	0.63±0.30 <sup>a</sup>	0.40±0.16	0.43±0.18	0.37±0.13	0.45±0.15
C16:0	3.81±1.12 <sup>b</sup>	5.31±0.66 <sup>b</sup>	7.46±0.86 <sup>*a</sup>	7.40±0.97 <sup>*b</sup>	5.79±0.43 <sup>b</sup>	11.4±1.48 <sup>*a</sup>	19.5±3.26 <sup>*c</sup>	15.4±2.14 <sup>*b</sup>	11.3±0.75 <sup>*a</sup>	5.10±1.31	5.24±1.72	4.97±0.35	4.53±0.99
C18:0	0.80±0.14 <sup>b</sup>	1.48±0.18 <sup>*a</sup>	1.55±0.59 <sup>*a</sup>	0.91±0.12 <sup>*b</sup>	1.01±0.37 <sup>*b</sup>	1.62±0.07 <sup>a</sup>	2.71±0.14 <sup>*c</sup>	2.14±0.17 <sup>*b</sup>	1.61±0.18 <sup>*a</sup>	0.39±0.09	0.40±0.14	0.45±0.12	0.46±0.19
<b>Total SATS</b>	4.57±1.51 <sup>b</sup>	7.18±0.74	9.80±1.39 <sup>a</sup>	8.75±1.13 <sup>c</sup>	7.17±0.79 <sup>b</sup>	14.2±1.57 <sup>8a</sup>	24.6±5.12 <sup>*c</sup>	18.5±2.52 <sup>*b</sup>	13.5±0.92 <sup>*a</sup>	6.18±1.60	6.36±2.08	6.02 ±0.55	6.73±2.71
<b>MUFA</b>													
C16:1n-7	0.15±0.05 <sup>*</sup>	0.41±0.11 <sup>*</sup>	0.56±0.37 <sup>*</sup>	0.44±0.04 <sup>*b</sup>	0.45±0.03 <sup>*b</sup>	1.22±0.15 <sup>a</sup>	4.25±0.69 <sup>*</sup>	4.37±0.68 <sup>*</sup>	3.24±0.52 <sup>*</sup>	0.55±0.15 <sup>*</sup>	0.73±0.24 <sup>*</sup>	0.87±0.11 <sup>*</sup>	1.73±0.54
C18:1n-9	0.81±0.18 <sup>*b</sup>	1.66±0.14 <sup>*</sup>	2.78±1.02 <sup>*a</sup>	0.91±0.11 <sup>*b</sup>	1.07±0.21 <sup>*b</sup>	2.44±0.12 <sup>*a</sup>	7.66±1.33 <sup>*</sup>	8.94±0.08 <sup>*</sup>	8.03±0.86 <sup>*</sup>	0.84±0.21 <sup>*</sup>	1.19±0.35 <sup>*</sup>	1.64±0.09 <sup>*</sup>	3.29±0.36
C18:1n-7	0.46±0.12 <sup>*</sup>	0.81±0.08 <sup>*</sup>	1.32±0.64 <sup>*</sup>	0.70±0.09 <sup>*b</sup>	0.87±0.09 <sup>*b</sup>	2.27±0.30 <sup>a</sup>	3.76±0.46 <sup>*b</sup>	4.92±0.13 <sup>*a</sup>	4.60±0.58 <sup>*</sup>	0.63±0.15 <sup>*</sup>	0.84±0.26 <sup>*</sup>	1.16±0.10 <sup>*</sup>	2.34±0.57
C20:1n-9	0.33±0.12 <sup>*</sup>	0.20±0.03	0.19±0.07	0.07±0.00 <sup>*b</sup>	0.10±0.02 <sup>b</sup>	0.22±0.04 <sup>a</sup>	1.33±0.07 <sup>*b</sup>	1.11±0.22 <sup>*b</sup>	0.82±0.10 <sup>*a</sup>	0.08±0.03	0.08±0.03	0.08±0.02	0.18±0.08
<b>Total MUFA</b>	1.69±0.45 <sup>*</sup>	3.08±0.22 <sup>*</sup>	4.85±1.71 <sup>*</sup>	2.15±0.20 <sup>*b</sup>	2.48±0.32 <sup>*</sup>	6.15±0.55 <sup>a</sup>	17.0±2.38 <sup>*</sup>	18.4±2.73 <sup>*</sup>	16.7±1.43 <sup>*</sup>	2.10±0.54 <sup>*</sup>	2.84±0.86 <sup>*</sup>	3.76±0.27 <sup>*</sup>	8.42±2.76
<b>N6 PUFA</b>													
C18:2	0.45±0.14	0.57±0.03	0.57±0.19	8.86±0.58 <sup>*c</sup>	6.54±0.73 <sup>*b</sup>	4.98±0.64 <sup>*a</sup>	2.95±0.52 <sup>*b</sup>	2.27±0.17 <sup>*a</sup>	1.85±0.23 <sup>*a</sup>	1.67±0.15 <sup>*c</sup>	1.39±0.64 <sup>*b</sup>	0.88±0.17 <sup>*a</sup>	0.60±0.22
C18:3	ND	ND	ND	2.24±0.17 <sup>*c</sup>	2.71±0.34 <sup>*b</sup>	0.10±0.01 <sup>a</sup>	ND	ND	ND	ND	ND	ND	ND
C20:2	ND	ND	ND	0.67±0.09 <sup>*b</sup>	0.39±0.04 <sup>*b</sup>	0.45±0.04 <sup>*a</sup>	ND	ND	ND	ND	ND	ND	0.21±0.00
C20:3	0.01±0.01 <sup>*</sup>	0.04±0.03	0.04±0.04	0.34±0.04 <sup>*b</sup>	0.36±0.08 <sup>*b</sup>	0.80±0.10 <sup>*a</sup>	0.20±0.04 <sup>*</sup>	0.22±0.03 <sup>*</sup>	0.21±0.04 <sup>*</sup>	0.04±0.01 <sup>*</sup>	0.05±0.01 <sup>*</sup>	0.06±0.00	0.09±0.03
C20:4	0.13±0.03 <sup>b</sup>	0.24±0.04 <sup>a</sup>	0.33±0.04 <sup>*a</sup>	1.51±0.15 <sup>*b</sup>	1.46±0.13 <sup>*b</sup>	2.95±0.45 <sup>*a</sup>	0.92±0.10 <sup>*b</sup>	0.80±0.11	0.68±0.09 <sup>a</sup>	0.15±0.03	0.14±0.04	0.16±0.01	0.17±0.07
C22:5	ND	ND	ND	0.06±0.01 <sup>*b</sup>	0.07±0.01 <sup>*b</sup>	0.16±0.02 <sup>*a</sup>	ND	ND	ND	ND	ND	ND	ND
<b>Total N6</b>	0.73±0.26	0.85±0.05	0.94±0.15	13.6±0.77 <sup>*c</sup>	11.5±0.81 <sup>*b</sup>	9.62±1.09 <sup>*a</sup>	4.07±0.52 <sup>b</sup>	3.29±0.31 <sup>*a</sup>	2.74±0.35 <sup>*a</sup>	1.86±0.68	1.29±0.82	1.30±0.45	0.92±0.35
<b>N3 PUFA</b>													
C18:3	ND	ND	ND	ND	ND	ND	ND	ND	ND	3.79±0.88 <sup>*c</sup>	1.83±0.57 <sup>*b</sup>	0.85±0.11 <sup>*a</sup>	ND
C20:3	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.58±0.12 <sup>*b</sup>	0.40±0.13 <sup>*a</sup>	0.27±0.03 <sup>*a</sup>	ND
C20:5	ND	ND	ND	ND	ND	ND	0.55±0.09 <sup>*</sup>	0.51±0.10 <sup>*</sup>	0.43±0.04 <sup>*</sup>	0.81±0.17 <sup>*</sup>	0.80±0.24 <sup>*</sup>	0.77±0.11 <sup>*</sup>	ND
C22:5	ND	ND	ND	0.03±0.01 <sup>*a</sup>	0.02±0.00 <sup>*b</sup>	0.03±0.00 <sup>*a</sup>	0.04±0.00 <sup>*</sup>	0.04±0.01 <sup>*</sup>	0.04±0.00 <sup>*</sup>	0.06±0.02 <sup>*</sup>	0.06±0.02 <sup>*</sup>	0.07±0.01 <sup>*</sup>	ND
C22:6	0.03±0.03 <sup>*b</sup>	0.11±0.02	0.14±0.03 <sup>a</sup>	0.25±0.05 <sup>*b</sup>	0.18±0.02 <sup>*b</sup>	0.39±0.03 <sup>*a</sup>	0.50±0.06 <sup>*</sup>	0.51±0.05 <sup>*</sup>	0.48±0.08 <sup>*</sup>	0.09±0.02 <sup>b</sup>	0.11±0.03	0.18±0.03 <sup>a</sup>	0.14±0.06
<b>Total N3</b>	0.03±0.03 <sup>*b</sup>	0.11±0.02	0.14±0.03 <sup>a</sup>	0.28±0.06 <sup>*b</sup>	0.20±0.02 <sup>b</sup>	0.42±0.03 <sup>*a</sup>	1.09±0.15 <sup>*</sup>	1.06±0.15 <sup>*</sup>	0.95±0.11 <sup>*</sup>	5.32±1.20 <sup>*b</sup>	3.21±0.98 <sup>*a</sup>	2.14±0.28 <sup>*a</sup>	0.15±0.07
<b>PUFA</b>													
LC PUFA	0.79±0.33	0.96±0.07	1.13±0.13	13.9±0.77 <sup>*c</sup>	11.7±0.82 <sup>*b</sup>	10.0±1.11 <sup>*a</sup>	5.17±0.65 <sup>*b</sup>	4.35±0.45 <sup>*</sup>	3.69±0.47 <sup>*a</sup>	7.18±1.88 <sup>*c</sup>	4.50±1.48 <sup>*b</sup>	3.44±0.48 <sup>*a</sup>	1.07±0.37
LC /PUFA	0.16±0.06 <sup>*b</sup>	0.39±0.04 <sup>a</sup>	0.56±0.13 <sup>a</sup>	2.84±0.06 <sup>*b</sup>	2.47±0.26 <sup>*b</sup>	4.96±0.71 <sup>*a</sup>	2.21±0.15 <sup>*</sup>	2.08±0.29 <sup>*</sup>	1.84±0.23 <sup>*</sup>	1.72±0.36 <sup>*</sup>	1.57±0.46 <sup>*</sup>	1.50±0.18 <sup>*</sup>	0.46±0.11
LC /PUFA	0.18±0.03 <sup>*b</sup>	0.40±0.08 <sup>a</sup>	0.49±0.07 <sup>a</sup>	2.84±0.06 <sup>*b</sup>	2.47±0.26 <sup>*b</sup>	4.96±0.71 <sup>*a</sup>	2.21±0.15 <sup>*</sup>	2.08±0.29 <sup>*</sup>	1.84±0.23 <sup>*</sup>	1.72 ± 0.36 <sup>*</sup>	1.57±0.46 <sup>*</sup>	1.50±0.18 <sup>*</sup>	0.46±0.11
<b>CLA</b>													
c9t11	0.65±0.10 <sup>*c</sup>	0.46±0.07 <sup>*b</sup>	0.22±0.90 <sup>*a</sup>	ND	ND	ND	11.1±0.32 <sup>*c</sup>	5.89±1.06 <sup>*b</sup>	2.77±0.24 <sup>*a</sup>	ND	ND	ND	ND
t10c12	4.85±0.77 <sup>*c</sup>	3.76±0.57 <sup>*b</sup>	2.60±0.29 <sup>*a</sup>	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
<b>Phospholipids</b>	46.12±12.41	73.02±25.03	69.65±27.00	43.14±6.92	46.45±5.11	42.15±4.42	64.57±3.87	55.97±5.72	61.99±3.03	30.33±3.03 <sup>*</sup>	32.12±2.94 <sup>*</sup>	25.28±2.85 <sup>*</sup>	65.32±23.98

