University of Alberta

Alteration of liver fat metabolism following irinotecan plus 5-fluorouracil treatment

by

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A thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of

Master of Science
in
Nutrition and Metabolism

Department of Agricultural, Food and Nutritional Science

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Fall 2011
Edmonton, Alberta

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Abstract

This study determined how chemotherapy treatment for colorectal cancer alters hepatic fat metabolism. Livers were isolated from tumor-bearing animals after one and two cycles of chemotherapy consisting of irinotecan plus 5-fluorouracil. Fatty acid amounts and composition within triglycerides (TG) and phospholipids (PL) as well as gene expression were assessed. Total TG did not change and total PL were higher after 7 days following treatment. Docosahexaenoic acid became undetectable after the treatment at 5 and 7 days, respectively. Following one cycle, the alterations appeared temporary. After second cycle, total TG were lower and n-3 fatty acids were also lower causing n-6/n-3 ratio to be higher. Chemotherapy altered 13 genes of 44, however not all genes within a single pathway were affected. The alterations in gene expression did not necessarily parallel the observations in fatty acids. This study is important to develop identify target pathways to circumvent negative effects of chemotherapy-associated steatohepatitis.

Acknowledgements

I am thankful to my supervisor Dr Vera Mazurak for her moral as well as academic support with a thoughtful advice, encouragement and providing all the facilities to conduct my Master's research. I got self confidence, positive thoughts and enthusiasm to do research because of all time support from her for these two academic years. I would like to thank my committee members Dr Vickie Baracos and Dr Rene Jacobs for providing valuable comments and suggestions to improve my research project from a different perspective. I would like to thank my external examiner Dr Jason Yap, for providing feedbacks for my research, which will help me to improve.

I would like to thank Kelly, Abha and Randy for the technical assistance, without them my research would not be possible. Thanks go to my lab mates Alaa, Maryam and Taylor for sharing good times together. I want to thank AFNS department for creating supportive environment for an international student, like me. I never felt myself alone here, as there was always someone to help when I asked. I want to thank all those people who made my stay happy in Edmonton. I will always have good memories, inspirations and positive thoughts from here.

I would like to thank my dear parents, sweet husband and the caring family members, for understanding me and giving love and care for life and a support for my study. I miss you all at this great moment!

I would like to thank all those people who supported me directly or indirectly throughout this life journey because of which I am here and proud of myself.

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List of Abbreviations

Δ	delta
5-FU	5-fluorouracil
ACAT 1	acetyl-CoA acetyl transferase 1
ACC 1	acetyl coenzyme A Carboxylase 1
ACLY	ATP citrate lyase
ACOX	acyl coenzyme A oxidase
ACS	American Cancer Society
ADRP	adipose differentiated related protein
ANOVA	analysis of variance
ANSA	8a-nilino-1-naphthalene-sulphonic acid
APO A1	apolipoprotein A1
APO B	apolipoprotein B
ATP	adenosine triphosphate
C18:2n-6	linoleic acid
C18:3n-3	linolenic acid
C20:4n-6	arachidonic acid
C20:5n-3	eicosapentaenoic acid
C22:6n-3	docosahexaenoic acid
$CaCl_2$	calcium chloride
CCS	Canadian Cancer Statistics
CD 36	cluster of differentiation 36
CD 68	cluster of differentiation 68
CHK α	choline kinase alpha
CHK β	choline kinase beta
CPT-11	irinotecan
CRC	colorectal cancer
DGAT	diglyceride acyl transferase
DNA	deoxyribonucleic acid
EFA	essential fatty acid
FABP	fatty acid binding protein

FAD2	fatty acid desaturase 2
FAS	fatty acid synthase
FAT	fatty acid translocase
FATP	fatty acid transporter protein
FDA	Food and Drug Administration
FNB	Food and Nutrition Board
F-UMP	5 floroxyuridine mono-phosphate
GC	Gas chromatography
GPAT	glycerol-3-phosphate acyl transferase
GPX1	glutathione peroxidase 1
GSR	glutathine reductase
HDL	high density lipoprotein
HMGCR	hydroxyl-methyl glutaryl-CoA reductase
HSPA5	heat shock protein 5
I-FABP	intestinal-fatty acid binding protein
INSIG 1	insulin induced gene 1
KOH	potassium hydroxide
LCAD	long-chain-acyl-CoA dehydrogenase
LDL	low density lipoprotein
LDLR	low-density lipoprotein receptor
L-FABP	liver fatty acid binding protein
MCAD	medium chain acyl- Coenzyme a dehydrogenase
ME1	maleic enzyme 1
MGAT2	alpha-1, 6-mannosyl-glycoprotein 2-beta-N-acetyl glucosaminyl transferase
mL	milliliter
MTP	microsomal triglyceride transfer protein
MUFA	monounsaturated fatty acids
n-3 FA	n-3 fatty acids
ND	not distinct
nm	nanometer

