University of Alberta

What Happens to Essential Fatty Acids in Cancer and During Chemotherapy Treatment?

by

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Abstract

Cachexia, a multifactorial wasting syndrome that involves the loss of muscle and fat despite adequate nutritional intake, often results in patients having a reduced quality of life, a poor response to treatment and a decreased survival time. Patients with lung and colorectal cancer are frequently affected by malnutrition and abnormalities in lipid metabolism may reduce availability of n-6 and n-3 fatty acids and exacerbate the cachectic condition.

We determined the total amount of plasma phospholipids as well as the amount of n-6 and n-3 fatty acids in plasma phospholipids of patients with advanced lung and colorectal cancer. In addition we determined the fatty acid composition and the amount of phospholipids and triglycerides in liver and plasma of rats that were injected with a colorectal tumor and treated with the chemotherapy drug Irinotecan.

Essential fatty acids were unexpectedly higher in patients with advanced cancer compared to healthy subjects. A fish oil diet maintained levels of essential fatty acids in rats treated with chemotherapy. This work is important in understanding fatty acid metabolism during the cancer trajectory and in defining points of potential intervention in patients with advanced cancer.

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List of Abbreviations

ANOVA analysis of variance

ANSA 8-anilino-1-naphthalene-sulfonic acid

ATP adenosine triphosphate

BF_3 boron trifluoride

BMI body mass index

$CaCl_2$ calcium chloride

cAMP cyclic adenosine monophosphate

CIU clinical investigation unit

CO_2 carbon dioxide

CPT-11 camptothecin-11

CRP c-reactive protein

CT computer tomography

DHA docosahexaenoic acid

DNA deoxyribonucleic acid

DXA dual-energy x-ray absorptiometry

EFA essential fatty acid

EPA eicosapentaenoic acid

GC gas chromatography

GCR gastric and colorectal

GLC gas liquid chromatography

Glut-4 glucose transporter protein

HDL high density lipoprotein

HSL hormone sensitive lipase

IL-1 interleukin-1

IL-6 interleukin-6

KOH potassium hydroxide

LA linoleic acid

LNA linolenic acid

LDL low density lipoprotein

MAP mitogen-activated protein

mRNA messenger ribonucleic acid

MUFA monounsaturated fatty acid

n-3 omega 3 fatty acid

n-6 omega 6-fatty acid

PGSGA patient generated subjective global assessment

PL phospholipid

PUFA polyunsaturated fatty acid

REE resting energy expenditure

SD standard deviation

SFA saturated fatty acids

SN-38 7-ethyl-10-hydroxy-camptothecin

SN-38G glucuronide

TEE total energy expenditure

TG triglyceride

TNF-α tumor necrosis factor alpha

TopoI topoisomerase I

TMN tumor; node; metastasis

VLDL very low density lipoprotein

Chapter 1

1.1 Lung and Colorectal Cancer

The National Cancer Institute of Canada reports that in 2007, there were approximately 159,900 new cases of cancer diagnosis and 72,700 deaths from cancer (NCIC cancer statistics, 2007). Lung cancer is the leading cause of cancer death in both men and women. Mortality from lung cancer continues to rise in women (NCIC cancer statistics, 2007). Smoking is the leading cause of lung cancer making it the most preventable respiratory disease worldwide. Other factors such as radon, second-hand smoke and exposure to asbestos also contribute. Lung cancer is increasing to epidemic proportions in developing countries whereas people in the developed world are becoming more aware of the health risks associated with lung cancer (Spiro et al., 2005).

Lung cancer is initially detected through abnormal chest radiographs and based on these results, a low dose computer tomography (CT) scan is done. CT detects pulmonary nodules with greater sensitivity than chest radiographs and therefore is a better tool for lung cancer diagnosis (Spiro et al., 2005). There are seven stages of lung cancer ranging from stage IA which represents small circumscribed tumors presenting no invasiveness or metastasis to stage IV where distant metastasis is observed (Mountain et al., 2000). Lung cancer can present as four different types: squamous cell carcinoma, adenocarcinoma (including bronchio-alveolar carcinoma) and finally, large and small cell carcinoma (Mountain et al., 2000).

In 2007, colorectal cancer was responsible for approximately 8,700 deaths in Canada and was second to lung cancer as the leading cause of cancer death in both men

and women (NCIC cancer statistics, 2007). Diet, obesity and physical inactivity increase the risk of developing colorectal cancer in addition to family history, excessive alcohol consumption and smoking. A fecal occult blood test is a screening tool used to determine whether the presence of blood in the stool is indicative of colorectal cancer. When results from a fecal occult blood test are positive, diagnostic testing which may include a digital rectal exam, a colonoscopy, a barium enema, a sigmoidoscopy or a CT scan may be required to confirm diagnosis.

Colorectal cancer has four distinct stages. The first stage, 0, involves the lining of the mucosa of the colon and is restricted to polyps which can ultimately be removed via colonoscopy. The stages increase in severity until stage IV is reached which indicates cancer has spread to other organs within the body. To accurately stage the disease, the specialist implies TNM descriptors (T=primary tumor, N=regional lymph nodes and M=distant metastasis) which are each assigned a number ranging from 0 to 4 indicating the size and location of the primary tumor, the involvement of the lymph nodes and finally whether or not distant metastasis has been detected.

Regardless of cancer type, the extent of disease at diagnosis is directly related to treatment options, response to treatment and recurrence after complete treatment (Mountain et al., 2000). Surgical resection, radiation therapy, chemotherapy or a combination of treatment modalities are currently used to manage the disease. Significant weight loss has been observed in patients with either lung and colorectal cancer regardless of whether patients are undergoing active treatment or are receiving palliative care (Hansell et al., 1986, Holroyde et al., 1984, Dewys et al., 1980, reviewed

by Bruera E, 1997). This is of considerable importance because weight loss greater than 5% of usual body weight has been associated with decreased response to treatment, compromised quality of life and a poor final outcome (Dewys et al., 1980, Lavin et al., 1980, Inui et al., 2002).

1.2 Metabolic Problems in Patients with Cancer

High mortality rates (Agteresch et al., 2000, Jatoi et al., 2001) and significant risk for malnutrition (Martin et al., 1999) are prevalent in patients with lung and colorectal cancer. Cachexia is a multifactorial wasting syndrome involving loss of muscle and fat despite adequate nutritional intake (MacDonald et al., 2003). It is characterized by hypercatabolism and the inadvertent breakdown of skeletal and adipose tissue. Cachexia is frequently accompanied by anorexia, early satiety and overall weakness (Baracos et al., 2004). Patients with advanced lung and colorectal cancer frequently present with cachexia. Cancer cachexia has also been associated with a reduced quality of life, poor response to treatment and decreased survival time (DeWys et al., 1980). Weight loss in cancer patients is often, but not always, accompanied by hypermetabolism which accelerates wasting by increasing the energy deficit that is present due to anorexia and/or early satiety. Reduced food intake and higher metabolic rate are frequently precursors of cachexia which leads to a significant depletion of adipose tissue (Bruera et al., 2000, Persson et al., 2005). One study reported weight loss in cancer patients was more closely correlated to increased resting energy expenditure (REE) as opposed to a reduced dietary intake (Van Cutsem et al., 2005). Other studies have shown that lung and pancreatic cancer patients experiencing cachexia have an elevated REE coupled with decreased total

energy expenditure (TEE) due to a reduction in physical activity (Moses et al., 2004). Compared to healthy subjects, significantly greater REE despite a lower body weight and body fat mass has been reported in cancer patients (Barber et al., 1999)

Tumor type may influence the elevated metabolic rate associated with body wasting. Fredrix et al., (1991) compared REE in patients with gastric and colorectal (GCR) cancer to patients with lung cancer and healthy age-matched controls. They reported that patients with lung cancer had an elevated REE compared to healthy controls ($p<0.001$) whereas patients with GCR cancer had a normal REE (21.0±2.0kcal/day/kg of BW). When patients with lung cancer underwent curative surgery, their REE was similar to that of healthy subjects at the 12 month follow-up visit (Fredrix et al., 1991). These findings would suggest that the tumor is somewhat responsible for elevated energy expenditure in cancer patients.

A suggested mechanism behind elevated REE in patients with cancer can be linked to the competition for nutrients between the host and the tumor. Both the host and the tumor require nutrients for energy however the tumor may preferentially metabolize glucose due to the overexpression of enzymes in the glycolytic pathway thereby preventing the host from obtaining adequate amounts of energy (Delano & Moldawer, 2006). The changes in metabolic rate may also be attributed to the mobilization of substrates for energy through lipolysis and proteolysis (Emery, 2005). For example, the tumor releases factors such as lipid mobilizing factor that stimulate lipolysis and mobilizes fat within the host (Sanders et al., 2004, Beck et al., 1987) when energy from fats is not required. This contributes to the depleted lipid stores. Another mechanism that

may contribute to wasting is futile cycling. A futile cycle is a set of opposing reactions that act simultaneously whereby one reaction is driven by ATP hydrolysis. This results in the use of energy substrates with no net yield of energy (Klein et al., 1990, Wolfe et al., 1987). If fatty acid synthesis and β-oxidation were to occur concurrently, this would be a futile cycle that could potentially contribute to fat wasting in patients with advanced cancer.

1.3 Fatty Acid Metabolism

Digestion of lipids occurs throughout the gastrointestinal tract, beginning in the stomach. Gastric lipase begins hydrolysis of triglycerides which become coated with bile salts in the duodenum to increase the availability of fat to digestive enzymes. Pancreatic lipase further hydrolyses the triglycerides that are coated with bile salts to diacylglycerols, monoacylglycerols and free fatty acids. These intermediates in digestion combine once again with bile salts to form micelles. Fats are then delivered to intestinal mucosal cells via passive diffusion or through specific fatty acid transport proteins located in the membrane. Once in the mucosal cell, triglycerides are reformed from the hydrolyzed products and packaged into chylomicrons which then enter the lymphatic system. Chylomicrons circulate through the lymph and remain in the abdominal region until they are released into the blood steam to supply fuel to nearby tissues. Lipoprotein lipase cleaves free fatty acids from chylomicrons which are absorbed by the tissue cells. Chylomicron remnants are removed from circulation by the liver and fatty acids are repackaged into triglycerides. The newly synthesized triglycerides in addition to the triglycerides formed from endogenous fatty acid synthesis within the liver are packaged into very low density lipoproteins (VLDL) and released into circulation. Lipoprotein

lipase found within muscle and adipose tissue cleaves the TG within the VLDL to release free fatty acids and glycerol. These products can be taken up by myocytes and used as energy through β-oxidation thereby releasing CO_2 and ATP or by adipocytes which reform and store the fatty acids as triglycerides (Gurr et al., 2002).

In a fasted state blood glucose concentrations fall, slowing insulin release and triggering the secretion of glucagon. Glucagon activates hormone sensitive lipase which catalyzes release of free fatty acids from triglycerides within adipose tissue. Fatty acids enter the bloodstream bound to serum albumin and are carried throughout the body. The glycerol portion of cleaved triglycerides is utilized by the liver and enters the glycolytic pathway where it can either be oxidized for energy or be used for gluconeogenesis. Once inside the liver mitochondria, fatty acids undergo β-oxidation which releases one molecule of acetyl-CoA for every two carbons of the fatty acid.

Abnormalities in lipid metabolism of patients with advanced cancer may be attributed to increased lipolysis and/or fat oxidation or decreased lipogenesis (Delano & Moldawer, 2006, Klein et al., 1990, reviewed by Agustsson et al., 2007). This has been determined through animal and cell culture studies therefore little is known about the deranged metabolism of patients with cancer. Currently two studies exist that look at the fatty acids in patients with advanced cancer (Zuijdgeest-Van Leeuwen et al., 2002, Pratt et al., 2002). These studies describe the changes in fatty acids however no mechanisms have been established to explain why these changes occur.

1.4 Importance of Lipids

Lipids are essential for survival and proper functioning of living organisms. Not only are fats an important source of energy (triglycerides), they are also structural components of cell membranes in the body (phospholipids) and serve as signaling molecules to stimulate the release of hormones and cytokines. Lipids are mainly obtained from the diet in vegetable oils and animal products, however they can also be synthesized within the organism in response to high energy levels.

The essential fatty acids, linoleic (LA) and linolenic (LNA) acid cannot be synthesized by humans therefore they must be obtained through the diet. LA and LNA undergo a series of desaturation and elongation reactions to produce longer chain and more highly polyunsaturated fatty acids that contribute to the structural integrity of membranes or become biologically active metabolites (Figure 1). N-6 fatty acids, particularly arachidonic acid, are predominantly found in membranes of most cells whereas long chain n-3 fatty acids are found in high abundance in nervous tissue (Gurr et al., 2002).

LA and LNA compete for desaturation by the same Δ6-desaturase enzyme. N-3 fatty acids have a higher affinity for the enzyme, however the transition through the n-3 pathway is generally lower because of the small proportions in the diet. Arachidonic acid, an n-6 fatty acid, is largely found in meat and is a precursor for pro-inflammatory eicosanoids. N-3 PUFA's are found in certain species of fatty cold water fish such as herring and mackerel, rapeseed oil and soybean oil.