Values are expressed as mean ( $\mu\text{g}$  fatty acid/mg protein)  $\pm$  SD of 4 replications. \* denotes significant ( $P < 0.05$ ) difference compared to control. The letters (*a*, *b* and *c*) denote a significant ( $P < 0.05$ ) difference between treatment according to ANOVA (analysis of variance) analyses, where *a*, *b* and *c* are all different. FA = fatty acid, mM = treatment concentration in mM, SATS = saturated fatty acids, MUFA = monounsaturated fatty acids, N6 PUFA = omega 6 polyunsaturated fatty acids, N3 PUFA = omega 3 polyunsaturated fatty acids, PUFA = polyunsaturated fatty acids, FA containing 2 or more double bonds, LCPUFA = long chain polyunsaturated fatty acids greater than 20 carbons in length, LC/PUFA = the ration between long chain FA and PUFA, CLA = conjugated linoleic acid, C9T11 = *cis*9-*trans*11 CLA, T10C12 = *trans*10-*cis*12 CLA, ND = not detected.

**Table 5.2: Fatty acid analysis ( $\mu\text{g}$  fatty acid/mg protein) of the phosphatidylethanolamine (PE) phospholipid fraction in HepG2 cells**

FA	<i>t10c12 CLA</i>			LA			<i>c9t11 CLA</i>			ALA			Control 0
	0.5	0.15	0.075	0.6	0.3	0.15	0.2	0.1	0.05	0.3	0.15	0.075	
<b>SATS</b>													
<b>C14:0</b>	0.06±0.03	0.04±0.01	0.05±0.02	0.13±0.02 <sup>*b</sup>	0.11±0.02 <sup>*</sup>	0.08±0.01 <sup>a</sup>	0.16±0.03 <sup>*b</sup>	0.10±0.02 <sup>a</sup>	0.07±0.02 <sup>a</sup>	0.03±0.03	0.06±0.03	0.05±0.01	0.06±0.03
<b>C16:0</b>	0.90±0.17	1.00±0.09	1.63±0.71	2.24±0.41 <sup>*</sup>	1.62±0.09 <sup>b</sup>	2.51±0.54 <sup>*a</sup>	3.10±0.47 <sup>*b</sup>	2.78±0.28 <sup>*b</sup>	1.88±0.21 <sup>*a</sup>	0.98±0.39	1.13±0.26	1.02±0.05	1.11±0.37
<b>C18:0</b>	0.84±0.17 <sup>b</sup>	1.23±0.07	1.40±0.16 <sup>a</sup>	2.16±0.32 <sup>*b</sup>	2.08±0.31 <sup>*b</sup>	3.08±0.34 <sup>*a</sup>	2.97±0.48 <sup>*b</sup>	2.87±0.12 <sup>*</sup>	2.21±0.38 <sup>*a</sup>	1.35±0.25	1.29±0.30	1.03±0.03	1.06±0.32
<b>Total SATS</b>	1.81±0.35	2.28±0.17	4.04±1.67	4.53±0.22 <sup>*</sup>	3.81±0.38 <sup>*b</sup>	5.68±0.77 <sup>*a</sup>	6.24±0.93 <sup>*b</sup>	5.76±0.41 <sup>*b</sup>	4.16±0.29 <sup>*a</sup>	2.36±0.66	2.53±0.59	2.11±0.04	2.11±0.04
<b>MUFA</b>													
<b>C16:1n-7</b>	0.11±0.04 <sup>*b</sup>	0.19±0.03 <sup>*</sup>	0.39±0.18 <sup>*a</sup>	0.24±0.02 <sup>*b</sup>	0.23±0.02 <sup>*b</sup>	0.45±0.06 <sup>a</sup>	1.37±0.22 <sup>*</sup>	1.32±0.13 <sup>*</sup>	1.16±0.21 <sup>*</sup>	0.17±0.08 <sup>*</sup>	0.27±0.07	0.28±0.02	0.43±0.13
<b>C18:1n-9</b>	0.98±0.21 <sup>*</sup>	1.41±0.11	1.72±0.04	1.34±0.18 <sup>*b</sup>	1.73±0.15 <sup>b</sup>	3.02±0.28 <sup>a</sup>	5.33±0.71 <sup>*</sup>	5.70±0.24 <sup>*b</sup>	4.33±0.45 <sup>*a</sup>	1.09±0.25 <sup>*</sup>	1.38±0.33	1.39±0.09	2.27±0.76
<b>C18:1n-7</b>	0.18±0.08 <sup>*</sup>	0.28±0.02 <sup>*</sup>	0.58±0.22 <sup>*</sup>	0.78±0.12 <sup>b</sup>	0.91±0.09 <sup>b</sup>	2.37±0.91 <sup>*a</sup>	1.65±0.23	1.97±0.04 <sup>*</sup>	1.70±0.24 <sup>*</sup>	0.55±0.11 <sup>*</sup>	0.65±0.17	0.66±0.02	1.15±0.40
<b>C20:1n-9</b>	0.17±0.02	0.11±0.01	0.18±0.04	0.08±0.01 <sup>b</sup>	0.12±0.02 <sup>b</sup>	0.28±0.06 <sup>*a</sup>	1.16±0.15 <sup>*b</sup>	0.88±0.08 <sup>*a</sup>	0.69±0.10 <sup>*a</sup>	0.09±0.04	0.10±0.02	0.09±0.02	0.13±0.05
<b>Total MUFA</b>	1.40±0.43 <sup>*</sup>	2.00±0.13 <sup>*</sup>	2.84±0.41	2.43±0.31 <sup>b</sup>	2.99±0.23 <sup>b</sup>	6.13±1.17 <sup>*a</sup>	9.51±1.27 <sup>*</sup>	9.77±0.57 <sup>*</sup>	7.87±0.90 <sup>*</sup>	1.90±0.48	2.39±0.57	2.43±0.10	3.98±1.29
<b>N6 PUFA</b>													
<b>C18:2</b>	0.24±0.18	0.17±0.01	0.23±0.06	5.95±0.36 <sup>*c</sup>	3.59±0.97 <sup>*b</sup>	2.00±0.56 <sup>*a</sup>	1.52±0.21 <sup>*b</sup>	1.03±0.31 <sup>*a</sup>	1.06±0.25 <sup>*</sup>	1.85±0.65 <sup>*</sup>	1.38±0.62 <sup>*</sup>	1.47±0.44 <sup>*</sup>	0.26±0.09
<b>C18:3</b>	ND	ND	ND	2.54±0.39 <sup>*b</sup>	2.85±0.17 <sup>*b</sup>	0.04±0.01 <sup>*a</sup>	ND	ND	ND	ND	ND	ND	ND
<b>C20:2</b>	ND	ND	ND	0.75±0.15 <sup>*b</sup>	0.36±0.03 <sup>*a</sup>	0.33±0.05 <sup>*a</sup>	ND	ND	ND	ND	ND	ND	ND
<b>C20:3</b>	0.02±0.02 <sup>b</sup>	0.05±0.01	0.06±0.03 <sup>a</sup>	0.36±0.07 <sup>*b</sup>	0.31±0.03 <sup>*b</sup>	0.53±0.05 <sup>*a</sup>	0.52±0.17 <sup>*b</sup>	0.21±0.01 <sup>a</sup>	0.18±0.03 <sup>a</sup>	0.05±0.01	0.05±0.01	0.05±0.00	0.10±0.05