PCK1	phosphoenol pyruvate carboxykinase 1
PL	phospholipid
PPAR-α	peroxisome proliferator-activated receptor alpha
PUFA	polyunsaturated fatty acids
Q-PCR	quantitative polymerase chain reaction
RNA	ribonucleic acid
ROS	reactive oxygen species
RT-PCR	reverse transcriptase polymerase chain reaction
SCD1	stearoyl-CoA desaturase 1
SEM	standard error mean
SFA	saturated fatty acid
SLC27A5	solute carrier family 27 fatty acid transporter, member 5
SPSS	Statistical Package for the Social Sciences
SREBF	sterol regulatory element binding transcription factor 1
SREBP1-c	sterol regulator element binding protein
TG	triglyceride
TLC	thin layer chromatography
TMN	tumor; node; metastases
TNF-α	tumor necrosis factor-alpha
Topo I	topoisomerase I
UCP	uncoupling protein
VLCAD	very long chain acyl-coenzyme A dehydrogenase
VLDL	very low density lipoprotein
WHO	World Health Organization
μg	microgram
μL	microlitre

Chapter 1: Introduction and literature review

1.1 Colorectal cancer

Colorectal cancer (CRC) results from uncontrolled growth of cells within the colon or rectum. CRC is a major cause of morbidity and mortality throughout the world (1). The incidence of CRC is most abundant in industrialized countries and the highest rates are reported in Australia, New Zealand, Canada, United States, and parts of Europe (2). In Canada, CRC is the second leading cause of cancer death (3). Risk factors for CRC that cannot be controlled include age, race, and family history and the modifiable factors include tobacco use, low physical activity, moderate alcohol consumption and poor diet (4) that includes a high intake of red and processed meats (5). The probability of developing CRC increases dramatically with age; in persons less than 40 years of age, the risk is 1 in 1678; in contrast, the risk is 1 in 25 in persons 60–79 years of age (6). Although the relationship between CRC and environmental factors is complex, risk may be lowered by a lifestyle that includes regular exercise, as well as a diet low in fat and high in fruits and vegetables.

CRC begins as a benign adenomatous polyp, which develops into an advanced adenoma with a high-grade dysplasia and then progresses to an invasive cancer (7). The most commonly used staging system for CRC is TNM system (T=primary tumor; N=regional lymph nodes; M=metastasis); (8). The numbers 0-4 in the T, N, and M classification provides details of each factor with higher number indicating increasing severity. Stage IV, advanced cancer indicates the cancer has spread to other organs. CRC survival is highly dependent upon stage of disease at diagnosis, and typically ranges from a 90% 5-year survival rate for cancers detected at the localized stage; 70% for regional; to 10% for people diagnosed for distant metastatic cancer (6,9).

Current methods of screening for CRC include fecal occult blood test, flexible sigmoidoscopy, barium enema, and colonoscopy (10). Early detection and removal of adenomatous polyps through regular screening could reduce the mortality rate by 50% (11). The most commonly used methods to treat CRC are

surgery, radiation, chemotherapy, biotherapy (immunotherapy) and stem cell transplantation. However, several treatment therapies for CRC may cause toxic side effects. The drug used for the treatment may impact the metabolism of various nutrients, such as fat; however, this has not been well defined.

1.2 Cancer chemotherapy

With the emergence of new chemotherapy drugs and targeted agents, there has been great improvement in the prognosis of patients with metastatic CRC (12). Regardless of drug used, rapidly dividing non-cancerous cells are affected, which gives rise to a range of toxic side effects. Single agent chemotherapy may remain the treatment choice for some tumors though combination chemotherapy is frequently prescribed.