1.5 Essential Fatty Acid Deficiency

A low dietary intake of essential fats causes a deficit that is easily remedied by supplementation or by increasing consumption in the diet providing there are no

underlying problems in the metabolic pathway described above. Improper fat digestion or malabsorption can also contribute to fatty acid deficiency. Pancreatic insufficiency can prevent secretion of pancreatic lipase which is essential in the initial stages of digestion. Failure to produce bile salts would prevent formation of micelles thereby preventing fats from being delivered to the intestinal mucosa. Malabsorption problems may be caused by the impaired formation of chylomicron or VLDL. This would lead to an accumulation of fat either in the enterocytes or in the liver thus preventing lipids from being delivered to tissues within the body.

The desaturase and elongase enzymes, located in the liver, must be fully functional to efficiently produce longer chain polyunsaturated fatty acids of the n-6 and n-3 families. When Δ6 and Δ5-desaturase activity was impaired by feeding fumonisin B_1 mycotoxin to rats, this resulted in a significant increase in C18:2n-6 and C18:3n-6 and a significant decrease in C20:4n-6 and C22:6n-3 over a 21 day period (Gelderblom et al., 2002). These findings show that without the desaturase enzymes, production of new fatty acids is not possible. Although LA and LNA may be present, functional aberrations of the enzymes in the metabolic pathway prevent the formation of arachidonic acid, EPA and DHA. Interestingly, studies have shown the conversion of LNA to DHA in humans to be less than 0.5% (Plourde M & Cunnane S, 2007). To overcome this inefficient conversion, it may be necessary to eat foods or supplements containing DHA as opposed to LNA which would increase plasma and tissue levels of DHA (Plourde M & Cunnane S, 2007).

Treatment with chemotherapy may also cause an essential fatty acid deficiency. Irinotecan (7-ethyl-10-[4-(1-piperidino)-1-piperidino] carbonyloxy-camptothecin) (CPT-11, Camptosar®) was introduced into the clinic in the late 1980s and is currently used in

the treatment of colorectal cancer (Sakata et al., 1994, Shimada et al., 1993, Rothenberg 2001, Rothenberg et al., 1999) and in combination therapy for the treatment of non-small cell lung cancer (Yanaihara et al., 2007). Irinotecan is a water soluble, semi-synthetic derivative of camptothecin, a natural plant alkaloid (Vanhoefer et al., 2001, Fuchs et al., 2006, Xu et al., 2006). This anti-neoplastic agent is converted to its most cytotoxic active metabolite SN-38 (7-ethyl-10-hydroxy-camptothecin) by carboxylesterase which cleaves the bulky piperidino side-chain of irinotecan (Vanhoefer et al., 2001, Xu Y & Villalona-Calero M., 2002) making it 1000 times more potent (Xu Y & Villalona-Calero M., 2002). Irinotecan inhibits topoisomerase I (Topo1) (Xu Y & Villalona-Calero M., 2002), a cellular enzyme that relieves torsional strain in the DNA helix during replication and transcription by causing single-strand breaks. By binding to cellular TopoI-DNA complexes, irinotecan causes irreversible damage as it creates double-stranded DNA breaks leading to cell death (Xu Y & Villalona-Calero M., 2002). The liver deactivates SN-38 to SN-38 glucuronide (SN-38G) (Xu Y & Villalona-Calero M., 2002). Glucuronidation, is responsible for the accumulation of SN-38 in the intestines which increases susceptibility to gut toxicity and diarrhea, (Gupta et al., 1994, Shimada et al., 1996) the major dose limiting factor of treatment with irinotecan. Toxic anti-cancer drugs circulating through the liver may potentially contribute to dysregulation of fatty acid packaging and distribution. Enzymes necessary for fatty acid synthesis which are naturally found in the liver may be affected by the toxicity of the drug or by additional strain on the liver to efficiently metabolize the chemotherapeutic agents.

1.6 Assessment of Essential Fatty Acid Status

To establish whether or not a patient has adequate essential fatty acids, it is necessary to determine amounts of n-6 and n-3 fatty acids in the blood. This can be accomplished using several different methods. Analyzing food frequency questionnaires, 24-hour recalls or food records are useful to assess the dietary intake of nutrients or food groups. Unfortunately, problems such as over or under-reporting may influence the results of the tests (Beydoun et al., 2007) because inaccurate amounts of food are being recorded. Additional problems may include difficulty with memory recall or altered eating patterns during the period of dietary assessment (Cantwell, 2000)

Several clinical signs may be indicative of an essential fatty acid deficiency. Diets low or free from essential fatty acids produce skin abnormalities such as scaly dermatitis in humans as well as dermatosis and an increased permeability to water in rats (Ziboh et al., 2002). In the clinical setting, it is difficult to determine if skin abnormalities are caused by an insufficiency in essential fats because various other diseases can also result in skin irregularities.

Finally, the most effective method of determining fatty acid status is through biological markers. Biomarkers of fatty acid composition are independent measures of intake and can be measured in various blood fractions such as erythrocytes and plasma (Cantwell, 2000, Sun et al., 2007). Depending on which blood fraction is used, this will determine the time period of fatty acid intake (Cantwell, 2000). One study showed that n-3 fatty acids were rapidly incorporated into serum within 5 days of a fish oil supplementation whereas it took approximately 1 month for n-3 fatty acids to appear in erythrocytes (Katan et al., 1997). Serum triglycerides reflect fatty acid intake of the last meal eaten whereas phospholipids represent the last several days to weeks and

erythrocytes are reflective of the past month (Cantwell., 2000, Sun et al., 2007). By measuring composition of plasma phospholipids in newly diagnosed patients with advanced cancer, one can determine if total amounts of these fats are within the normal range (PL = 600 - 800μg/ml, TG = 1.7 - 2.3mmol/L) in addition to short term dietary intake of fatty acids.

1.7 Fatty Acid Status in Cancer Patients

Little research has been done on alterations in fatty acid metabolism underlying the wasting syndrome in patients with advanced cancer. Evidence suggests there are abnormalities in lipid metabolism of patients with advanced cancer such as increased lipolysis and fatty acid oxidation (Klein et al., 1990, reviewed by Agustsson et al., 2007) in addition to elevated circulating triglycerides (Grunfeld et al., 1996, Klein et al., 1990, Rossi-Fanelli et al., 1995). These aberrations may cause a reduction in the availability of n-6 and n-3 fatty acids within the body and exacerbate the cachectic condition (Zuijdgeest-Van Leeuwen et al., 2002, Pratt et al., 2002).

The fatty acid status of newly diagnosed patients with advanced cancer is not known, however, studies have been done on patients in more advanced states. One study reports total plasma phospholipids in patients with advanced cancer approximately 3 months from death and entering palliative care who lost 5% or more of their pre-illness weight to be 30% lower than in healthy subjects (Pratt et al., 2002). Several other studies have reported the percentage of n-3 (EPA, DHA) and n-6 (arachidonic acid) fatty acids within the plasma phospholipids of patients with advanced cancer (Barber et al., 1999, Bruera et al., 2003, Fearon et al., 2003, Moses et al., 2004, Read et al., 2007) however the total amount or composition of their fatty acids are not known. Assessing the amounts of

fat in blood plasma from the time of diagnosis throughout the cancer trajectory would provide valuable information in determining when fatty acids become depleted. This would help define nutrient metabolism and requirements for fats throughout the cancer trajectory.

1.8 Factors Influencing Lipid Metabolism

Patients diagnosed with lung or colorectal cancer are at risk of malnutrition. This is a result of many factors including tumor factors or as a result of treatment, hormonal disturbances and/or the presence of proinflammatory cytokines (Table 1).

1.9 Summary

Patients with advanced lung and colorectal cancer often experience unintentional weight loss (Kahlid et al., 2007) which can be attributed to a myriad of factors. Although weight loss can occur in both muscle and adipose tissue, changes related to adipose tissue have been poorly addressed to date. More specifically, the amount and composition of fatty acids in patients with advanced cancer are not known in this particular population. The fatty acid status of newly diagnosed patients with advanced cancer must be established to aid in determining when fatty acids become depleted during the cancer trajectory. Information on whether or not patients with advanced cancer are deficient in essential fats or at what point do fatty acids become depleted and if there is a requirement for specific fats throughout the disease is urgently needed.

The specific effects of chemotherapy on fatty acids remains poorly defined. In animal and cell culture models, n-3 fatty acids have been shown to enhance tumor response to treatment by increasing cytotoxicity to tumor cells (Guffy et al., 1984, Colas et al., 2006) and to protect normal cells from the cytotoxic effects of anti-cancer agents

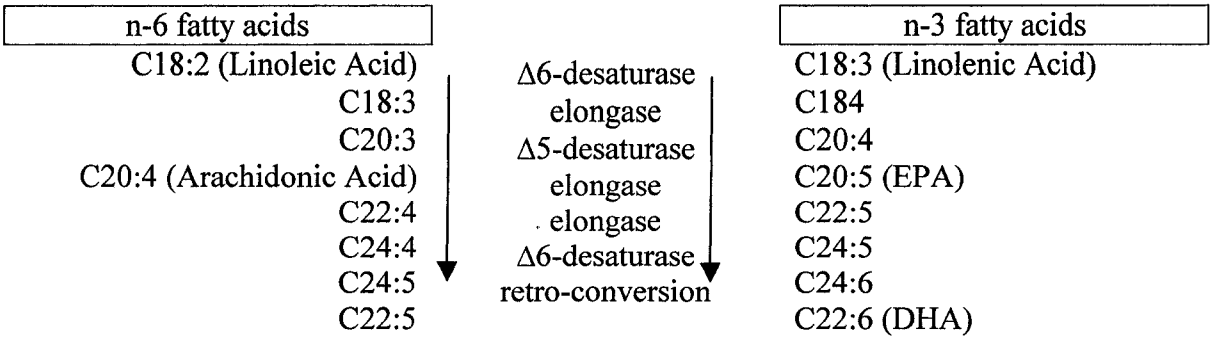
(Tsai et al., 1997, Grammaticos et al., 1994). Supplementation with n-3 fatty acids may be required if the body is unable to synthesize sufficient amounts of necessary long chain n-3 fatty acids to support physiological function. It would be useful to determine how long chain polyunsaturated fatty acids are affected by toxic drugs to establish whether supplementation with diets rich in polyunsaturated fatty acids would be of value. Additionally, it would be important to assess whether or not EPA and DHA should be fed directly to the patients if there is a decreased capacity to synthesize these fats from precursor n-3 fatty acids.

1.10 Tables and Figures

Table 1 Some factors involved in wasting of adipose tissue

	Normal	Cancer Cachexia
Insulin	-binds to cell surface receptors on fat cells causing tyrosine phosphorylation thereby activating insulin receptors (Arner., 2005) -signal proteins such as insulin-like receptor substrates activate phosphatidyl inositol 3-kinase which in turn activates phosphodiesterase 3A (Arner., 2005) -intracellular cascade results in ↓ CAMP which ↓ HSL activity (Arner., 2005) -glucagon ↑ CAMP which activated HSL and ↑ lipolysis (Carmen et al., 2006)	-↑ production of HSL due to enhanced gene activity in fat cells causes enhanced lipolysis in cachetic patients (Agustsson et al., 2007, Thompson et al., 1993) -↓ in Glut-4 mRNA in fat of cachectic mice which suggests an altered glucose metabolism (Bing et al., 2006) -reduced circulating concentrations of insulin (Barber et al., 2001) -elevated glucose production accompanied by glucose intolerance and insulin resistance with respect to adipose tissue and liver (Argiles et al., 1999)
Cortisol	-depresses Δ6-desaturase activity (Gurr et al., 2002) -promotes gluconeogenesis and fat mobilization -↓ effectiveness of insulin	-elevated levels of cortisol (Simons et al., 1999, Barber et al., 2004)
IL-6	-produced by adipocytes (Pan et al., 2007)	-primary mediator involved in inducing cancer cachexia in the C-26.IVX cell line derived from colon-26 (C-26) undifferentiated carcinoma in mice (Strassmann et al., 1992)
TNF-α	-produced by adipocytes (Pan et al., 2007) but is not released into circulation, instead it acts as a local factor (Arner P. 2005) -TNF-α stimulates MAP kinases through a receptor thereby causing a ↓production of perilipin (Arner., 2005) -perilipin allows HSL to hydrolyze the adipocyte (Arner P. 2005) -a reduction in insulin sensitivity coupled with hyperglycemia was observed when tumor necrosis factor was administered to healthy individuals (Van der Poll et al., 1991)	-inhibits lipogenesis and lipoprotein lipase activity thereby inducing hypertriglyceridemia (Green et al., 2004)
IL-1	-pro-inflammatory cytokine produced by adipocytes -IL1-β ↓ cellular lipid content and induces insulin resistance (Lagathu et al., 2006) -can stimulate lipolysis in humans and cultured adipocytes (Green et al., 2004, Mulligan et al., 1991)	-involved in weight-loss associated with cancer (Barber et al., 2001)
CRP	-produced by hepatocytes and regulated by IL-6 (Heikkila et al., 2007) -marker of chronic inflammation (Heikkila et al., 2007)	-tumor growth can cause tissue inflammation resulting in ↑ levels CRP (Heikkila et al., 2007)

Figure 1 Metabolic pathway of essential fatty acids (Sprecher *et al.*, 1995)



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Chapter 2

Research Plan

2.1 Rationale

Cachexia is a multifactorial wasting syndrome involving loss of muscle and fat despite adequate nutritional intake (MacDonald et al., 2003). Patients with advanced lung and colorectal cancer frequently present with cachexia. High mortality rates (Agteresch et al., 2000, Jatoi et al., 2001) and a significant risk for malnutrition (Martin et al., 1999) is prevalent in this population. Cancer cachexia has also been associated with a reduced quality of life, poor response to treatment and decreased survival time (DeWys et al., 1980). While several alterations in metabolism exist, little research has been done on derangements in fatty acid metabolism underlying the wasting syndrome.