<b>C20:4</b>	0.53±0.15	0.70±0.06	0.54±0.2	2.03±0.46 <sup>*b</sup>	2.42±0.20 <sup>*b</sup>	4.48±0.36 <sup>*a</sup>	1.42±0.23 <sup>*</sup>	1.62±0.03 <sup>*</sup>	1.23±0.25 <sup>*</sup>	0.42±0.07 <sup>*</sup>	0.44±0.10 <sup>*</sup>	0.43±0.02 <sup>*</sup>	0.76±0.21
<b>C22:4</b>	ND	ND	ND	0.28±0.07 <sup>*b</sup>	0.20±0.02 <sup>*a</sup>	0.20±0.03 <sup>*a</sup>	ND	ND	ND	ND	ND	ND	ND
<b>C22:5</b>	ND	ND	ND	0.32±0.09 <sup>*b</sup>	0.40±0.05 <sup>*b</sup>	0.67±0.08 <sup>*a</sup>	ND	ND	ND	ND	ND	ND	ND
<b>Total N6</b>	0.83±0.56	0.91±0.27	0.83±0.14 <sup>*</sup>	12.2±1.23 <sup>*b</sup>	10.1±1.23 <sup>*a</sup>	8.28±0.79 <sup>*a</sup>	3.46±0.56 <sup>*</sup>	2.86±0.29 <sup>*</sup>	2.48±0.52	2.32±0.74	1.87±0.64	2.01±0.37	1.70±0.65
<b>N3 PUFA</b>													
<b>C18:3</b>	ND	ND	ND	ND	ND	ND	ND	ND	ND	2.32±0.53 <sup>*c</sup>	1.03±0.23 <sup>*b</sup>	0.47±0.09 <sup>*a</sup>	ND
<b>C20:3</b>	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.49±0.08 <sup>*c</sup>	0.25±0.06 <sup>*b</sup>	0.12±0.01 <sup>*a</sup>	ND
<b>C20:5</b>	0.10±0.02	0.04±0.01 <sup>*</sup>	0.03±0.02 <sup>*</sup>	ND	ND	ND	0.65±0.04 <sup>*b</sup>	0.59±0.03 <sup>*b</sup>	0.39±0.09 <sup>*a</sup>	0.98±0.19 <sup>*</sup>	1.09±0.26 <sup>*</sup>	0.99±0.07 <sup>*</sup>	0.09±0.05
<b>C22:5</b>	ND	ND	ND	0.08±0.02	0.09±0.01	0.11±0.02	0.13±0.02	0.15±0.00 <sup>*b</sup>	0.11±0.01 <sup>a</sup>	0.25±0.05 <sup>*</sup>	0.23±0.06 <sup>*</sup>	0.17±0.01	0.11±0.02
<b>C22:6</b>	0.14±0.06 <sup>*</sup>	0.23±0.03 <sup>*</sup>	0.21±0.08 <sup>*</sup>	0.54±0.05 <sup>*b</sup>	0.61±0.04 <sup>*b</sup>	0.88±0.09 <sup>*a</sup>	0.87±0.10	1.02±0.07 <sup>*b</sup>	0.79±0.05 <sup>a</sup>	0.42±0.08 <sup>*</sup>	0.49±0.13 <sup>*</sup>	0.56±0.04	0.70±0.08
<b>Total N3</b>	0.13±0.08 <sup>*</sup>	0.27±0.03 <sup>*</sup>	0.26±0.07 <sup>*</sup>	0.62±0.07	0.70±0.04	0.99±0.11	1.80±0.42 <sup>*</sup>	1.76±0.09 <sup>*</sup>	1.39±0.30	4.47±0.93 <sup>*b</sup>	3.09±0.73 <sup>*a</sup>	2.31±0.16 <sup>*a</sup>	0.85±0.42
<b>PUFA</b>	0.96±0.64 <sup>*</sup>	1.19±0.10 <sup>*</sup>	1.09±0.20 <sup>*</sup>	12.8±1.29 <sup>*b</sup>	10.7±1.26 <sup>*a</sup>	9.26±0.87 <sup>*a</sup>	5.27±0.87 <sup>*</sup>	4.62±0.29 <sup>*</sup>	3.87±0.83	6.76±1.67 <sup>*b</sup>	4.96±1.11 <sup>*</sup>	4.26±0.59 <sup>a</sup>	2.72±0.68
<b>LC PUFA</b>	0.57±0.32 <sup>*</sup>	1.02±0.09 <sup>*</sup>	0.86±0.25 <sup>*</sup>	4.37±0.90 <sup>*b</sup>	4.30±0.37 <sup>*b</sup>	7.22±0.62 <sup>*a</sup>	3.74±0.74 <sup>*</sup>	3.59±0.11 <sup>*</sup>	2.80±0.58 <sup>*</sup>	2.62±0.49 <sup>*</sup>	2.55±0.62	2.31±0.13	1.76±0.44
<b>LC /PUFA</b>	0.18±0.03 <sup>*b</sup>	0.18±0.03 <sup>*b</sup>	0.40±0.08 <sup>a</sup>	0.49±0.07 <sup>a</sup>	2.84±0.16	2.47±0.24	4.96±0.22 <sup>*b</sup>	2.21±0.13 <sup>a</sup>	2.08±0.07 <sup>a</sup>	1.84±0.52	1.72±0.49	1.57±0.46	1.50±0.18
<b>CLA</b>													
<b>c9t11</b>	0.43±0.10 <sup>*b</sup>	0.28±0.03 <sup>*</sup>	0.26±0.13 <sup>*a</sup>	ND	ND	ND	9.98±0.99 <sup>*c</sup>	4.89±0.40 <sup>*b</sup>	2.02±0.31 <sup>*a</sup>	ND	ND	ND	ND
<b>t10c12</b>	1.76±0.56 <sup>*</sup>	1.26±0.15 <sup>*</sup>	1.25±0.64 <sup>*</sup>	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
<b>Phospholipids</b>	22.16±7.42 <sup>*b</sup>	36.63±8.03	43.08±12.13 <sup>a</sup>	29.70±4.15 <sup>*</sup>	21.82±4.64 <sup>*</sup>	28.11±3.30 <sup>*</sup>	38.62±12.93	33.23±7.93	33.22±8.48	23.12±5.45 <sup>*</sup>	28.76±7.71 <sup>*</sup>	23.27±2.04 <sup>*</sup>	43.03±7.46

Values are expressed as mean ( $\mu\text{g}$  fatty acid/mg protein)  $\pm$  SD of 4 replications. \* denotes significant ( $P < 0.05$ ) difference compared to control. The letters (*a, b and c*) denote a significant ( $P < 0.05$ ) difference between treatment according to ANOVA (analysis of variance) analyses, where *a, b and c* are all different. FA = fatty acid, mM = treatment concentration in mM, SATS = saturated fatty acids, MUFA = monounsaturated fatty acids, N6 PUFA = omega 6 polyunsaturated fatty acids, N3 PUFA = omega 3 polyunsaturated fatty acids, PUFA = polyunsaturated fatty acids, FA containing 2 or more double bonds, LCPUFA = long chain polyunsaturated fatty acids greater than 20 carbons in length, LC/PUFA = the ration between long chain FA and PUFA, CLA = conjugated linoleic acid, C9T11 = *cis9-trans11 CLA*, T10C12 = *trans10-cis12 CLA*, ND = not detected.



**Table 5.3: Fatty acid ratios ( $\mu\text{g}$  fatty acid/mg protein) of the PC and PE phospholipid fractions in HepG2 cells following treatment for 48 hours**

FA Dose (mM)	t10c12 CLA			LA			c9t11 CLA			ALA			Control
	0.5	0.15	0.075	0.6	0.3	0.15	0.2	0.1	0.05	0.3	0.15	0.075	0
<b>Phospholipid ratio PC/PE</b>	2.22±0.65	1.96±0.29	1.75±0.99	1.48±0.35	1.98±0.40	1.51±0.19	1.85±0.58	1.98±0.80	1.69±0.38	1.34±0.18	1.15±0.18	1.09±0.07	1.63±0.41
<b>PC FA ratios</b>													
<b>N6/N3</b>	13.3±6.85* <i>b</i>	7.90±1.08	5.90±2.88 <i>a</i>	51.5±12.3* <i>b</i>	58.7±6.01* <i>b</i>	22.8±1.97* <i>a</i>	3.74±0.31* <i>b</i>	3.13±0.24*	2.89±0.06* <i>a</i>	0.34±0.05*	0.42±0.30*	0.62±0.22*	5.76±0.45
<b>P/S</b>	0.15±0.03	0.13±0.01	0.15±0.05	1.61±0.20* <i>b</i>	1.65±0.13* <i>b</i>	0.71±0.05* <i>a</i>	0.21±0.02	0.24±0.01*	0.27±0.04*	1.16±0.04* <i>b</i>	0.72±0.15* <i>a</i>	0.57±0.07* <i>a</i>	0.15±0.06
<b>AA/EPA</b>	NA	NA	NA	NA	NA	NA	1.70±0.37*	1.60±0.22*	1.56±0.11*	0.18±0.01	0.18±0.02	0.20±0.03	NA
<b>16:1/16:0</b>	0.04±0.00	0.08±0.01	0.12±0.00	0.06±0.01 <i>b</i>	0.08±0.01 <i>a</i>	0.11±0.00 <i>a</i>	0.22±0.01	0.28±0.01	0.29±0.03	0.11±0.00	0.14±0.00	0.17±0.01	0.29±0.02
<b>18:1/18:0</b>	1.01±0.07*	1.13±0.11*	1.32±0.18*	1.00±0.01*	1.11±0.20*	1.51±0.14*	2.59±0.06	3.91±0.30	4.99±0.30	2.13±0.02	0±0.81	5.10±0.77	3.39±0.81
<b>18:1/16:0</b>	0.12±0.02	0.15±0.02	0.20±0.05	0.09±0.00*	0.15±0.01*	0.20±0.02*	0.19±0.01	0.29±0.02	0.41±0.06	0.12±0.00	0.16±0.01	0.23±0.02	0.53±0.11
<b>Desaturation index</b>	0.49±0.22*	0.43±0.03*	0.57±0.09*	0.25±0.01*	0.35±0.01*	0.44±0.04*	0.70±0.04* <i>b</i>	0.99±0.03*	1.23±0.11* <i>a</i>	0.34±0.00* <i>c</i>	0.45±0.02* <i>b</i>	0.62±0.03* <i>a</i>	1.30±0.22
<b>Delta 6 S</b>	0.45±0.14	0.57±0.03	0.57±0.19	8.89±0.57* <i>c</i>	6.56±0.73* <i>b</i>	5.01±0.64* <i>a</i>	2.99±0.53* <i>b</i>	2.31±0.18* <i>a</i>	1.89±0.24* <i>a</i>	5.52±1.53* <i>b</i>	2.99±1.12* <i>a</i>	2.01±0.45 <i>a</i>	0.61±0.30
<b>Delta 6 P</b>	0.05±0.02* <i>b</i>	0.11±0.02 <i>a</i>	0.19±0.10 <i>a</i>	2.54±0.17* <i>c</i>	2.93±0.36* <i>b</i>	0.65±0.06* <i>a</i>	0.50±0.06*	0.51±0.05*	0.48±0.08*	0.09±0.0* <i>c</i>	0.11±0.01 <i>b</i>	0.18±0.03 <i>a</i>	0.15±0.07
<b>Delta 6 S/P</b>	11.17±4.59* <i>b</i>	5.29±0.82 <i>a</i>	3.68±0.62 <i>a</i>	3.50±0.19 <i>b</i>	2.25±0.44 <i>b</i>	7.77±1.17* <i>a</i>	5.90±0.41 <i>b</i>	4.56±0.14	3.99±0.17 <i>a</i>	63.9±4.81* <i>c</i>	26.55±7.81 <i>b</i>	11.57±2.85 <i>a</i>	4.97±3.48
<b>PE FA ratios</b>													
<b>N6/N3</b>	7.12±2.36* <i>b</i>	3.36±0.17 <i>a</i>	3.29±0.82 <i>a</i>	19.6±0.26* <i>c</i>	16.9±1.48* <i>b</i>	8.40±0.78* <i>a</i>	1.96±0.35	1.63±0.19	1.79±0.03	0.51±0.06*	0.63±0.26*	0.84±0.14*	2.22±1.01
<b>P/S</b>	0.46±0.31*	0.53±0.03*	0.31±0.16*	2.84±0.29* <i>b</i>	2.82±0.30* <i>b</i>	1.64±0.10* <i>a</i>	0.85±0.11	0.80±0.03	0.96±0.12	2.89±0.16* <i>b</i>	1.99±0.35* <i>a</i>	1.93±0.28* <i>a</i>	1.01±0.30
<b>AA/EPA</b>	23.4±6.7* <i>b</i>	17.9±2.06*	14.3±5.55 <i>a</i>	NA	NA	NA	1.90±0.55*	2.7±0.13*	3.2±0.16*	0.43±0.01*	0.40±0.01*	0.43±0.02*	10.78±2.5
<b>16:1/16:0</b>	0.12±0.03*	0.19±0.03*	0.17±0.05*	0.11±0.02*	0.14±0.01*	0.18±0.02*	0.44±0.02 <i>b</i>	0.47±0.01	0.62±0.14* <i>a</i>	0.17±0.02	0.28±0.01	0.28±0.01	0.40±0.09
<b>18:1/18:0</b>	1.16±0.14*	1.15±0.05*	1.25±0.15*	0.62±0.01* <i>b</i>	0.84±0.11* <i>a</i>	0.98±0.05* <i>a</i>	1.80±0.08	1.98±0.02	1.98±0.15	0.80±0.04	1.34±0.06	1.34±0.06	1.98±0.35
<b>18:1/16:0</b>	0.20±0.06*	0.28±0.01*	0.28±0.08*	0.36±0.10* <i>b</i>	0.56±0.05*	0.93±0.15 <i>a</i>	0.54±0.05* <i>e</i>	0.72±0.08*	0.91±0.14	0.60±0.13	0.71±0.12	0.71±0.12	1.08±0.29
<b>Desaturation index</b>	0.65±0.23*	0.88±0.02*	0.84±0.07*	0.54±0.07* <i>b</i>	0.79±0.04*	1.07±0.10* <i>a</i>	1.53±0.03	1.70±0.03	1.89±0.07	0.81±0.02	0.94±0.04	1.19±0.07	1.68±0.32
<b>Delta 6 S</b>	0.42±0.39	0.17±0.01*	0.23±0.06*	6.31±0.35* <i>c</i>	3.87±0.98* <i>b</i>	2.32±0.65* <i>a</i>	1.65±0.23*	1.28±0.28*	1.18±0.28*	4.43±1.23* <i>b</i>	2.64±0.71* <i>a</i>	2.20±0.46* <i>a</i>	0.71±0.28
<b>Delta 6 P</b>	0.14±0.6*	0.23±0.03*	0.21±0.08*	3.41±0.52* <i>b</i>	3.77±0.22* <i>b</i>	1.60±0.17* <i>a</i>	0.87±0.10	1.02±0.07	0.88±0.18	0.42±0.08	0.49±0.13	0.56±0.04	0.64±0.31
<b>Delta 6 S/P</b>	2.60±1.61*	0.73±0.07	1.34±0.85	1.88±0.28	1.02±0.22	1.46±0.36	1.91±0.26	1.17±0.32	1.35±0.06	10.3±0.87* <i>c</i>	5.58±1.52* <i>b</i>	3.76±0.82* <i>a</i>	1.44±0.98
<b>AA PC/PE</b>	0.25±0.04 <i>b</i>	0.35±0.03 <i>b</i>	0.57±0.27* <i>a</i>	0.76±0.13*	0.60±0.04*	0.66±0.10*	0.66±0.13*	0.50±0.07*	0.55±0.06*	0.35±0.01*	0.33±0.06*	0.31±0.15*	0.20±0.06

Values are expressed as mean ( $\mu\text{g}$  fatty acid/mg protein)  $\pm$  SD of 4 replications. \* denotes significant ( $P < 0.05$ ) difference compared to control. The letters (*a, b and c*) denote a significant ( $P < 0.05$ ) difference between treatment according to ANOVA (analysis of variance) analyses, where *a, b* and *c* are all different. FA = fatty acid, mM = treatment concentration in mM, N6 PUFA = omega 6 polyunsaturated fatty acids, N3 PUFA = omega 3 polyunsaturated fatty acids, PUFA = polyunsaturated fatty acids, FA containing 2 or more double bonds, CLA = conjugated linoleic acid, C9T11 = *cis9-trans11* CLA, T10C12 = *trans10-cis12* CLA, N6/N3 = omega 6/omega 3 ratio, P/S = polyunsaturated/saturated fatty acid ratio, AA/EPA = arachidonic acid/eicosapentanoic acid ratio, C20:4n6/C20:5n3, Delta 6 S = substrate for  $\Delta$ -6 desaturase, Delta 6 P = product of  $\Delta$ -6 desaturase, Delta 6 S/P = ratio of  $\Delta$ -6 desaturase substrate/product, NA = not available, EPA is undetected.