The fatty acid status of newly diagnosed patients with advanced cancer is not known. One study however, reports total plasma phospholipids in patients with advanced cancer who lost 5% or more of their pre-illness weight were 30% lower than in healthy subjects (Pratt et al., 2002). Assessing the amounts of fat in blood plasma from the time of diagnosis throughout the cancer trajectory would provide valuable information in determining when fatty acids become depleted. This would help define nutrient metabolism and requirement for fats throughout the cancer trajectory.

Chemotherapy may be a contributing factor to alterations in lipid metabolism in patients with advanced cancer. Patients undergoing chemotherapy treatments have very low levels of long chain polyunsaturated fatty acids in plasma phospholipids (Pratt et al., 2002) which may be attributed to cytotoxic agents interfering with fatty acid metabolism

(Marra et al., 1986). N-3 fatty acids have been shown to protect host cells from the cytotoxic effects of anti-cancer agents (Tsai et al., 1997, Grammaticos et al., 1994) while increasing cytotoxicity to tumor cells (Guffy et al., 1984, Colas et al., 2006). Whether depletions in plasma fatty acids are attributed to abnormalities within the liver caused by the anti-cancer drugs are not known however, a fish oil diet may increase or maintain the amounts of fat, particularly n-3 fatty acids within the blood.

2.2 Research Objectives and Hypotheses

1. Essential Fatty Acid Status in Newly Diagnosed Patients with Advanced Cancer

The primary objective of this thesis research is to characterize the amount and composition of fatty acids in the plasma of newly diagnosed patients with advanced cancer.

Objective: To determine the total amount of plasma phospholipids as well as the amount of n-6 and n-3 fatty acids within the plasma phospholipids of newly diagnosed patients with advanced lung and colorectal cancer. A lipid panel was also determined by analyzing plasma cholesterol, HDL and LDL.

Hypotheses: It is hypothesized that:

a) Compared to healthy age matched controls, newly diagnosed patients with advanced lung and colorectal cancer will have:

(i) a reduction in total plasma phospholipids

(ii) lower n-6 and n-3 fatty acids including EPA and DHA

b) The lipid and inflammatory components in the plasma of newly diagnosed patients with advanced cancer will relate to length of survival. These components include:

(i) total plasma triglycerides

(ii) total plasma phospholipids

(iii) C-reactive protein

2. The Effects of Irinotecan and Diet on an Animal Model of Colorectal Cancer

This research will determine the effects of a chemotherapy drug, irinotecan, on the fatty acid amount and composition in the liver and plasma of rats bearing a colorectal tumor and fed a control or a fish oil diet.

Objective: Determine the fatty acid composition and amount of phospholipids and triglycerides in liver and plasma of rats that have been injected with a colorectal tumor and treated with the chemotherapy drug irinotecan.

Hypothesis: It is hypothesized that:

Chemotherapy may be a contributing factor to alterations in lipid metabolism therefore rats treated with irinotecan will have:

(i) decreased amounts of fatty acids and lower PUFA in liver triglycerides and phospholipids

(ii) amounts and compositions of n-3 fatty acids, specifically EPA and DHA in plasma phospholipids and triglycerides will be similar to untreated rats when fed a fish oil diet

Chapter 3

Essential Fatty Acid Status in Newly Diagnosed Patients with Advanced Lung and Colorectal Cancer

3.1 Introduction

Patients with locally advanced or metastatic lung and colorectal cancer are at high risk for malnutrition and frequently suffer from cachexia. Evidence suggests there are abnormalities in the lipid metabolism of patients with advanced cancer such as increased lipolysis and fatty acid oxidation (Klein et al., 1990, reviewed by Agustsson et al., 2007) in addition to elevated circulating triglycerides (Grunfeld et al., 1996, Klein et al., 1990, Rossi-Fanelli et al., 1995). These aberrations may cause a reduction in the availability of n-6 and n-3 fatty acids within the body and exacerbate the cachectic condition (Zuijdgeest-Van Leeuwen et al., 2002; Pratt et al., 2002).

Several studies have reported the percentage of n-3 (EPA, DHA) and n-6 (arachidonic acid) fatty acids within the plasma phospholipids of patients with advanced cancer (Barber et al., 1999, Bruera et al., 2003, Fearon et al., 2003, Moses et al., 2004, Read et al., 2007) however the total amount or composition of their fatty acids are not known. Without this information, it is impossible to determine whether or not the total fats are depleted or at what point during the cancer trajectory did the decrease in fatty acids occur.

Composition of fatty acids within plasma phospholipids reflects are markers of fats consumed in the diet (Dougherty et al., 1987, Phillips et al., 1967). EPA and DHA, key fatty acids associated with weight maintenance and/or gain in patients with advanced cancer experiencing unintentional weight loss (Barber et al., 1999, Fearon et al., 2003, Fearon et al., 2006, Wigmore et al., 1996) can easily be detected in plasma phospholipids. The analysis of plasma phospholipids will reveal whether or not newly diagnosed patients with advanced cancer have lower amounts of fatty acids in their plasma during the early stages of cancer.

Compared to healthy age matched controls, it was hypothesized that patients with advanced lung and colorectal cancer will have a reduction in total plasma phospholipids. Lower amounts of n-6 and n-3 fatty acids including EPA and DHA and total plasma triglycerides are also expected. This study was designed to analyze the fatty acid composition of plasma phospholipids in newly diagnosed patients with advanced cancer. Changes within lipid components of plasma such as cholesterol, HDL and LDL in addition to body composition parameters were also assessed to determine whether or not a relationship exists between adipose tissue wasting and time of death.

3.2 Subjects and Study Design

Protocols for the study of all subjects were reviewed and approved by the Research and Ethics Board of the Alberta Cancer Board. All participants provided written informed consent. Three hundred and ninety eight (n=398) patients with advanced cancer (n=179 lung; n=149 colorectal) were approached for the study. Fifty-one patients (n=51) consented to participate (n=24 lung; n=27 colorectal) however only forty-three (n=43) subjects provided blood samples (n=22 lung; n=21 colorectal). Inclusion criteria

included: (1) locally advanced or metastatic non-small cell lung cancer or colorectal cancer; (2) experiencing weight loss; (3) at risk for involuntary weight loss; (4) women cannot be pregnant (exposure to radiation); (5) ability and willingness to provide written, informed consent. Patients were asked to arrive fasted at the Human Nutrition Research Unit at the University of Alberta. Blood samples were obtained by venous catheter usually in the non-dominant arm and centrifuged for 10 minutes at 704 X g. Plasma was separated and immediately frozen for further analysis.

Of the 43 patients with advanced cancer, 22 returned for a follow-up visit (n=12 colorectal; n=10 lung) approximately 8-10 weeks after their initial visit. Patients who did not return for a follow-up visit were either non-compliant (n=1), uninterested (n=7), ill (n=4) or had too much going on (n=9). Lipid panels as well as body composition parameters were reassessed. Time to death was also recorded throughout the study and is ongoing.

Additional plasma samples from patients with advanced lung and colorectal cancer were obtained from McGill University. Samples from eighty three (n=83) patients with advanced cancer (n=35 lung; n=18 colorectal) were frozen and shipped on dry ice from McGill University, Montreal, Quebec to the University of Alberta.

Patients with advanced cancer were compared to healthy, age-matched controls (n=17). Healthy subjects gave written informed consent and ethics approval was provided by the Human Ethics Review Committee of the Royal Victoria Hospital. Subjects were admitted to the McGill University Health Centre - Royal Victoria Hospital's Clinical Investigation Unit (CIU) and were screened by medical history, physical examination, and laboratory investigation previously described by Morais et al., 1997. The participants

were not taking medications and vitamin supplements were stopped the week prior to admission. Venous blood samples were drawn from fasting healthy subjects and centrifuged at 2000 X g at 4°C for 15min. Once plasma was separated, multiple aliquots were stored at -20°C. Frozen plasma samples were shipped on dry ice from McGill University, Montreal, Quebec to the University of Alberta.

3.3 Methods

3.3.1 Fatty Acid Analysis

3.3.1.1 Lipid Extraction

Plasma samples were removed from the freezer and 50μL were thawed and put into methylation tubes with 2mL of a 2:1 chloroform-methanol mixture and vortexed. Calcium chloride (400μL) was added to each tube which was then capped, vortexed and stored at 4°C overnight to separate.

The following day, the clear bottom layer containing the fatty acids, was removed and added to clean methylation tubes. An 86:14:1 chloroform-methanol-water mixture (1mL) was added to the original test tube which was then vortexed and once again allowed to separate. After phase separation, the bottom fraction was extracted and pipetted into clean methylation tubes which were then dried under nitrogen gas. Once dry, 100μL of chloroform was added and sample was vortexed.

3.3.1.2 Thin Layer Chromatography

Thin layer chromatography plates (Silica Gel G, 20 x 20cm, 250 microns, Analtech Inc., Newark, DE) were heat activated for one hour at 110°C. Samples prepared in chloroform were spotted in duplicate columns on the plates. Solvent tanks were lined with filter paper (Fisher, Whitby, ON) and an 80:20:1 petroleum ether-ethyl ether-glacial

acetic acid solvent mixture saturated the tanks for one hour prior to adding the plates. When the sample reached the top of the plate (approx. 25min), plates were dried in the fume hood and sprayed with 0.1% ANSA (8-anilino-1-naphthalene-sulfonic acid) to visualize the various lipid classes. Ultraviolet light was used to identify the phospholipid and triglyceride bands which were subsequently scraped and added to clean methylation tubes.

3.3.1.3 Methylation

Phospholipid (PL) samples were methylated using 2mL hexane, 1mL boron trifluoride (BF_3) and 50μL C17:0 (100μg/mL). Samples were boiled on the dry bath for 1 hour at 110°C and then left to cool at room temperature. Triglyceride (TG) samples were methylated using 1mL potassium hydroxide (KOH) in methanol with 100μL C15:0 (100μg/mL) and were left to boil at 110°C on the dry bath. After one hour, 2mL of hexane, 1mL of BF_3 and 50μL of C17:0 (100μg/mL) were added and boiling resumed for an additional hour at 110°C. Once the PL and TG samples were cool, distilled water (1mL) was added to each tube. Tubes were vortexed and allowed to separate in the fridge overnight. Once separated, the top layer was removed and added to a gas chromatography (GC) vial. The vial was dried under nitrogen and 200μL of pure hexane was added and the sample was vortexed. The hexane was pipetted into a glass insert which was returned to the GC vial and the sample was stored at -80°C until analysis.

3.3.1.4 Gas Liquid Chromatography

Samples were analyzed by automated gas liquid chromatography (GLC) (Varian star 3400cx, Varian Instruments, Georgetown ON) on a fused silica BP20 capillary column (25 m x 0.22 mm internal diameter, SGE Instruments). Fatty acid methyl esters

were separated by an automated gas-liquid chromatograph, Varian model 3400cx equipped with a Star Chromatography Workstation data system and a Varian 8200 autosampler (Varian Instrument Company, Georgetown Ontario). The system used a bonded phase fused silica capillary column, BP20: 25 m x 0.25 OD SGE product. Helium was used as the carrier gas at a flow rate of 30ml/min using a split injector (28:1). The GLC oven temperature of 90°C was increased to 170°C at 20°C/min and held for 23 minutes followed be a second stage temperature increase to 230°C at 4/minute for a total analysis time of 40 minutes. These conditions separate saturated, monounsaturated and polyunsaturated fatty acids between 6 and 24 carbon chain lengths and peaks were compared to known standards to determine the μg of each fatty acid.

3.3.2 Blood Analysis

Blood samples were collected in heparinised tubes and analyzed by Dynacare Medical Laboratories (Edmonton, Alberta) for analysis of the lipid panel. Amount of triglycerides, HDL, LDL and cholesterol were measured by plasma assays using the ADIVA® 1650 Chemistry system. Serum samples of cortisol and insulin were also analyzed by Dynacare Medical Laboratories using the ADIVA® Centaur™ System whereas C-reactive protein was measured in plasma using the IMMAGE® Immunochemistry Systems and Calibrator 5 Plus.

3.3.3 Nutritional Assessment and Body Composition Parameters

The Patient Generated Subjective Global Assessment (PGSGA) is a nutritional assessment screening tool for patients with cancer that identifies malnourished patients based on a numerical scale. Dual-energy X-ray absorptiometry (DXA) was used for body

composition analysis as described by Mourtzakis et al., 2007 and resting energy expenditure was also measured using indirect calorimetry.

3.3.4 Statistical Analysis

Data are reported as means ± SD. The results were evaluated with a oneway ANOVA to identify significant differences between multiple samples. When significant overall differences were observed, a post-Hoc analysis was done using the Bonferonni model. Differences in EPA and DHA were measured using an unpaired t-test. Statistical significance was assured when the p value was <0.05. All statistical analyses were conducted using SPSS 15.0 for Windows.