## 5.5. Discussion

This study explores the effect of *c9t11* CLA, *t10c12* CLA, LA and ALA on fatty acid incorporation, phospholipids and cholesterol in the membranes of HepG2 cells. The cellular membrane consists of a phospholipid bilayer. Each phospholipid contains two FA chains and it is well documented that the types of FA they contain affect membrane fluidity, the transfer of nutrients across the membrane and intercellular communication (Spector and Yorek, 1985). The FA composition of the cell membrane can easily be modified by dietary intake of fatty acids.

For the purpose of this study, cells were exposed to FA conjugated to BSA in the culture medium. This clearly affected the FA profile of the cell membrane after 48 hour incubation. All supplemented FA incorporated well into the cell membrane of HepG2 cells. The treatment concentrations were based on the ATP IC<sub>50</sub>, BrdU IC<sub>50</sub> results and a concentration positioned between the two IC<sub>50</sub>s as defined in Chapter 4 - Table 4.1, were chosen. The FAs were incorporated into the PC and PE membrane fractions in a dose dependent manner. Thus, as the treatment concentration increased, the amount of FA detected in the sample increased proportionally and vice versa. The fatty acids were however incorporated better into the PC fraction than the PE phospholipid fraction of the cell membrane. Based on supplementation concentration, LA and *c9t11* CLA were the best incorporated fatty acids and *t10c12* was the least best incorporated fatty acid. CLA isomers did not affect the phospholipid concentration of the PC fraction, which was reduced dose dependently in the PC phospholipid fraction of HepG2 cell membranes. Regarding the effect on membrane composition, the *t10c12* CLA seems to have a lesser effect than *c9t11* CLA in that the *t10c12* CLA mostly has effect on MUFA while *c9t11* CLA seems to affect all FA groups, i.e. SATS, MUFA, N6, N3 and this

pattern seems consistent for PC and PE. As expected, ALA increased N3 FA and LA increased the composition of N6 FA, which is consistent in both PC and PE.

FA supplementation did not significantly affect the cholesterol content in the cell membranes of HepG2 cells. This finding was consistent with a previous study that looked at the effect of CLA isomers and LA on the distribution of cholesterol in lipid rafts and non-raft lipids (Subbaiah et al., 2011).

CLA alters the desaturation index and  $\Delta 9$ -desaturase activity in HepG2 cells and *t10c12* CLA has the greater effect than *c9t11* CLA (Subbaiah et al., 2011). CLA reduces the expression and activity of  $\Delta 9$  desaturase, the enzyme involved in converting SATS to MUFA by catalysing the formation of a *cis*-double bond at carbon-9 position of the SATS (Zhang et al., 2010). The desaturation ratios that can be identified in the present study are 16:1n7/16:0, 18:1n9/18:0 and 18:1n7/16:0. All desaturation ratios have been greatly reduced following FA supplementation by all FA. The most outspoken effect in the desaturation of SATS to MUFA was observed in samples treated with *t10c12* CLA and LA. The *t10c12* CLA also showed low  $\Delta 6$  (desaturation of 18:2n6 to 18:3n6) and  $\Delta 5$  (desaturation of 18:3n6 to 18:4n6) desaturase activity. These findings are consistent with previous studies indicative of the FA metabolism altering effect of *t10c12* CLA, and increased prostaglandin formation (Eder et al., 2002).

N6 FA cannot be converted to N3 FA in humans because of the absence of the  $\Delta 15$ -desaturase enzyme. Cancer cells are known to produce FA to maintain cell integrity through *de novo* synthesis (Berquin et al., 2011). The process involves the conversion of Acetyl-CoA leading to the formation of C18:1n9 and C20:4n3. This could explain the result of treatment with *c9t11* CLA in Table 5.1 and 5.2. C18:1n9 is significantly higher than the control and the long chain N3 FA also increased significantly, indicating a possible response of *de novo* FA synthesis.

C20:4n6 (Arachidonic acid, AA) and C20:5n3 (Eicosapentaenoic acid, EPA) are important precursors of lipid mediator molecules involved in inflammation, proliferation, apoptosis and angiogenesis. These FA are substrates for a number of enzymes belonging to COX and LOX families in the production of prostaglandins, thromboxanes and leukotrienes (Berquin et al., 2011). EPA and DHA are precursors for anti-inflammatory lipid mediators while AA is a precursor for pro-inflammatory lipid mediators (Azrad et al., 2013). The results show that *c9t11* CLA increase AA, EPA and DHA in both PC and PE cellular membrane fractions, suggesting an antagonistic effect with regard to pro- and anti-inflammatory lipid mediators. The *t10c12* CLA isomer showed insignificant suppression of AA, EPA and DHA, suggesting possible suppression in eicosanoid production, consistent with previous findings (Eder et al., 2002, Eder et al., 2003).

The results show that treatment with FA alters the lipid profile and FA metabolism of HepG2 cells. It is clear that the two CLA isomers, *c9t11* CLA and *t10c12* CLA, produced different outcomes with regard to the proliferation altering mechanism of each isomer. The *c9t11* CLA increased desaturation of PUFA and lowered the N6/N3 ratio, whereas *t10c12* suppressed desaturation of SATS and increase the N6/N3 ratio. AA and EPA were increased by *c9t11* and suppressed by *t10c12*, suggesting altering effects on lipid mediator production. Indicating that the anti-carcinogenic mechanism of *c9t11* CLA involves inflammation, apoptosis and cell proliferation, however the exact mechanism of *t10c12*CLA is yet to be established. Therefore further studies in cycle events and gene expression are required to fully uncover the anticancer effect of CLA.

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**CHAPTER 6**

## **6. General discussion and conclusion**

### **6.1. Introduction**

Cancer cells possess a number of characteristics that are vital to their immortality. These phenotypic characteristics contrast to what is considered normal characteristics of normal cells, i.e. altered pathways in growth signals, evasion of apoptosis and protection from cellular stress signals (Santos and Schulze, 2012). Cellular stress signals resulting from changes in cancer cell metabolism or unfavourable tumour microenvironment, such as hypoxia and glucose deprivation, initiate the cellular stress response to ensure the cell's survival (Kim et al., 2014). The cellular stress response includes: evasion of apoptosis, unfolded protein response and autophagy, which may contribute to carcinogenesis, tumour progression, and also treatment resistance, since most current anticancer treatments act by stimulating cell death pathways, like apoptosis in cancer cells. (Fulda, 2010).

Fatty acids are essential building blocks of cell membrane and play an important role in cellular energy and signalling molecules. The fatty acid composition of cell membranes has a significant effect on many cellular processes. Research suggests that altering the fatty acid profile of cellular membranes may play a role in human health (Bondia-Pons et al., 2007). Modifications to the lipid profile of cellular membranes alter membrane fluidity as well as many cellular functions, including transport of extracellular molecules via carrier-mediated transport, prostaglandin production and cell growth (Spector and Yorek, 1985).

The present study explored three different areas that could possibly affect the tumour development process. Firstly, the antioxidant activity of CLA isomers was studied. Antioxidants are chemicals that block the activity of free radicals and reactive oxygen species, which are highly reactive and have the potential to cause damage to cellular



components that may lead to cancer. Secondly, this study explored the cytotoxicity of CLA on HepG2 cells and addressed their effect on cell viability, apoptosis and cell proliferation. Finally, the effect of CLA on cell membrane fatty acid composition was evaluated and proposed possible effects that contribute to the anti-tumour properties of CLA.

## 6.2. Antioxidant activity (Chapter 3)

The antioxidant activity of *c9t11* CLA and *t10c12* CLA was evaluated by measuring the protection against iron-induced lipid peroxidation in rat liver microsomes by the TBARS method. When the microsomes were treated with varying concentrations of C18 FA's, there was a clear difference in the level of iron-induced lipid peroxidation. Each fatty acid showed varying degrees of protection, but certain groupings were evident. Mono-unsaturated FA displayed the greatest protection, which can be attributed to the availability of a single hydrogen bond for free radical attack thereby producing fewer lipid peroxides. Polyunsaturated fatty acids were clustered together, with the highest and lowest effect at each concentration level spanning less than 20%.

Each PUFA displayed a slight difference in effect as indicated by the different TBARS  $IC_{50}$  levels depicting protection against lipid peroxidation. In order of most to least protection, LA has the lowest  $IC_{50}$ , followed by *c9t11*, *t10c12* and finally ALA with the highest  $IC_{50}$ . This drove the conclusion, that the number and position of the double bond played an effectual role in the susceptibility to iron-induced lipid peroxidation. MUFA showed the highest protection and the lowest  $IC_{50}$ , PUFAs with 2 double bonds (LA and CLA) were in the middle, while PUFAs with three double bonds showed least protection and therefore the highest  $IC_{50}$  concentration. Linoleic acid and the 2 CLA isomers each have 2 double bonds

and differ only in their position and arrangement. The same can be said with ALA and GLA, with 3 double bonds differing in their position. These two groups did not produce the same effect, but effectual differences could be seen possibly linked to the distance of the double bond closest to the methyl end of the fatty acid chain. Linoleic acid (C18:2n6) has its first double bond at carbon position 6 from the methyl end, *c9t11* CLA at position 9 and *t10c12* at position 10. Similarly, the effect can be seen with ALA (C18:3n3), with its first double bond methyl end at carbon position 3, and GLA (C18:3n6), which has its first double bond at carbon position 6 from the methyl end. This led to the summation that the positions of the double bond also play a role in iron-induced lipid peroxidation.

Polyunsaturated fatty acids are highly susceptible to lipid peroxidation when exposed to free radicals. Free radicals attack the double bonds closest to the tail of the FA chain, thereby forming lipid peroxides. Lipid peroxidation plays an important role in cell membrane damage and increase in membrane permeability due to the presence of oxidized lipids (Wong-Ekkabut et al., 2007). Not only are the cell membranes affected, intracellular oxidative stress as well as irreversible oxidative damage also occurs, ultimately leading to cell death (Repetto et al., 2012). In this study we saw that *c9t11* and *t10c12* CLA isomers did not significantly affect iron-induced lipid peroxidation differently compared to supplementation with LA or ALA. If the outcome was to reduce lipid peroxidation, supplementation with MUFA would be the best option, but in the scope of this study, this was not the main objective. The present study showed that introducing CLA isomers to the cell membranes did not significantly differ from what was observed when introducing LA or ALA. This indicates that if CLA was introduced via the diet for example, has a similar effect to LA with regard to lipid peroxidation and the protection thereof.

### 6.3. Growth parameters (Chapter 4)

FAs possess multifaceted effects on the survival of cancer cells. They have been implicated in altering cancer cell proliferation and growth signals (Stephenson et al., 2013), and affecting apoptosis (Corsetto et al., 2011). In ideal situations, an effectual anticancer agent would be cytotoxic to only the cancerous cells and not healthy cells and tissue. The agent should decrease cell viability, increase apoptosis and retard the proliferation of these cells.

The present study showed the effect of *c9t11* CLA and *t10c12* CLA on cytotoxicity, cell viability, apoptosis and cell proliferation in HepG2 cells was determined and compared to effects elicited by LA and ALA. The results have shown that each FA that was used affected each parameter differently, but the outcomes were generally the same. These results were increased cytotoxicity, decreased cell viability, increased apoptosis and a decreased cancer cell proliferation at different levels for each FA. Between the 2 CLA isomers, *c9t11* CLA showed greater cytotoxicity than *t10c12* CLA, which is also reflected in cell viability. *c9t11* CLA reduced cell viability to a greater extent than *t10c12* CLA. All supplemented FAs reduced cell proliferation, however slight differences were evident with least effect observed with LA where no  $IC_{50}$  could be calculated. The *c9t11* CLA and ALA effect was the greatest and almost overlapped (Chapter 4; Figure 4.3). With regards to apoptosis, caspases are effector molecules in all the apoptotic pathways leading to DNA fragmentation, membrane blebbing and formation of apoptotic bodies (Tawa et al., 2004). Here again *c9t11* CLA showed a much greater effect on apoptosis fold increase than the *t10c12* CLA isomer. These findings suggest that different mechanisms of action exist between *c9t11* and *t10c12* CLA in reducing cancer cell growth parameters.

With regard to cytotoxicity, cell viability and cell proliferation, the effect between the 2 CLA isomers were not the same but showed similar trends. However, the effect on apoptosis was

substantially different. This could mean that *t10c12* CLA may possess anticancer properties that to a lesser extent involve apoptosis and more actively involve effects on cell growth involving cell cycle arrest and senescence. Of interest is that, *t10c12* CLA was shown to be a greater inhibitor of the genes regulating cell cycle and growth than *c9t11* CLA (Kelley et al., 2007).

#### 6.4. Lipid profile (Chapter 5)

Fatty acids play a major role in the structure and function of cellular membranes. Certain FAs are important for maintaining membrane structure and integrity and are involved in signalling and regulating the cell's response to its environment. Modifications to the lipid profile of the cell membrane have been shown to alter a number of cellular processes such as cell proliferation and prostaglandin production (Berquin et al., 2011).

HepG2 cells were supplemented with *c9t11* CLA, *t10c12* CLA, LA and ALA to determine effects on the membrane lipid profile. A distinct change in the FA composition of the cellular membrane was observed compared to unsupplemented control samples. In general, LA increased omega 6 (N6) fatty acids and ALA increased omega 3 (N3) FA, as predicted.

Supplementation with *t10c12* CLA reduced the MUFA of both PC and PE fractions of the cell membrane. LA, ALA and *t10c12* CLA also decreased the desaturation index in both PC and PE phospholipid fractions. This finding suggests that these FA may moderate the activity of  $\Delta 9$ -desaturase, which is the enzyme involved in converting SATS to MUFA. Tumour cells obtain most fatty acids, to maintain high levels of proliferation, by *de novo* synthesis. Therefore, increased expressions of biosynthetic enzymes that are required to produce the large amounts of FA that are essential for maintaining the cancer phenotype

(Mohammadzadeh et al., 2014). The  $\Delta 9$ -desaturase enzyme is an important regulatory enzyme in cellular *de novo* fatty acid synthesis and has been implicated in the proliferation of cancer cells (Luyimbazi et al., 2010).

When looking at the outcome of this study (Chapter 5), it is evident that supplementation with *c9t11* CLA significantly increased C20:4n6 (Arachidonic acid, AA) and C20:5n3 (Eicosapentaenoic acid, EPA) in both PC and PE phospholipid fractions of the cell membrane. AA and EPA are important precursors of lipid mediator molecules involved in inflammation, proliferation, apoptosis and angiogenesis. EPA is a precursors for anti-inflammatory lipid mediators while AA is a precursor for pro-inflammatory lipid mediators (Azrad et al., 2013).



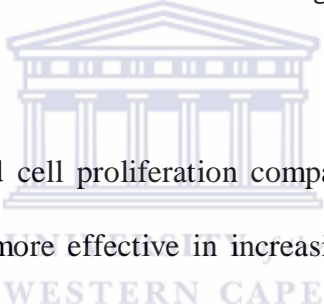
## 6.5. Conclusion

Cell proliferation, death and senescence control in normal mammalian cells and cancer cells are related to the regulation of metabolism, in particular the synthesis and remodelling of lipid modulators and structures (Igal, 2010). Elevated rate of aerobic glycolysis and an accelerated biosynthesis of macromolecules, including DNA, proteins and lipids, are characteristic in the metabolism in cancer cells (DeBerardinis et al., 2008). An essential aspect of the remodelled metabolism of transformed cancer cells is an increased rate of lipid biosynthesis (Baenke et al., 2013). Lipid metabolism modifications can influence many cellular processes, including cell growth, proliferation, differentiation and motility (Santos and Schulze, 2012).

It is shown in the present study that *c9t11* and *t10c12* CLA are incorporated into the cell membrane of HepG2 cells. Their incorporation has a consequential inhibiting effect on cell

proliferation of cancer cells that may be associated with the altered lipid profile of the cellular membrane.

The *c9t11* CLA isomer markedly increased apoptosis and the overall FA content of the cellular membrane. This increased a number of factors that contribute to the effect of programmed death seen on cancer cells. These include increased MUFA concentrations, which reduces overall susceptibility to lipid peroxidation, thereby preventing cellular stress and allowing the cells to follow their normal cycle towards cell death. Moreover, increasing the total FA content of the cell membrane suggest increase *de novo* synthesis of FA, that are required for the formation of new cells, but could also be implicated in the formation of apoptotic bodies, which are membranous bodies containing cell fragments that are formed at the end of the apoptosis process.

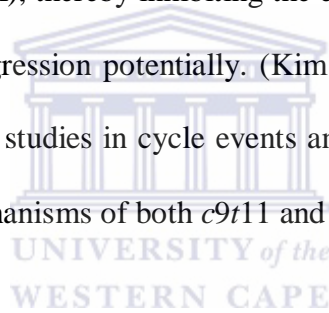


The *t10c12* CLA isomer reduced cell proliferation comparable to *c9t11* CLA (Chapter 4; Table 4.1); however *c9t11* was more effective in increasing apoptosis and decreasing cell viability, similar to ALA. This suggests that the cell proliferation reducing effect of *t10c12* CLA may be the result of a different mechanism. The *t10c12* CLA isomer showed to reduce delta-9 desaturase activity, which is an essential enzyme in *de novo* synthesis of FA, indicated by reduction in MUFA composition of the cell membrane of HepG2 cells. Limiting *de novo* FA synthesis effectively reduces the FA availability required for proliferation of new cells. This could explain the effect seen in the cell proliferation assay. EPAs are precursors to anti-inflammatory modulatory molecules that have been implicated in prostaglandin production, which could suggest another mechanism of action of CLA.