3.4 Results

The mean age of patients with colorectal cancer was 60 ± 11 years; mean BMI 27.7 ± 7.5 kg/m² and the mean age of patients with lung cancer was 66 ± 9 years; mean BMI 25.9 ± 4.0 kg/m² (Table 1). The mean age of the healthy participants was 70 ±5 years; mean BMI 24.8±3.0 kg/m².

3.4.1 Plasma Phospholipids

The amounts and proportions of fatty acids in plasma phospholipids in patients with advanced lung and colorectal cancer were similar to the healthy, older adult reference population however several differences within the n-6 and n-3 fatty acids were measured (Table 2). The amounts arachidonic acid and DHA were significantly higher in patients with lung and colorectal cancer compared to healthy subjects (Table 2). Additionally, EPA and total n-3 fatty acids were significantly higher in patients with colorectal cancer compared to healthy subjects (Table 2). Based on the large standard

deviation, the variation was greater in the cancer patient population vs. the healthy subjects when total SFA, MUFA and PUFA were calculated.

3.4.2 EPA and DHA

Levene's Test for equality of variances revealed most of the data was heterogeneous therefore we used the results from the t-test for unequal variances where applicable. EPA and DHA content of phospholipids in healthy subjects have a mean value of 3.9±4.7μg/mL and 14.6±12.6μg/mL respectively. Every fatty acid in patients with advanced cancer who had EPA values below 3.9±4.7μg/mL were significantly lower compared to those who had EPA levels above the reference value (Table 3). Every fatty acid except C16:0, C18:3n-3 and C20:2n-6 was significantly lower in patients with DHA below 14.6±12.6μg/mL compared to those with DHA above the reference value.

3.4.3 Lipid Panel

The majority (91%) of patients with cancer from the CCI in Edmonton had elevated HDL levels (>1.0mmol/L). In this same group of patients, cholesterol (>5.2mmol/L) and LDL (>3.4mmol/L) were elevated in 63% and 55% of the patients. Additionally, high cholesterol was characterized by high levels of LDL in 79% of the patients. Triglyceride levels below the reference value (<2.3mmol/L) was exhibited in 77% of the patients with advanced cancer (Figure 1). A lipid panel was not available for the patients from McGill therefore this data was not included in this section.

3.4.4 CRP and Days until Death

CRP levels in patients who passed away within 600 days of their last visit were not significantly different compared to patients who are still alive (Figure 2). A negative r

value indicates CRP levels increase as time to death approaches however no statistical differences were observed.

3.4.5 Triglycerides and Days until Death

Ninety percent (90%) of the patients who passed away within 600 days of their last visit had triglyceride levels below the reference value of 2.3mmol/L (Heart and Stroke Foundation) (Figure 3). No significant differences were found in the triglyceride levels of patients who passed away compared to those who are still alive. The mean level of triglycerides in patients who passed away within 600 days was 1.5mmol/L whereas those who are still alive (mean days alive = 850) have a mean value of 2.1mmol/L.

3.5 Discussion

This is the second report in the literature that describes fatty acid composition of plasma phospholipids in newly diagnosed patients with lung and colorectal cancer. Unlike the previous report by Pratt et al., (2002), depletions in n-6 and n-3 fatty acids were not evident in this group; instead arachidonic acid, EPA, DHA and total n-3 fatty acids were significantly higher in patients with advanced cancer compared to healthy subjects. Other populations of patients with advanced cancer have been reported to have lower levels of plasma phospholipids including n-3 and n-6 fatty acids (Pratt et al. 2002; Zuijdgeest-van Leeuwan et al., 2000). Pratt et al. (2002) examined plasma phospholipids in patients with advanced cancer who lost 5% or more of their pre-illness weight. They found that the total phospholipids in patients with advanced cancer were approximately 30% lower than healthy subjects and patients with advanced cancer also had the lowest levels of essential fatty acids in their plasma phospholipids. Zuijdgeest-van Leeuwan et

al., 2000 found total n-3 fatty acids, EPA and DHA in plasma phospholipids were lower in newly diagnosed, untreated patients with non-small cell lung cancer compared to healthy subjects. The reductions did not reach statistical significance because patients were newly diagnosed without a previous history of malignant disease. Zuijdgeest-van Leeuwan et al., 2000 also showed that total n-3 fatty acids and EPA were significantly lower in the plasma phospholipids of lung cancer patients with weight loss compared to those without weight loss. A high degree of variation was observed in the fatty acid composition of newly diagnosed patients with advanced cancer.

Fish oil supplements rich in essential fatty acids have shown to attenuate cancer cachexia and tumor growth in mice bearing the MAC16 tumor (Beck et al., 1991, Tisdale 1996). Additionally, when weight losing patients with advanced pancreatic cancer were given a nutritional supplement enriched with fish oil, weight loss was reversed (Barber et al., 1999). EPA and DHA are key n-3 fatty acids that have been associated with weight maintenance and weight gain in patients with advanced cancer experiencing unintentional weight-loss (Barber et al., 1999, Fearon et al., 2003, Fearon et al., 2006, Wigmore et al., 1996). When newly diagnosed patients with advanced cancer were categorized based on EPA and DHA levels compared to the reference value, most of the fatty acids were significantly lower in patients who had amounts of EPA or DHA below the reference value compared to those with EPA or DHA above the amount seen in healthy patients. Although the majority of the fatty acids were significantly lower, n-3 fatty acids were of particular interest. The lower amounts of these fatty acids may be indicative of aberrations within the n-3 pathway. Low levels of n-3 fatty acids may impair the ability to maintain lean body mass and exacerbate weight loss because supplementation with fish

oil has been associated with weight maintenance and weight gain (Barber et al., April 1999, Barber et al., 1999, Fearon et al., 2003, Wigmore et al., 1996).

High cholesterol levels in this patient population may be associated with high BMI (mean; 26.9kg/m^2). Approximately 57% of the newly diagnosed patients with advanced cancer were either overweight or obese and of those patients, 68% had cholesterol levels above the reference value. Several studies have shown that total cholesterol is higher in subjects with BMI's above 25 kg/m^2 (Lamon-Fava et al., 1996; Brown et al., 2000; Alexander 2001) and plasma HDL decreases significantly by increasing BMI (Lamon-Fava et al., 1996). These results however were not seen in our newly diagnosed advanced cancer population. Most of the patients in our study had HDL levels above the reference value which would not be expected in overweight or obese patients (Brown et al., 2000). HDL picks up and carries cholesterol through the bloodstream and into the liver for excretion or re-utilization. Subjects who are overweight are often at risk for having low levels of HDL's (Opie 2007) which would prevent the clearance of cholesterol within the body.

C-reactive protein (CRP), a marker of chronic inflammation, was not related to time of death in our patient population. CRP levels of patients who were closest to death had higher levels of CRP than those who were 600 days from death. These results were not significant however this trend may be strengthened to reach significance as more patients pass away. These findings are not consistent with those of Falconer et al., (1994) who reported that serum levels of CRP is a predictor of survival in patients with unresectable pancreatic cancer. Suh et al., (2006) also showed that CRP in terminally ill cancer patients was a useful indicator of predicted survival time. Furthermore, an elevated level

of CRP was found to be an independent prognostic indicator of survival in patients suffering from oesophageal cancer (Nozoe et al., 2001) colorectal cancer (Nozoe et al., 1998) and those with haematological malignancies (Kroschinsky et al., 2002). These studies support CRP as a useful marker to predict life expectancy. It would be beneficial to continue to measure CRP levels in our patients with advanced cancer to determine if this is true in our patient population as they approach death. With length of time to death being of considerable timeframe, CRP levels may not be a good marker of survival until patients approach death.

Although patients were generally overweight, plasma triglycerides within the normal range were measured within 600 days till death. Elevated circulating triglycerides (Grunfeld et al., 1996, Klein et al., 1990, Rossi-Fanelli et al., 1995) have been reported in patients with advanced cancer however this was not seen in newly diagnosed patients with advanced cancer. Ongoing analysis will aid in defining the relationship between lower triglycerides and survival. Data collected previously on patients with advanced cancer who were close to death showed decreased levels of plasma phospholipids (Pratt et al., 2002). It would be of value to determine if their plasma triglycerides were also depleted to determine if a stronger relationship exists between plasma triglyceride levels and survival.

In conclusion, the amounts of fatty acids or their composition in plasma phospholipids of newly diagnosed patients with advanced cancer were not what was predicted. These patients appeared to have a fatty acid profile similar to that found in healthy subjects; however the large variation in the data would suggest that patients with advanced cancer are a very heterogeneous population and a more standard measure can

be obtained when stratified by time to death. HDL's of the patients with advanced cancer was unexpectedly high considering the high mean BMI of this population. CRP and low levels of plasma triglycerides were two markers that could potentially relate to time of death and although CRP has previously been reported as an indicator of length of survival, TG levels may be novel to patients with advanced cancer. By establishing what is taking place in the body during the initial stages of cancer, this can be the starting point to mapping out the cancer trajectory. Once the point where cancer begins to affect the body is determined, this could ultimately assist in deciding treatment options as well as taking preventative measures to prevent undesirable outcomes such as uncontrolled body wasting.

3.7 Tables and Figures

Table 1 Characteristics of Patients with Advanced Cancer and Healthy Subjects

Subjects	N	Age (Years)	Gender	BMI (kg/m^2)
Lung Cancer	57	67 ± 8.6	F = 20 M = 37	25.2 ± 4.5
Colorectal Cancer	39	63 ± 13.1	F = 18 M = 21	27.1 ± 7.0
Healthy	17	70 ± 5.2	F = 10 M = 7	24.8 ± 3.0

Data are expressed as mean ± SD.

Table 2 Fatty acid content of plasma phospholipids in patients with advanced lung and colorectal cancer compared to healthy age-matched controls.

Fatty Acid	Colorectal (n=39)	Lung (n=57)	Healthy Subjects (n=17)	Overall p value
C16:0	295±94.7	274±90.4	246±59.6	.174
C18:0	130±38.9	132±46.2	130±31.5	.965
C18:1n-9	98±36.2	97±36.4	89±24.4	.637
C18:2n-6	173±63.5	173±59.9	170±40.2	.981
C20:4n-6	78±33.8	87±34.7	54±30.1	.003[abc]
C20:5n-3	8±6.5	9±7.7	4±4.7	.033[ab]
C22:6n-3	26±17.1	29±13.9	15±12.6	.004[abc]
Total($\mu g\ mL^{-1}$)	917±300	920±291	794±180	.246
∑SFA	442±134	430±135	393±88.9	.431
∑MUFA	142±57.0	142±51.1	130±30.7	.668
∑PUFA	333±127	347±118	272±85.2	.068
∑n-6	290±105	301±103	250±73.1	.195
∑n-3	37±22.9	40±19.9	21±17.2	.007[abc]

[a]Overall significant difference ($p<0.05$)
[b]Significant difference between healthy and lung cancer ($p<0.05$)
[c]Significant difference between healthy and colorectal cancer ($p<0.05$)

Amount of fatty acid (μg/mL) in plasma phospholipids (PL) of patients with advanced lung and colorectal cancer compared to healthy age-matched controls was determined using gas chromatography. Total plasma PL were calculated using 50 μg of C17:0 [0.1μg/μL] as the standard and are expressed as μg/mL of plasma. Data are expressed as means ± SD. Groups were statistically compared using a oneway ANOVA. Abbreviations: SFA, saturated fatty acids, MUFA, monounsaturated fatty acids, PUFA, polyunsaturated fatty acids.

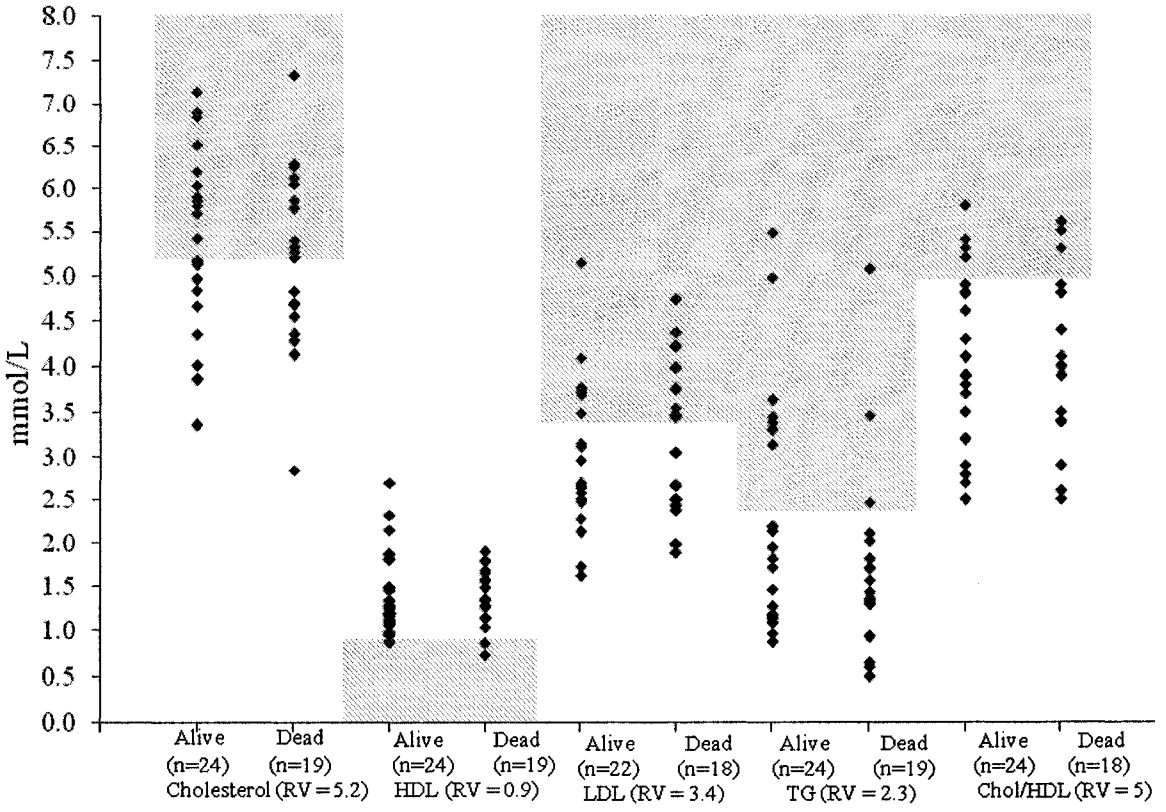
Table 3 EPA and DHA above and below the reference values in patients with advanced lung and colorectal cancer