When looking at Table 4.1 in Chapter 4, *c9t11* seems to mimic the effectiveness of ALA on of viability, cell proliferation and apoptosis in HepG2 cells. *c9t11* also increased N6 and N3 PUFA, potentially increasing cell susceptibility to decreased survival and growth despite the

increased SATS and MUFA. The antioxidative potential of *c9t11* CLA was greater than *t10c12* CLA; this in addition to *c9t11*'s greater effectiveness on cell viability and apoptosis, and the difference between the 2 CLA isomers could make *c9t11* a more effective anticancer agent.

The evidence exists in literature that reduction of cell proliferation exhibited by CLA may be attributed to the aggregation of cells in the S phase of the cell cycle and apoptosis via mechanisms involving the mitochondrial pathway (Miglietta et al., 2006). CLA also elicited cell cycle arrest in G1 phase and induced tumour suppressor protein accumulation (Kemp et al., 2003). Isomer specifically, *t10c12* indicated that *t10c12*-induced p21 (CIP1/WAF1) binds to cyclin dependent kinases (CDK), thereby inhibiting the enzyme activity, which contributed to the decrease in the G1-S progression potentially. (Kim et al., 2006). There are yet to be fully explained; therefore further studies in cycle events and gene expression are required to fully uncover the anticancer mechanisms of both *c9t11* and *t10c12* CLA isomers.



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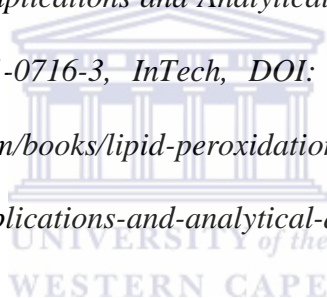


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12 April 2000

Dr WCA Gelderblom  
PROMEC  
PO Box 19070  
**TYGERBERG**  
7505

Dear Dr Gelderblom

### ECRA MEETING : 28 FEBRUARY 2000

Thank you for your letter of 21 February 2000. We discussed the issues you raised and should like to comment as follows:

- Provision will be made to discuss unclear issues or questions with relevant applicants when necessary.
- Animals used for "harvesting tissue" for supporting standard biochemical techniques are not classified as animal experiments and therefore need no ethical approval. ECRA, however, requests that a register be kept for this specific purpose containing the following information:
  - Date
  - Animal used
  - Number of animals
  - Tissue harvested
  - Purpose of procedure
  - Method of termination
  - Researcher's signature
- Changes to protocol while experiment is in progress: Any changes in procedures must be reported, in writing, to the Chairperson who will use his discretion as to whether the request needs to be discussed at committee level. This will especially apply when radical changes are introduced.

Yours sincerely

DR AJS BENADÉ  
CONVENOR : ECRA

**BCA (Pierce) Protein Determination Assay****Solutions required**

- 2% SDS Solution
- BCA Protein Reagent A
- BCA Protein Reagent B (4% CuSO<sub>4</sub>·5H<sub>2</sub>O)
- BSA Standard (1mg/ml)
- Distilled H<sub>2</sub>O

<b>2% SDS Solution</b>	<b>Reagent A</b>	<b>Reagent B</b>	<b>BSA</b>
Make your own	Purchase from Separations	Make your own	
2Mm EDTA		4% CuSO <sub>4</sub> ·5H <sub>2</sub> O	
20Mm NaHCO <sub>3</sub>			
2% Sodium dodecyl sulphate			

**Working Reagent preparation**

Mix Solution A and Solution B in a 50:1 ratio

**Standard preparation**

	<b>BSA(μl)</b>	<b>2% SDS(μl)</b>	<b>Working Reagent(μl)</b>
<b>Blank</b>	0	100	200
<b>S1</b>	5	95	200
<b>S2</b>	10	90	200
<b>S3</b>	20	80	200
<b>S4</b>	30	70	200
<b>S5</b>	40	60	200
<b>S6</b>	50	50	200

## Addendum 2

### Unknown samples

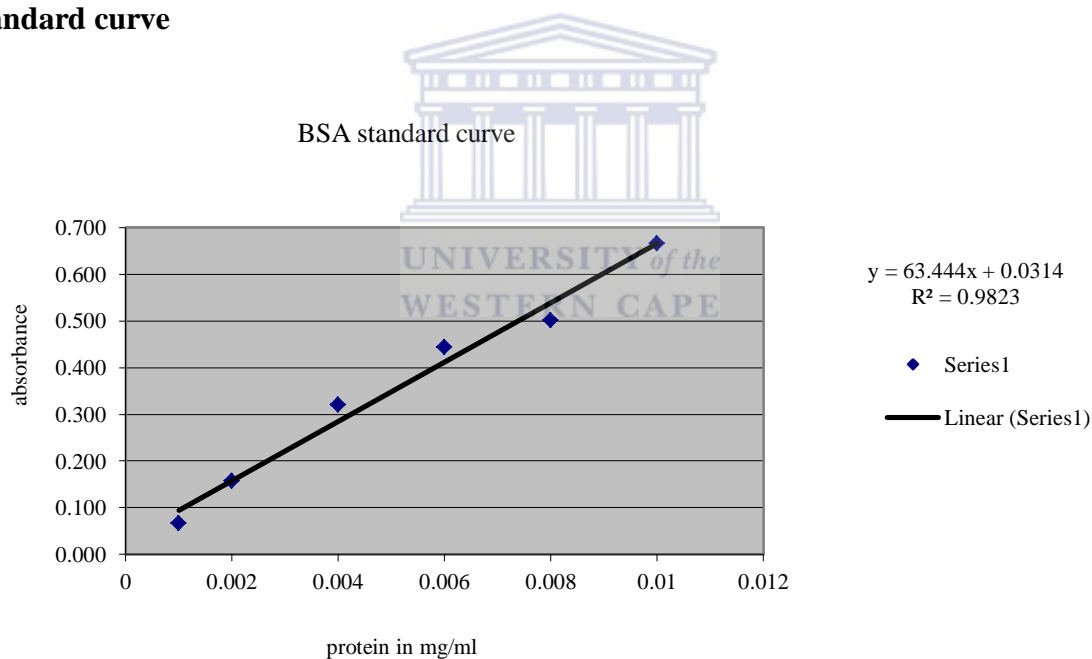
Sample( $\mu$ l)	2% SDS( $\mu$ l)	Working Reagent( $\mu$ l)
25	75	200

\*Smaller volume of sample can be used – just ensure that sample and SDS does not exceed 100 $\mu$ l.

### Procedure

1. Pipette each standard and sample into a microplate well.
2. Add the working reagent.
3. Cover plate and incubate at 37°C for 30 minutes.
4. Cool plate to room temperature.
5. Measure the absorbance at 562nm on plate reader.

### Standard curve



Equation:  $y = mx + c$

$y$  = Absorbance reading (nm)

$m$  = slope

$x$  = protein concentration (mg/ml)

$c$  = y-intercept

*Esterbauer and Cheeseman (1990), Methods Enzymol. 186: 407-421*

*Yen & Hsieh, (1998), J. Agric. Food Chem. 46: 3952-3957*

*Hu et al., J. Nutr. (1989), 119: 1574-1582*

### **ASSESSMENT OF LIPID PEROXIDATION:**

#### **MDA (Malondialdehyde) or TBARS ASSAY**

##### **REAGENTS:**

- TCA Reagent (10 % TCA, BHT [see below] & 1mM EDTA) – Make up fresh
- 0.67% TBA solution in distilled water – Make up fresh: Weigh out amount of TBA powder needed and mix with less than needed amount of water, heat at approx. 45°C-50°C until dissolved, cool to room temp. Then make up to desired volume in a volumetric flask.
- 1.15% KCl containing 0.01 M potassium phosphate buffer (pH 7.4)
- FeSO<sub>4</sub> solution (2.5mM ferrous sulphate; 69.5mg/100ml dist. water) – Make up fresh
- BHT (stock = 8g/100ml ethanol, kept in walk in fridge), add 0.125ml/100ml TCA-EDTA (BHT does not dissolve easily in an aqueous solution, will flocculate)

**NB: For all steps work on ice**

**Only make up the required amount of reagents (TCA, TBA, as required per assay)**

##### **Homogenate preparation**

- Homogenise tissue samples on ice in 19 volumes of 1.15% KCl in phosphate buffer using glass hand Dounce (dounce approx. 10 x) and store at -80°C.
- Microsomes (stored at -80°C) for determination of anti-oxidative activity should be dounced on ice before use (10x, loose dounce)
- Determine protein concentration

(NB: Dilute samples for protein assay with 2% SDS solution approx. 50-60x, microsomes 50x).