	EPA		DHA	
Fatty Acid	Below Reference (n=29)	Above Reference (n=59)	Below Reference (n=13)	Above Reference (n=75)
C16:0	238±58.8	303±98.1[a]	276±88.2	282±93.2
C18:0	105±23.9	145±44.9[a]	106±19.4	136±45.0[b]
C18:1n-9	83±17.6	105±40.5[a]	83±23.7	100±37.4
C18:1n-7	16±4.0	19±7.7[a]	14±3.3	19±7.1[b]
C18:2n-6	140±32.2	189±65.4[a]	142±40.1	178±62.4[b]
C18:3n-3	1±1.4	2±1.6[a]	1±1.9	2±1.5
C20:2n-6	2±1.5	4±4.2[a]	2±2.1	4±3.8
C20:3n-6	20±9.0	32±15.5[a]	17±7.5	30±14.9[b]
C20:4n-6	67±20.3	92±37.2[a]	57±21.2	88±34.3[b]
C20:5n-3	2±1.1	12±6.9[a]	4±2.9	10±7.5[b]
C22:4n-6	2±1.6	4±3.1[a]	2±2.8	3±2.7[b]
C22:5n-6	5±2.6	9±4.8[a]	4±1.8	8±4.8[b]
C24:0	4±3.1	7±5.5[a]	3±3.2	7±5.2[b]
C22:6n-3	19±9.3	32±15.8[a]	11±2.4	31±14.4[b]
Total($\mu g\ mL^{-1}$)	741±127	1007±312[a]	751±172	948±300[b]
∑SFA	358±73.3	472±142[a]	394±108	441±138
∑MUFA	120±25.6	152±59.6[a]	114±33.3	147±54.5[b]
∑PUFA	263±56.9	381±125[a]	242±54.1	359±121[b]
∑n-6	235±51.2	327±109[a]	222±52.1	310±105[b]
∑n-3	23±8.9	46±21.1[a]	17±3.9	43±20.4[b]

[a]Significant difference between EPA above and below the amounts in healthy subjects
[b]Significant difference between EPA above and below the amounts in healthy subjects

Amount of fatty acid (μg/mL) in plasma phospholipids of patients with advanced lung and colorectal cancer was determined using gas chromatography. Total plasma PL were calculated using 50 μg of C17:0 [0.1μg/μL] as the standard and are expressed as μg/mL of plasma. Data are expressed as means ± standard deviation. Groups were statistically compared using an unpaired t-test in the EPA and DHA groups respectively. Abbreviations: EPA, eicosapentaenoic acid, DHA, docosahexaenoic acid.

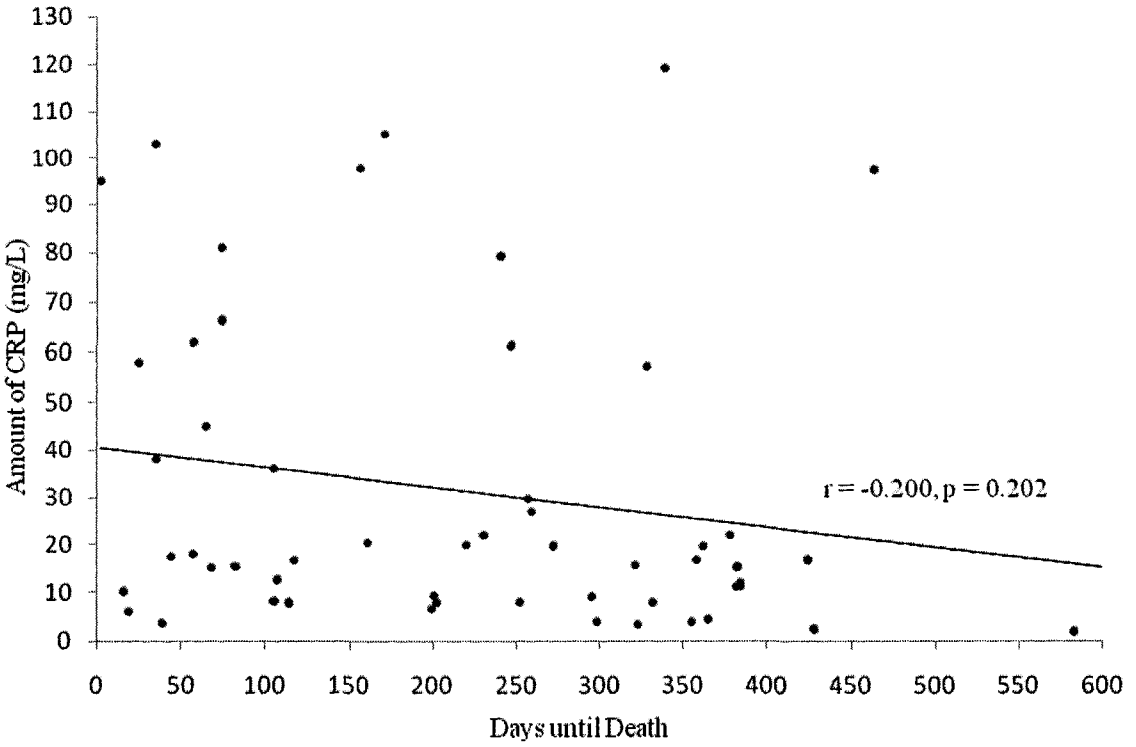
Figure 1 Lipid panel of patients with advanced lung and colorectal cancer.



Shaded areas indicate levels above or below the reference values for lipid components. Abbreviations: HDL, high density lipoprotein, LDL, low density lipoprotein, TG, triglycerides, Chol/HDL, cholesterol/high density lipoprotein, RV, reference value.

Fasting blood samples were collected in heparinised tubes and analyzed by Dynacare Medical Laboratories (Edmonton, Alberta) for analysis of the lipid panel. Amount of triglycerides, HDL, LDL and cholesterol were measured by plasma assays using the ADIVA® 1650 Chemistry system.

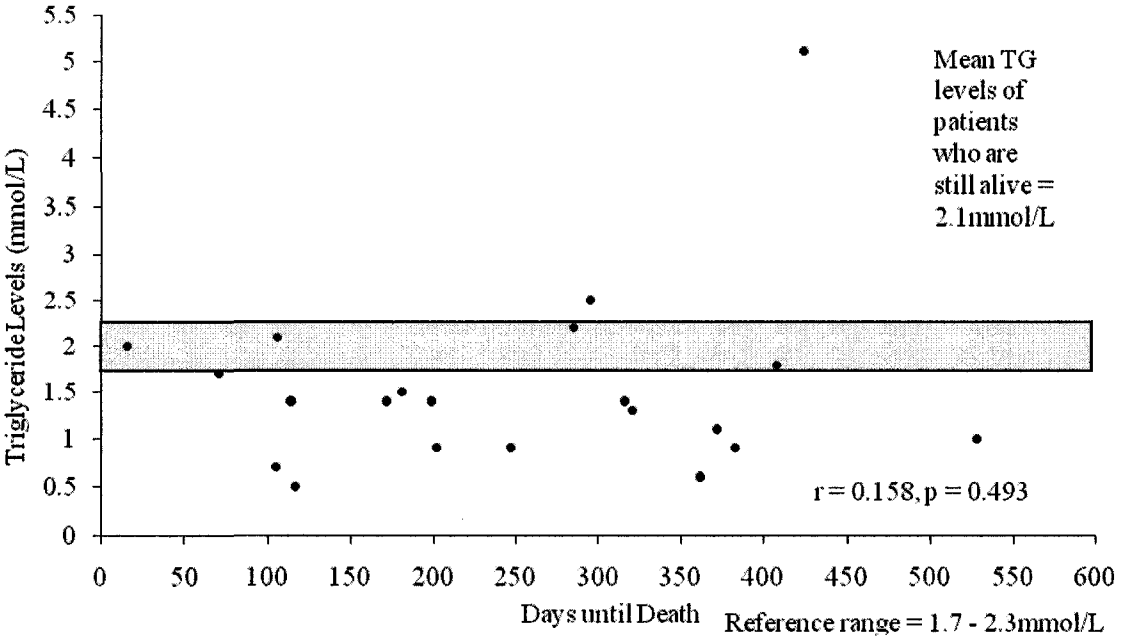
Figure 2 CRP levels of CCI patients from Edmonton with advanced cancer (n=55) at less than 600 days until death.



No association was present between CRP levels in patients with advanced cancer who passed away within 600 days of their last visit. Abbreviation: CRP, C-reactive protein.

Fasting blood samples were collected in heparinised tubes and analyzed by Dynacare Medical Laboratories (Edmonton, Alberta) for analysis of CRP. CRP was measured in plasma using the IMMAGE® Immunochemistry Systems and Calibrator 5 Plus.

Figure 3 Triglyceride levels of patients with advanced cancer (n=21) at less than 600 days until death.



Shaded are indicates triglyceride levels mmol/L below the reference value determined by the Heart and Stroke Foundation.

Blood samples were collected in heparinised tubes and analyzed by Dynacare Medical Laboratories (Edmonton, Alberta) for analysis of triglycerides. Triglycerides were measured by plasma assays using the ADIVA® 1650 Chemistry system.

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Chapter 4

Effects of Irinotecan (CPT-11) for Colorectal Cancer on the Fatty Acid Status of Rats Fed Fish Oil or Control Diets

4.1 Introduction

Chemotherapy may be a contributing factor to alterations in lipid metabolism characterized largely by the depletion of polyunsaturated fatty acids in patients with advanced cancer (Pratt et al., 2002). Plasma phospholipids of patients receiving chemotherapy (5-fluorouracil, adriamycin and cyclophosphamide) were reported to have very low levels of long chain PUFA, which are the precursors of the n-6 or n-3 fatty acids, indicating that these drugs may target PUFA and interfere with PUFA metabolism (Marra et al., 1986, Pratt et al., 2002). Whether these effects are specific to this drug combination or how long the fatty acids within plasma phospholipids remain depleted is not known therefore it would be of value to further investigate the effects of anti-cancer treatment on lipid metabolism. The toxic effects of chemotherapy drugs often prevent patients from receiving the optimal dose of anti-cancer therapy thus jeopardizing their survival. Furthermore, treatment related toxicities such as diarrhea frequently limit the dose of anti-neoplastic agents tolerated by the patient. Irinotecan (7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxy-camptothecin) (CPT-11, Camptosar®) is a common chemotherapeutic agent used for the treatment of both lung and colorectal cancer (Rothenberg 2001) and has been reported to decrease arachidonic acid, EPA, DHA and n-3 PUFA in the intestinal mucosal phospholipids of rats receiving treatment (Usami et al., 2006). The major side effect of irinotecan is chemotherapy-induced diarrhea which hospitalizes 1/3 of patients due to severe grade 3 or 4 diarrhea (Reviewed by Maroun et al., 2007). If the dose of chemotherapy can be increased while limiting

toxicities this could ultimately lead to better treatment options, increased survival time and improved quality of life. Experimental data shows that n-3 PUFA can inhibit cancer growth during several stages of cancer development, they can enhance chemotherapy induced tumor cell death and they can also reduce toxic side effects of chemotherapy on host tissue.

Newly synthesized fatty acids produced by the liver enter the circulatory system either in the form of very low density lipoprotein-triglyceride (VLDL-TG) or as phospholipids (Hellerstein et al., 1991) therefore it would be of value to measure the triglycerides and phospholipids in these two lipid pools. The objective of this study was to determine the fatty acid composition and the amount of phospholipids and triglycerides in plasma and whole liver of rats that have been injected with a colorectal tumor and treated with the chemotherapy drug, irinotecan. It was hypothesized that changes in plasma fatty acids may be attributed to abnormalities induced in the liver therefore decreased amounts and changes in composition of fatty acids in the liver and plasma triglycerides and phospholipids of rats treated with irinotecan is expected. Additionally, rats treated with irinotecan and fed a fish oil diet are expected to have amounts and compositions of n-3 fatty acids, specifically EPA and DHA that are similar to untreated rats.

4.2 Materials and Supplies

Animal use was reviewed and approved by the Institutional Animal Care Committee and conducted in accordance with the Guidelines of the Canadian Council on Animal Care. Female Fisher 344 rats (body weight, 150–180g), 11-12 weeks of age, were obtained from Charles River (QC, Canada). Rats were housed 2 per cage under aseptic

conditions including positive-air-pressured room, cages, bedding and filter tops. All handling was carried out under a laminar flow hood in a temperature (22°C) and light controlled (12h light) room. Throughout the study, water and food were available for *ad libitum* consumption. One week before chemotherapy was initiated rats were separated into individual housing. The chemically induced Ward colorectal carcinoma was provided by Dr. Y Rustum, Roswell Park Institute. Irinotecan was provided by Pfizer as a ready-to-use clinical formulation (20mg/ml). Atropine (0.6mg/ml), obtained from the hospital pharmacy, was in a clinical injectable formulation.

Experiment 1: Effects of Irinotecan 1 day vs. 7 days post infusion (Figure 1)

Rats (n=15) were provided a control diet *ad libitum* for 14 days prior to tumor implantation. Following this 2 week period, tumor pieces (~0.05g) were transplanted subcutaneously into the left flank of the rats via trocar under slight isoflurane anesthesia and allowed to grow. Once the tumor reached ~2.0cm^3 rats were randomly assigned to either receive treatment with irinotecan (n=9) or no treatment (n=6). Treatment groups were injected intravenously with 100mg/kg/day of irinotecan for 3 consecutive days. On the fourth day, rats were euthanized by CO_2 inhalation and livers were obtained. The remainder of the rats, both treated and untreated, were euthanized seven days following the last irinotecan injection and liver samples were taken. The liver samples were frozen immediately in liquid nitrogen then stored at -80°C until fatty acid analysis.

Experiment 2: Effects of Diet on Rats Treated with Irinotecan (Figure 2)

Rats (n=13) were provided a control diet *ad libitum* for 7 days then divided randomly into 2 groups. The first group (n=6) remained on the control diet whereas the second group (n=7) was fed a fish oil diet containing 3.2% (w/w) EPA and 0.8% (w/w)

DHA (Table 2) for 14 days. After 2 weeks, rats from the fish oil group (n=3) and rats from the control group (n=4) underwent tumor implantation as described in Experiment 1. Four rats from the fish oil group and 2 rats from the control group were not injected with tumors and were used as controls. These animals were compared to those that were injected with a tumor and treated with irinotecan while on the same diet. When the tumor reached ~2.0cm^3 animals were injected with 125mg/kg/day of irinotecan for 3 consecutive days as in Experiment 1. All rats, including those without a tumor, were euthanized by CO_2 asphyxiation 7 days following the last irinotecan injection. Whole blood was obtained via cardiac puncture and immediately put on ice. Tubes were centrifuged at 4°C, 3000g for 10min. Once the blood components were separated, plasma was extracted and frozen at -80°C until fatty acid analysis.

4.3 Methods

4.3.1 Fatty Acid Analysis

a) Whole Liver

To extract the lipids from each sample, approximately 0.2 g of frozen rat liver was weighed and homogenized with 1mL of calcium chloride ($CaCl_2$). Once a uniform mixture was obtained, 5mL of a 2:1 chloroform-methanol mixture was added to each tube. The tubes were then capped, vortexed and stored at 4°C overnight to separate.

Procedure continues as for plasma in section 3.3.1.1 (Chapter 3).

b) Rat Diets

Approximately 1.3g of each rat diet (powder) was transferred to large methylation tubes and 25mL of a 2:1 chloroform-methanol mixture was added. Tubes were then vortexed and centrifuged at 67 x g for 20min. Once separated, the top layer was poured into

separatory funnels and another 15mL of 2:1 chloroform-methanol was added to the methylation tubes. The tubes were once again vortexed and centrifuged for an additional 20min at 67 x g and the top layer was poured into the corresponding separatory funnels. Water (8mL) was added to the funnels and the lipid fraction found in the bottom layer was decanted into large clean methylation tubes. 100μL of the lipid was pippetted into small methylation tubes and the remaining solution in the large methylation tubes was dried under nitrogen gas. The large tubes were then weighed to determine the weight of the fat in the diet and the small methylation tubes were dried under nitrogen gas and 100μL of chloroform was added and the sample was vortexed.

Procedure continues as for plasma in section 3.3.1.2 (Chapter 3).

4.3.2 Statistical Analysis

Data are reported as means ± SD. Results from the first experiment were evaluated using a one way ANOVA to identify significant differences between multiple samples. When significant overall differences were observed, a post-Hoc analysis was done using the Bonferonni model. A 2 way univariate ANOVA was used in the second experiment to compare diet and treatment in plasma phospholipids and triglycerides. Statistical significance was assured when the p value was <0.05. All statistical analyses were conducted using SPSS 15.0 for Windows.

Sample sizes were small because rats were part of a larger study and only a small number were available for analysis. The sample size resulted in a very small power of <0.2. A power between 0.8 and 1.0 is ideal and to achieve this, the n would have to increase to 25 rats per group. Additional studies have been done with rats fed a fish oil

diet and treated with irinotecan and samples are waiting to be analyzed thereby increasing the sample size.

4.4 Results

4.4.1 Experiment 1: Triglycerides

Total saturated fatty acids (SFA) were significantly lower in rats killed 1 day post-irinotecan treatment compared to the non treated group (controls) (Table 3). This difference in total SFA's was due largely to lower C16:0 and significantly lower amounts of C18:0 (Table 3).

4.4.2 Experiment 1: Phospholipids

There was a significant increase in C22:4n-6 and C22:5n-6 in rats killed 7 days after the last dose of irinotecan compared to either the control group or those killed 1 day post-irinotecan treatment (Table 5). In addition, C20:4n-6 was significantly higher in rats killed 7 days after the last dose of irinotecan compared to the controls (Table 5). Finally, C16:0 was also significantly higher in rats killed 1 day after treatments when compared to the controls and rats killed 7 days after treatment.

4.4.3 Experiment 2: Diet

Significant differences that were attributed to diet were found between C20:4n-6 and C20:5n-3 in both plasma phospholipids and triglycerides. Rats fed a fish oil diet had significantly lower C20:4n-6 compared to those on the control diet whereas C20:5n-3 was significantly higher (Table 5 and Table 6). C22:6n-3 was significantly higher in plasma triglycerides of rats fed the fish oil diet compared to those on the control diet (Table 6).

4.4.4 Experiment 2: Treatment

Treatment with irinotecan was associated with significantly lower amounts of C18:0, C18:2n-6, C20:5n-3, total saturated fatty acids and total phospholipids in plasma phospholipids of rats treated with chemotherapy compared to the untreated group (Table 5). Treatment with chemotherapy was not associated with any differences in plasma triglycerides.

4.5 Discussion

Although significant differences were not found in total liver triglycerides of rats treated with irinotecan, they did decrease by 54% after 3 days of treatment. During this time, food intake decreased significantly in rats treated with irinotecan compared to the untreated group ($p<0.01$). Pair feeding studies are underway to determine if food intake is accountable for the lower liver triglyceride levels. Reductions in total liver triglyceride fatty acids were only temporary because 7 days later, the amount of triglycerides returned to levels comparable to those found in the untreated group. These findings would indicate that while the initial insult with chemotherapy may have short term deleterious effects on hepatic lipids, by 7 days, fatty acids are replenished either by de novo biosynthesis in the liver or are obtained from other sources within the body such as adipose tissue or through dietary intake.

Literature on chemotherapy effects on fatty acid metabolism is limited. A single study by Usami et al., 2006 reports a significant decrease ($p<0.01$) in total plasma triglycerides in rats treated with 60mg/kg/day of irinotecan 3 days following 4 consecutive days of treatment (Usami et al., 2006). Whether or not the plasma triglycerides returned to levels that were similar before treatment with chemotherapy is

unknown. Interestingly, the total plasma triglycerides from the rats in our study were not significantly different 7 days following chemotherapy treatment.

Total saturated fatty acids within the liver triglycerides were significantly lower in rats that were killed 1 day post-treatment compared to the untreated group. These alterations were brief and lasted approximately one week because 7 days after treatment, total SFA's were virtually the same as those that were not treated. Changes in C16:0 and C18:0 were the most remarkable as they decreased by almost 50 and 70% respectively after the initial insult. These results suggest chemotherapy may have prevented de novo fatty acid synthesis. C16:0 (palmitic acid) is the principle product of fatty acid biosynthesis and also the precursor for other long chain fatty acids. Although C16:0 was not significantly lower, C18:0 (stearic acid) a product of C16:0 elongation, decreased significantly after treatment with chemotherapy (Lehninger et al.,1993). Irinotecan may have prevented the elongation of C16:0 thereby contributing to the lower total SFA's.

Linoleic acid (C18:2n-6) is required for the synthesis of C20:4n-6 and other intermediates within the n-6 series such as C22:4n-6 and C22:5n-6. Results from our study indicate that the amount of linoleic acid did not change in liver phospholipids however there was a significant increase in C20:4n-6, C22:4n-6 and C22:5n-6 when rats were killed 7 days after treatment with irinotecan. It is difficult to determine if the increase in C22:4n-6 and C22:5n-6 is significant because in healthy humans, they comprise less than 1% of the total phospholipids (Pratt et al., 2002). An excess of C20:4n-6 however may be harmful because it induces a pro-inflammatory response. Arachidonic acid (C20:4n-6), largely found in membrane phospholipids, is a precursor of pro-inflammatory eicosanoids which produce cyclooxygenase products such as

prostaglandin and thromboxanes thereby inducing an inflammatory state (Calder PC., 2007).

Rats fed a fish oil diet had significantly lower amounts of C20:4n-6 and significantly higher amounts of C20:5n-3 in their plasma phospholipids and triglycerides compared to rats fed a control diet. Additionally, the plasma triglycerides of rats fed a fish diet also had significantly higher amounts of C22:6n-3 compared to those on a control diet. It would appear that preference for desaturation was given to the n-3 fats because n-3 fatty acids have a higher affinity for the Δ6-desaturase enzyme than n-6 fatty acids. By feeding a diet high in n-3 fatty acids, this could reduce inflammation and possibly provide chemo-protective effects by protecting normal cells from the cytotoxic effects of anti-cancer agents (Baracos et al., 2004, Grammaticos et al., 1994, Tsai et al., 1997).

Although total plasma phospholipids were lower in rats fed a fish oil diet compared to those on a control diet, the fish oil maintained EPA levels similar to untreated rats despite the effects of chemotherapy. These findings indicate that the depletion of EPA can be overcome by supplementation and that dietary intervention may have some application in the clinical setting. Patients undergoing treatment with chemotherapy may initially have a fatty acid status similar to the healthy population. However, fatty acids may become depleted following several cycles of chemotherapy. Supplementation with essential fatty acids may be most beneficial prior to treatment to prevent the depletion as opposed to trying to replete the loss. In conclusion, we found that treatment with chemotherapy temporarily changed the amounts of fatty acids in rat liver triglycerides. After 7 days, fatty acids within the triglycerides returned to levels

comparable to those seen in untreated animals. Finally, the fish oil diet maintained EPA levels in the plasma phospholipids and triglycerides of irinotecan treated rats that were comparable to those left untreated.

4.6 Tables and Figures

Table 1 Composition of the experimental diets fed to rats

Ingredient (g/100g of diet)			Control	Fish oil
Constant portion	Modified AIN-76 basal mix 70g/100g	Casein	25.2	25.2
		Methionine	0.25	0.25
		Corn starch	23.7	23.7
		Glucose	13.95	13.95
		Vitamins AIN 76	1	1
		Minerals AIN 76	5	5
		Inositol	0.6	0.6
		Choline	0.3	0.3
Variable portion	Lipids 20g/100g	Soybean stearine	15.22	13.84
		Linseed oil	0.4	0.4
		Sunflower oil	0	3.46
		Safflower oil	4.38	0
		Fish oil	0	2.3
	Fibers 10g/100g	Cellulose	10	10
Total			100	100

All diets contained 262 g protein and 15.48 MJ of energy per kg. The constant portion consisted of the pre-mixed modified AIN-76 basal ingredients (Harlan Teklad, Madison, WI). The variable portion was formulated to allow the addition of selected fat elements. Other ingredients were supplied: soybean stearine (ICN Biomedicals Inc., Cleveland, OH), safflower oil (Canadian Superstore, President's Choice, AB), linseed oil (Planet Organic, Gold Top, AB) and fish oil (Ocean Nutrition Canada, Dartmouth, NS).

Table 2 Fatty acid composition of the experimental rat diets

Fatty Acid	Control	Fish oil
	(Percent fatty acids)	
C14:0	2.2	2.8
C16:0	21.1	21.0
C18:0	44.4	42.6
C18:1n-9	4.6	4.5
C18:2n-6	23.4	18.8
C18:3n-3	1.1	0.2
C 20:5n-3	nil	3.2
C 22:5n-3	nil	0.2
C 22:6n-3	nil	0.8
∑SFA	70.2	69.0
∑MUFA	4.8	6.6
∑PUFA	24.5	24.0
∑n-6	23.4	19.1
∑n-3	1.1	5.0

Fatty acid composition was determined by fatty acid analysis using gas chromatography as discussed Section 4.3.1 b) in the methods section.