**METHOD**

- Use 1mg protein per unknown sample/microsomes

Note: Use 1mg protein per test tube, therefore 0.5ml sample must contain 2mg protein. This will equate to 1mg protein per 1ml total test tube reaction volume.

- Run 2x blanks through the procedure containing sample buffer only (1.15% KCl in phosphate buffer)
- Dilution of tea samples may need to be pre-determined, a range of dilutions should be used to determine the IC<sub>50</sub> value

Treatment with iron (Table 1)

- Vortex and incubate at 37°C for 1 hr in a shaking waterbath.
- Thereafter, place tubes on ice and follow the assay procedure.

**Table 1 pipetting scheme for testing antioxidant potential of tea/oil etc. samples using microsomes**

	<i>Antioxidant</i>	<i>KCl-buffer</i>	<i>FeSO<sub>4</sub></i>	<i>Microsomes (2mg/ml)</i>	<i>Total volume</i>
Reagent blank	-----	0.8ml	0.2ml	-----	1ml
Microsomes blank	-----	0.5ml	-----	0.5ml	1ml
Positive control	-----	0.3ml	0.2ml	0.5ml	1ml
Samples (teas, oils, etc.)	0.1ml	0.2ml	0.2ml	0.5ml	1ml

**Table 2 pipetting scheme for determination of TBARS in tissue homogenates**

	<i>protein</i>	<i>KCl-buffer</i>	<i>FeSO<sub>4</sub></i>	<i>Homogenate</i>	<i>Total volume</i>
Reagent blank	-----	0.8ml	0.2ml	-----	1ml
Samples as is (liver homogenates)	±10mg/ml	0.7ml	0.2ml	0.1ml	1ml

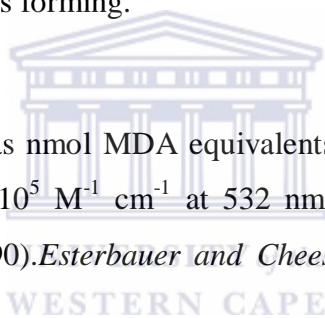


### Addendum 3

#### Assay procedure:

- add 2ml TCA reagent (10% TCA with BHT & EDTA) to each tube (Addition of EDTA and BHT to the TCA reagent prevents further oxidative damage during the assay procedure)
- Vortex and centrifuged at 2000 rpm for 15 min.
- Take off 2ml of the supernatant, add to a new test tube and add 2 ml 0.67% TBA solution
- Vortex and heat in capped tubes at 90°C for 20 min in a water-bath.
- Allow mixture to cool (ice bath)
- Measure absorbency at 532 nm on spectrophotometer, use distilled water as blank and read sample blanks as well as unknown samples. **Use glass 2ml or 4ml cuvettes.** Plastic cuvettes get bubbles forming.

Lipid peroxidation is expressed as nmol MDA equivalents per mg protein, using the molar extinction coefficient of  $1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$  at 532 nm for MDA or the standard curve (Esterbauer and Cheeseman, 1990). *Esterbauer and Cheeseman (1990), Methods Enzymol. 186: 407-421*



#### Addendum 4

Product	size	Cat.#
CellTiter-Glo® Luminescent Cell Viability Assay	10 ml	G7570

Substrate is sufficient for 100 assays at 100µl/assay in 96-well plates or 400 assays at 25µl/assay in 384-well plates. Includes:

- 1 × 10ml CellTiter-Glo® Buffer
- 1 vial CellTiter-Glo® Substrate (lyophilized)

Mix together before use.

#### Protocol:

1. Prepare opaque-walled multiwell plates with mammalian cells in culture medium 100µl per well for 96-well plates.
2. Prepare control wells containing medium without cells to obtain a value for background luminescence.
3. Add the test compound to experimental wells, and incubate according to culture protocol.
4. Equilibrate the plate and its contents at room temperature for approximately 30 minutes.
5. Add a volume of CellTiter-Glo® Reagent equal to the volume of cell culture medium present in each well (e.g., add 100µl of reagent to 100µl of medium containing cells for a 96-well plate, or add 25µl of reagent to 25µl of medium containing cells for a 384-well plate).
6. Mix contents for 2 minutes on an orbital shaker to induce cell lysis.
7. Allow the plate to incubate at room temperature for 10 minutes to stabilize luminescent signal.
8. Record luminescence.

Note: Instrument settings depend on the manufacturer. An integration time of 0.25–1 second per well should serve as a guideline.

## Addendum 5

Product	size	Cat.#
Caspase-Glo®3/7 Assay	100ml	ZZG8092

Each system contains sufficient reagents for 1,000 assays at 100µl per assay in a 96-well plate or 4,000 assays of 25µl per assay in a 384-well plate. Includes:

- 1 × 100ml Caspase-Glo® 3/7 Buffer
- 1 bottle Caspase-Glo® 3/7 Substrate (lyophilized)

Mix together before use.

### Standard Protocol for Cells Cultured in a 96-Well Plate

1. Before starting the assay, prepare the Caspase-Glo®3/7 Reagent. Allow the reagent to equilibrate to room temperature. Mix well.
2. Remove 96-well plates containing cells from the incubator and allow plates to equilibrate to room temperature.
3. Add 100µl of Caspase-Glo®3/7 Reagent to each well of a white-walled 96-well plate containing 100µl of blank, negative control cells or treated cells in culture medium.
4. Gently mix contents of wells using a plate shaker at 300–500rpm for 30 seconds. Incubate at room temperature for 30 minutes to 3 hours, depending upon the cell culture system.
5. Measure the luminescence of each sample in a plate-reading Luminometer as directed by the luminometer manufacturer.

**CHOLESTEROL DETERMINATION**

Adapted from – Richmond (1973). Clin. Chem., 19: 1350-1356.

– Smuts *et al.* (1994). Cor. Art. Dis. 5: 331-338

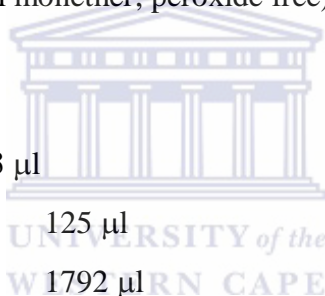
**Reagents:****(1) Cholesterol Colour Reagent:**

To one liter volumetric flask add the following:

KH <sub>2</sub> PO <sub>4</sub>	22,1827 g
K <sub>2</sub> HPO <sub>4</sub> .3H <sub>2</sub> O	8,4445 g
KI	19,9212 g
NaN <sub>3</sub>	9,7515 g
Alkylbenzyltrimethylammonium chloride	100 mg
Ammonium molybdate	12,4 mg
Triton X-100 (polyethylene glycol monether; peroxide free)	2 g

**(2) Cholesterol Reagent B:**

Cholesterol esterase	83.3 µl
Cholesterol oxidase	125 µl
3M NaCl	1792 µl

**Method:**

- (1) Use 20 µl of the lipid sample from CMS extraction used for TLC spotting.
- (2) Add 50 µl CM, vortex.
- (3) Add 300 µl 1% Tritin-X100 (peroxide free), vortex.
- (4) Evaporate emulsion under N<sub>2</sub>, vortex until clear.
- (5) Add 1.7 ml cholesterol reagent.
- (6) Standards:
  - 10 µl from 4 different cholesterol standard concentrations
  - 300 µl 1% Triton-X100
  - Add 1.7 ml cholesterol reagent
- (7) Carry 1 ml over from (5) and (6) into small sample tubes and add 20 µl cholesterol Reagent B.
- (8) Add 20 µl 3 M NaCl to original tubes from (5) and (6).
- (9) Incubate at room temperature.

(10) Read at 365 nm with both VIS and UV bulbs on. Read each sample together with its respective blank.

**Cholesterol content calculation:**

Colon polyps/mucosa

$$C_{\text{cholesterol}} = \frac{f_{\text{standard}} \times f_{\text{aliquot}} \times (100/\text{CLW}) \times \text{absorbance}_{\text{sample}}}{m_{\text{colon\_protein}} [\text{mg}]}$$

RBC membranes

$$C_{\text{cholesterol}} = \frac{f_{\text{standard}} \times f_{\text{aliquot}} \times \text{absorbance}_{\text{sample}}}{C_{\text{protein}}}$$

- $f_{\text{standard}}$  average of n values:  $m_{\text{standard}} [\mu\text{g}] / \text{absorbance}_{\text{standard}}$
- $f_{\text{aliquot}}$   $V_{\text{total CMS of sample}} [\mu\text{l}] / V_{\text{aliquot of CMS extract spotted for TLC}} [\mu\text{l}]$
- CLW colon lipid weight (mg)
- $m_{\text{colon\_protein}} [\text{mg}]$  mg sample protein per 100 mg colon protein dry weight
- $C_{\text{protein}}$  RBC protein concentration (mg per ml)
- unit:  $\mu\text{g cholesterol per mg protein}$

This letter serves to inform the reader that necessary corrections have been made to the MSc thesis titled: *The Modulating Effect of Conjugated Linoleic Acid CLA on Cancer Cell Survival in vitro.*

Lyle Arendse

15 February 2015