Table 3 Effects of Irinotecan on rat liver triglyceride fatty acid composition 1 day vs. 7 days post infusion

Fatty Acid	Not Treated with Chemotherapy (n=6)	Treated with Chemotherapy (100mg/kg 1 day after chemo) (n=3)	Treated with Chemotherapy (100mg/kg 7 days after chemo) (n=5)	Overall p value
C16:0	833±288	432±26.9	678±166	.064
C16:1	43±25.7	19±5.1	32±10.3	.216
C18:0	290±70.8	93±5.2	233±35.8	.001[abc]
C18:1n-9	709±284	375±27.0	627±141	.108
C18:1n-7	57±22.6	36±3.3	55±14.2	.243
C18:2n-6	483±188	292±41.1	395±85.8	.168
C18:3n-3	4±1.2	4±1.4	7±1.7	.014[abd]
C20:2n-6	5±2.0	3±0.6	4±1.1	.315
C20:3n-6	8±3.9	7±0.4	8±3.0	.713
C20:4n-6	48±25.2	57±12.9	59±25.8	.732
C20:5n-3	6±3.4	5±1.9	6±2.6	.863
C22:4n-6	11±5.1	12±1.8	14±6.0	.603
C22:5n-6	5±2.4	6±0.3	8±2.7	.194
C22:6n-3	28±20.7	45±14.5	25±11.1	.258
Total($\mu g\ mL^{-1}$)	2555±860	1403±103	2172±447	.069
∑SFA	1133±330	535±23.9	919±194	.017[ac]
∑MUFA	808±330	430±35.2	714±165	.120
∑PUFA	613±241	438±63.4	540±129	.403
∑n-6	575±224	385±47.6	502±118	.297
∑n-3	38±24.5	53±16.0	37±14.8	.506

[a]Significant overall difference
[b]Significant difference between 1 day and 7 days
[c]Significant difference between 1 day and no treatment
[d]Significant difference between 7 days and no treatment

Amount of fatty acid (μg/g) in rat liver triglycerides either treated or not treated with chemotherapy. 100μL of C15:0 standard [0.1μg/μL] was used to determine the μg of each fatty acid. Data is expressed as means ± standard deviation. Significant differences ($p<0.05$) were determined using a oneway ANOVA.

Table 4 Effects of Irinotecan on rat liver phospholipid fatty acid composition 1day vs. 7 days post infusion

Fatty Acid	Not Treated with Chemotherapy (n=6)	Treated with Chemotherapy (100mg/kg 1 day after chemo) (n=3)	Treated with Chemotherapy (100mg/kg 7 days after chemo) (n=5)	Overall p value
C16:0	1715±127	2134±176	1738±76	.001[abc]
C16:1	35±14.3	25±21.4	23±19.8	.513
C18:0	5639±1838	4652±403	5639±707	.538
C18:1n-9	464±133	536±121	548±62.3	.430
C18:1n-7	148±30.9	197±21.7	178±22.0	.051
C18:2n-6	1308±262	1454±288	1346±170	.693
C18:3n-3	32±10.1	30±4.5	42±6.8	.103
C20:2n-6	20±4.0	27±13.7	19±3.9	.304
C20:3n-6	108±46.3	89±8.10	90±19.0	.617
C20:4n-6	2637±374	2886±243	3661±892	.047[ad]
C20:5n-3	85±33.1	66±6.5	82±28.8	.649
C22:4n-6	43±6.7	36±1.7	64±16.4	.009[abd]
C22:5n-6	52±12.8	44±30.8	129±28.6	.000[abd]
C22:6n-3	1018±200	1332±274	1369±400	.160
Total($\mu g\ mL^{-1}$)	13407±1508.4	13602±619.1	15037±1962.4	.248
∑SFA	7458±1758	6881±497	7487±734	.783
∑MUFA	646±159	758±78.4	748±73.7	.303
∑PUFA	5302±795	5964±326	6801 ±1494	.108
∑n-6	4167±659	4535±157	5309±1086	.100
∑n-3	1135±202	1428±282	1493±419	.181

[a]Significant overall difference
[b]Significant difference between 1 day and 7 days
[c]Significant difference between 1 day and no treatment
[d]Significant difference between 7 days and no treatment

Amount of fatty acid (μg/g) in rat liver phospholipids either treated or not treated with chemotherapy. 50μL of C17:0 standard [0.1μg/μL] was used to determine the μg of each fatty acid. Data is expressed as means ± standard deviation. Significant differences ($p<0.05$) were determined using a t-test. Values in rows not sharing common superscript are significantly different.

Table 5 Effects of diet on rat plasma phospholipid fatty acids treated with Irinotecan

	Fish Oil Diet		Control Diet		
Fatty Acid	Not Treated with Chemotherapy / No Tumor (n=4)	Treated with Chemotherapy (125mg/kg 7 days after chemo) (n=3)	Not Treated with Chemotherapy / No Tumor (n=2)	Treated with Chemotherapy (125mg/kg 7 days after chemo) (n=4)	Overall p value
C16:0	287±76.8	241±13.0	273±3.2	246±44.2	.760
C18:0	720±101	431±57.7	670±21.3	498±143	.358[b]
C18:1n-9	45±6.1	52±7.8	37±2.8	55±17.5	.397
C18:1n-7	11±1.4	12±2.7	13±0.1	15±4.0	.959
C18:2n-6	267±48.4	174±23.7	295±33.4	186±33.9	.715[b]
C20:4n-6	176±31.4	138±27.4	279±63.5	251±116	.907[a]
C20:5n-3	32±11.2	16±5.6	12±1.6	1±2.5	.586[ab]
C22:6n-3	65±17.6	58±17.3	54±14.9	74±27.0	.291
Total($\mu g\ mL^{-1}$)	1613±247	1136±113	1631±92.8	1343±382	.559[b]
∑SFA	1012±175	678±66.8	942±18.0	747±189	.477[b]
∑MUFA	56±7.3	64±10.5	50±2.6	69±21.3	.494
∑PUFA	545±94.9	395±77.2	639±113	527±185	.809
∑n-6	448±69.7	320±55.7	573±97.0	451±156	.964
∑n-3	97±26.7	75±22.6	66±16.5	76±29.3	.314

[a]Differences can be associated to diet
[b]Differences can be associated to treatment with chemotherapy

Amount of fatty acid (μg/mL) in rat plasma (50μL) phospholipids. Rats were either treated or not treated with chemotherapy and on a fish oil or a control diet. 50μL of C17:0 standard [0.1μg/μL] was used to determine the μg of each fatty acid. Data is expressed as means ± standard deviation. Significant differences ($p<0.05$) were determined using a t-test. Values in rows not sharing common superscript are significantly different.

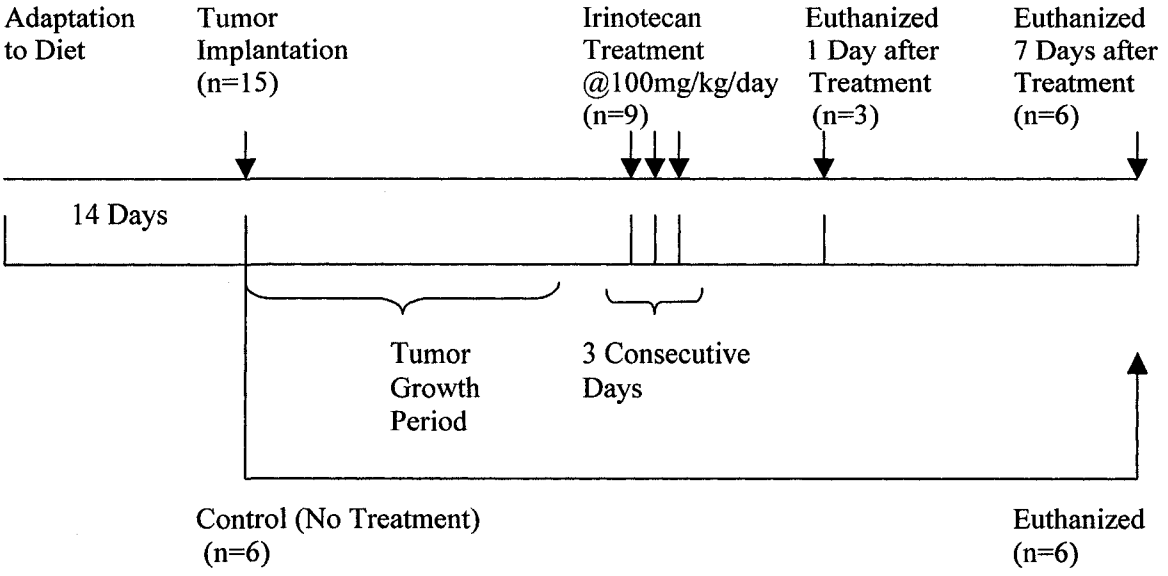
Table 6 Effects of diet on rat plasma triglyceride fatty acids treated with Irinotecan

	Fish Oil Diet		Control Diet		
Fatty Acid	Not Treated with Chemotherapy / No Tumor (n=4)	Treated with Chemotherapy (125mg/kg 7 days after chemo) (n=3)	Not Treated with Chemotherapy / No Tumor (n=2)	Treated with Chemotherapy (125mg/kg 7 days after chemo) (n=4)	Overall p value
C16:0	125±23.3	136±42.2	167±13.8	234±134	.563
C16:1	ND	ND	ND	41±51.8	.265
C18:0	133±48.1	113±17.9	139±18.4	135±39.4	.708
C18:1n-9	99±36.3	138±69.4	114±7.4	171±122	.849
C18:1n-7	4±5.1	6±10.2	3±4.1	18±13.4	.262
C18:2n-6	116±56.1	119±56.2	179±28.3	166±92.0	.839
C20:4n-6	3±6.6	2±3.4	22±1.4	23±15.9	.859[a]
C20:5n-3	11±12.3	11±9.8	ND	ND	.958[a]
C24:0	2±4.6	ND	ND	ND	.472
C22:6n-3	9±10.6	14±13.7	ND	ND	.625[a]
Total($\mu g\ mL^{-1}$)	505±172	539±213	628±16.6	791±379	.678
∑SFA	261±72.6	249±57.1	306±4.6	369±172	.574
∑MUFA	103±40.4	144±79.2	116±11.5	230±98.1	.405
∑PUFA	141±84.2	146±81.6	205±23.5	193±112	.864
∑n-6	121±62.8	121±58.5	205±23.5	189±107	.855
∑n-3	20±22.7	25±23.2	ND	4±8.3	.940

ND Fatty acid was not detected
[a]Differences can be associated to diet

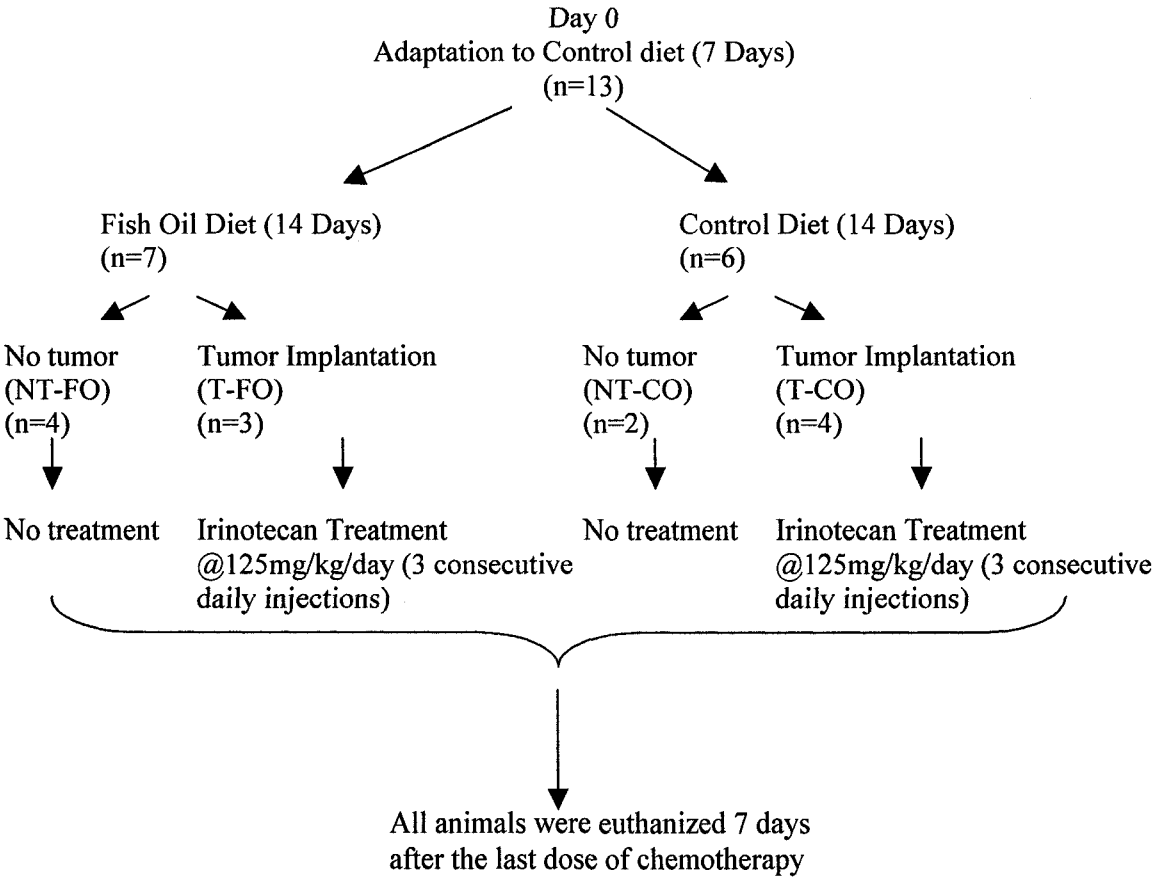
Amount of fatty acid (μg/g) in rat plasma (50μL) triglycerides. Rats were either treated or not treated with chemotherapy and on a fish oil or a control diet. 50μL of C17:0 standard [0.1μg/μL] was used to determine the μg of each fatty acid. Data is expressed as means ± standard deviation. Significant differences ($p<0.05$) were determined using a t-test. No significant differences were found.

Figure 1 Experiment 1: Effects of Irinotecan 1 day vs. 7 days post infusion



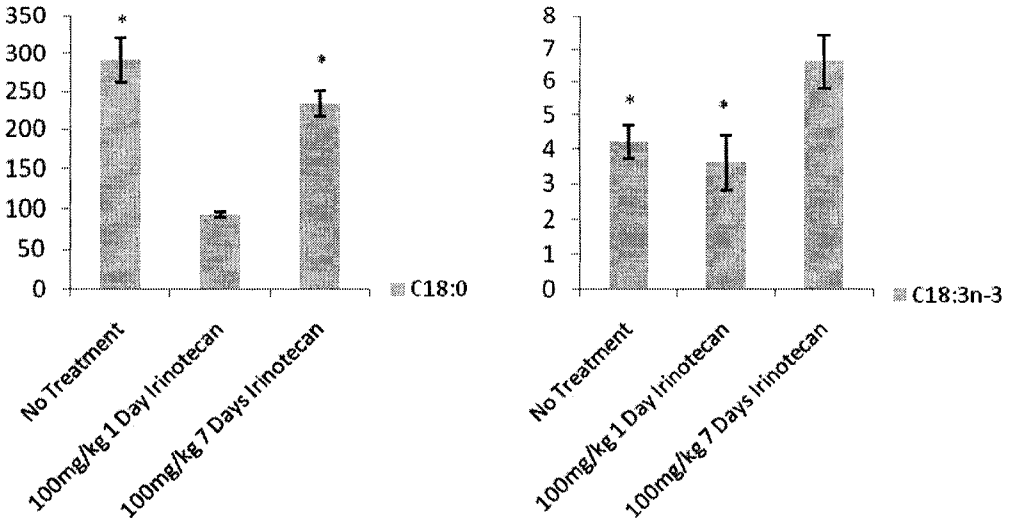
*Liver samples were taken for phospholipid and triglyceride analysis.

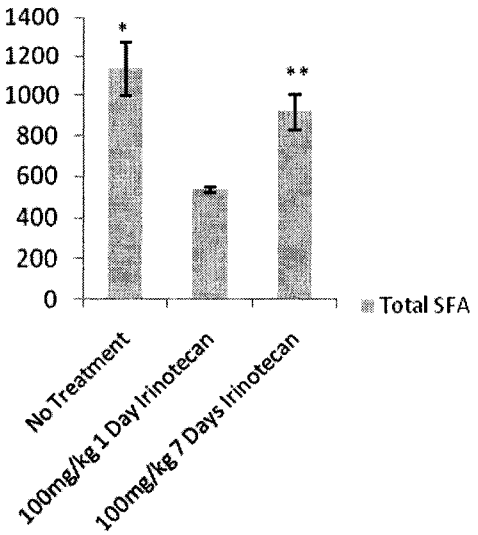
Figure 2 Experiment 2: Effects of Diet on Rats Treated with Irinotecan



*Plasma samples were taken for phospholipid and triglyceride analysis.

Figure 3 Significantly different fatty acids in rat liver triglycerides

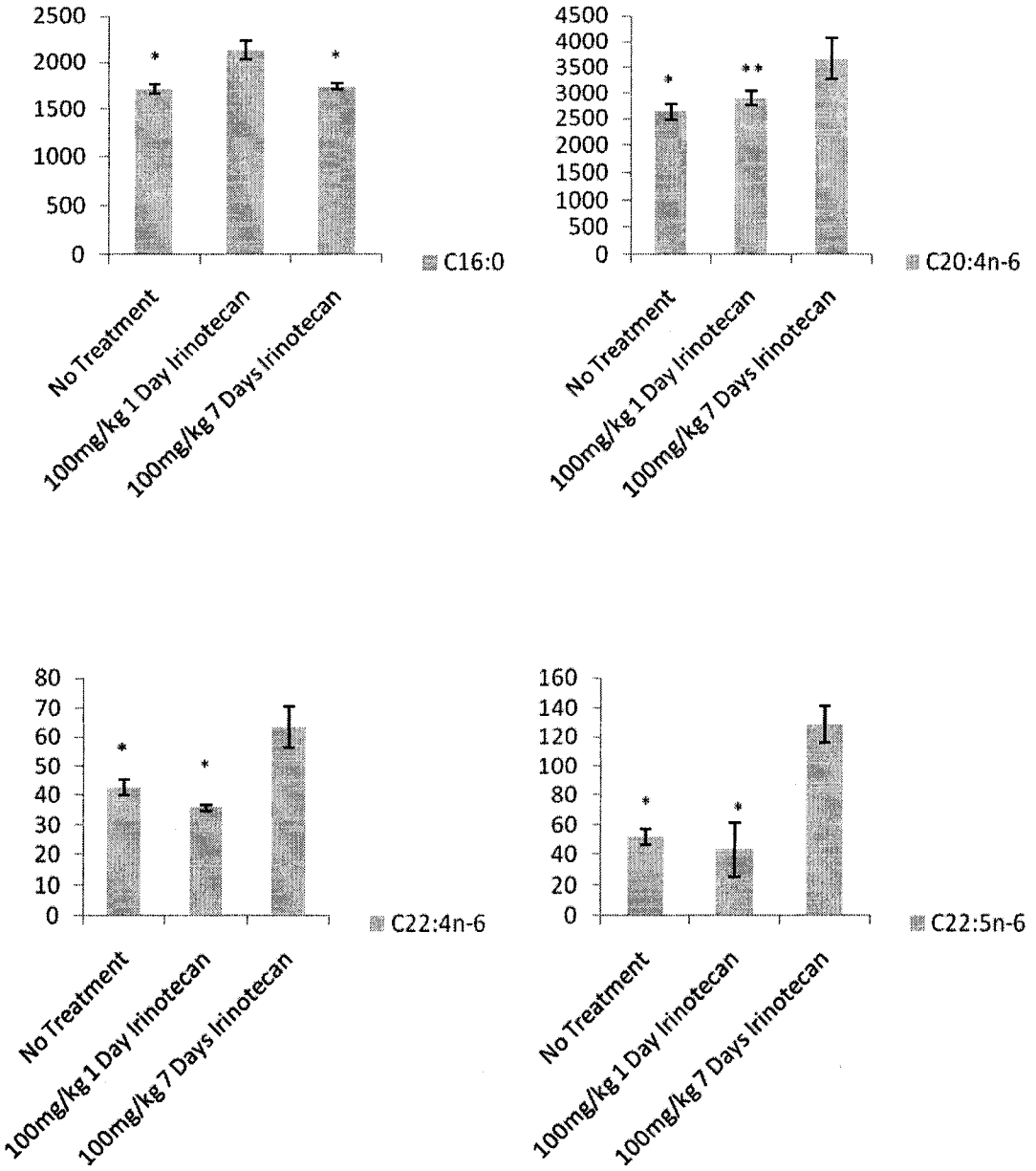




*Significantly different

**Not significantly different

Figure 4 Significantly different fatty acids in rat liver phospholipids



*Significantly different
**Not significantly different

4.7 References

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Chapter 5

5.1 Conclusion

This pilot study has revealed the fatty acid status in newly diagnosed patients with advanced lung and colorectal cancer. To further strengthen this data, a longitudinal study would be beneficial to observe the fatty acids in patients with advanced cancer over a longer period of time. This information would be important in understanding fatty acid metabolism during the cancer trajectory. Based on results from previous studies, fatty acids in phospholipids of patients with advanced cancer are depleted compared to healthy subjects. It would appear that this only holds true for patients with advanced cancer who are close to time of death, as this work showed no difference in several fatty acids between patients with advanced cancer and healthy subjects. The n-3 fatty acids are of particular interest, especially EPA and DHA. These two key fatty acids have been associated with weight maintenance and/or gain in patients with advanced cancer experiencing unintentional weight loss (Barber et al., 1999, Fearon et al., 2003, Fearon et al., 2006, Wigmore et al., 1996). By maintaining levels of EPA and DHA similar to those found in healthy subjects, undesired weight loss in patients with advanced cancer, may be attenuated, quality of life improved and survival prolonged (Bauer et al., 2005, Wigmore et al., 1996). Although it is common for patients with cancer to supplement with fish oil only five patients with advanced cancer (n = 2 lung; n = 3 colorectal) from Edmonton reported taking 3-6-9 omega supplements.

Our animal study showed that irinotecan had several different effects on liver and plasma fatty acids in rats. Although total saturated fats were decreased, arachidonic acid

and other n-6 intermediates increased after treatment with irinotecan. Interestingly, a diet rich in n-3 fatty acids was associated with decreased levels of arachidonic acid and increased levels of EPA and DHA. This information is valuable in telling us what might be happening to the fatty acids in patients receiving chemotherapy treatment. Additionally, it would seem that essential fatty acids can be maintained during treatment with chemotherapy by feeding a diet high in n-3 fats.

The largest limitation of the animal study was the small sample size. The rats were part of a larger study and only a small number were available for fatty acid analysis. The sample size resulted in a very small power of <0.2. A power between 0.8 and 1.0 is ideal and to achieve this, the n would have to increase to 25 rats per group. Additional studies have been done with rats fed a fish oil diet and treated with irinotecan. These samples are waiting to be analyzed to increase the sample size and amplify the power of this study.

Low total plasma phospholipids have been reported in patients with advanced cancer who lost >5% of their pre-illness body weight. Although this was not seen in our newly diagnosed patients with advanced cancer, depletions may be evident as the disease progresses. While our patients were newly diagnosed with an average BMI above the healthy range, they were still losing weight or at risk for weight-loss. Over time, these people may suffer from cachexia and become emaciated, malnourished and eventually die. By establishing a timeline and mapping out the cancer trajectory indicating when fatty acids become depleted, this could ultimately determine at what time fatty acid supplementation would be optimal to prevent a decrease in fatty acids. The information

learned from this work will be important in defining points of potential intervention in patients with advanced cancer.

Appendix

Complete List of Fatty Acids in Patients with Advanced Cancer and Healthy Subjects

Fatty Acid	Colorectal (n=39)	Lung (n=57)	Healthy Subjects (n=17)	Overall p value
C16:0	294±94.7	273±90.4	245±59.6	.174
C16:1	8±4.4	6±4.6	4±2.2	.031[ac]
C17:1	2±1.5	3±2.3	1±1.5	.076
C18:0	129±38.9	132±46.2	130±31.5	.965
C18:1n-9	98±36.2	97±36.4	89±24.4	.637
C18:1n-7	19±8.1	18±6.2	20±5.7	.574
C18:2n-6	173±63.5	173±59.9	170±40.2	.981
C18:3n-6	2±2.2	1±1.6	1±1.1	.194
C18:3n-3	2±1.8	1±1.4	3±2.7	.018[ab]
C20:0	2±1.8	3±2.4	3±1.3	.192
C20:1	2±1.7	2±1.4	3±1.3	.000[abc]
C20:2n-6	4±5.5	3±1.7	2±2.1	.089
C20:3n-6	27±16.2	29±14.0	17±9.6	.010[ab]
C20:4n-6	78±33.8	87±34.7	54±30.1	.003[ab]
C20:3n-3	1±2.2	1±1.7	0±0.5	.360
C20:5n-3	8±6.5	9±7.7	4±4.7	.033[ab]
C22:0	4±4.0	7±6.1	4±3.6	.005[ad]
C22:1	2±1.9	3±2.5	2±2.3	.173
C22:2	2±2.4	4±10.3	0±0.6	.150
C22:4n-6	3±2.5	3±2.8	2±1.0	.069
C22:5n-6	7±4.1	8±5.1	5±3.8	.156
C24:0	5±3.7	6±5.8	4±1.9	.212
C22:6n-3	26±17.1	29±13.9	15±12.6	.004[abc]
C24:1	11±13.1	14±10.2	10±4.9	.203
Total($\mu g\ mL^{-1}$)	917±300	920±291	794±180	.246
∑SFA	442±134	430±135	393±88.9	.431
∑MUFA	142±57.0	142±51.1	130±30.7	.668
∑PUFA	333±127	347±118	272±85.2	.068
∑n-6	290±105	301±103	250±73.1	.195
∑n-3	37±22.9	40±19.9	21±17.2	.007[abc]

[a]Overall significant difference ($p<0.05$)
[b]Significant difference between healthy and lung cancer ($p<0.05$)
[c]Significant difference between healthy and colorectal cancer ($p<0.05$)
[d]Significant difference between lung cancer and colorectal cancer ($p<0.05$)

Amount of fatty acid (μg/mL) in plasma phospholipids (PL) of patients with advanced lung and colorectal cancer compared to healthy age-matched controls was determined using gas chromatography. Total plasma PL were calculated using 50 μg of C17:0 [0.1μg/μL] as the standard and are expressed as μg/mL of plasma. Data are expressed as means ± SD. Groups were statistically compared using a oneway ANOVA. Abbreviations: SFA, saturated fatty acids, MUFA, monounsaturated fatty acids, PUFA, polyunsaturated fatty acids.