1.3 Irinotecan plus 5-fluorouracil

5-Fluorouracil (5-FU), a potent agent against solid tumors, was introduced in 1957 for clinical use and has remained the most effective single-agent treatment for advanced CRC (13). 5-FU is widely used in the treatment of a range of cancers including CRC, breast cancers and cancers of the aero-digestive tract (14). *In vivo*, 5-FU is converted to the active metabolite 5-fluoroxyuridine mono-phosphate (F-UMP); replacing uracil, F-UMP incorporates into RNA and inhibits RNA processing, thereby inhibiting cell growth (14). Another active metabolite, 5-5-fluoro-2'-deoxyuridine-5'-O-monophosphate, inhibits thymidylate synthase, resulting in the depletion of thymidine triphosphate, one of the four nucleotide triphosphates used in the *in vivo* synthesis of DNA (14). For more than three decades, the therapeutic options for patients with advanced CRC have been almost exclusively based on 5-FU (15). 5-FU based chemotherapy has been associated with multiple toxicities, including nausea and vomiting, cytopenias secondary to bone marrow suppression, palmar-plantar erythrodysesthesia (hand-foot syndrome); (14) and cardiotoxicity (16).

Irinotecan (CPT-11), an anti-neoplastic drug was introduced for clinical use after FDA approval in 1996 (17). It is a semi-synthetic, water-soluble derivative of camptothecin, which is a cytotoxic alkaloid, extracted from plants such as

Camptotheca acuminata (18). CPT-11 has shown activity against colorectal, esophageal, gastric, non-small cell and small cell lung cancers, leukemia and lymphomas, as well as malignant gliomas (17). CPT-11 is a potent inhibitor of topoisomerase I (TopoI); (19), 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, CPT-11 causes irreversible damage as it creates double-stranded DNA breaks leading to cell death (19).

Recently, the combinations of 5-FU plus CPT-11 (20-21) or oxaliplatin (22) have improved response rates to 50%. There appears to be a survival advantage for 5-FU plus CPT-11. Although investigations on efficacy and toxicity effects with CPT-11 and 5-FU treatment *in vitro* and *in vivo* studies are numerous (21, 23-25), the drugs' toxic effect on the alteration of fat metabolic processes has not been investigated in detail.

1.4 Dietary fat

Dietary fat contains more than twice the energy per gram (on average, 9 kcal) as protein and carbohydrates and generally comprises 20-35 % of total energy intake (26). Fat is an important source of energy in the form of triglycerides (TG) and structural and functional component of cell membranes as phospholipids (PL). Fat also serves as numerous regulatory roles such as signaling molecules and provides sources for prostanoids and steroids to stimulate the release of hormones and cytokines. Dietary fat is incorporated into cells and tissues thereby affecting membrane structure and cell function (27). Therefore, an understanding of how dietary fat affects cellular function and metabolic processes has a potential application for prevention and treatment of diseases (28).

There is an optimal requirement for essential fatty acids (EFA) such as linoleic acid (C18:2n-6) and linolenic acid (C18:3n-3) as they cannot be synthesized within body. C18:2n-6 and C18:3n-3 undergo a series of de-saturation and elongation reactions (**Figure 1-1**) to produce longer chain polyunsaturated fatty acids (PUFA), which contribute to the structural integrity of membranes or become biologically active metabolites. C18:2n-6 and C18:3n-3 compete with

each other for de-saturation and elongation through the same enzymes. The n-3 fatty acids have a higher affinity for the enzyme however the transition through the n-3 pathway is generally lower because n-3 comprises a smaller proportion of the diet (29). Good sources of long-chain n-3 fatty acids are fatty cold water fish such as mackerel, tuna, salmon, sardines and menhaden. Good sources of C18:2n-6 are safflower oil, sunflower seeds, pine nuts, sunflower oil and corn oil. Good sources of C18:3n-3 are soybean, canola and linseed oil, flaxseed and walnuts.

Although mammals have the necessary enzymes to make long-chain PUFA from the parent PUFA, only ≈5% of C18:3n-3 is converted to eicosapentaenoic acid (C20:5n-3) and <0.5% of C18:3n-3 is converted to docosahexanoic acid (C22:6n-3), as determined from *in vivo* studies in humans (30). Due to the low conversion of this pathway, even large amounts of dietary C18:3n-3 have a negligible effect on plasma C22:6n-3 and parallel to this, C18:2n-6 has a negligible effect on plasma arachidonic acid (C20:4n-6); (30). Despite this inefficient conversion, C18:3n-3 and C20:5n-3 have a potential role in human health that could be independent of their metabolism to C22:6n-3 (30).

1.5 Fat metabolism

Digestion of dietary fat begins in the stomach and occurs throughout the gastrointestinal tract. Gastric lipase secreted in the stomach begins hydrolysis of TG, producing free fatty acids and diglycerides. In response to cholecystokinin, bile from the gall bladder and lipase from pancreas are released enabling hydrolysis of TG and diglycerides into monoglycerides and free fatty acids. The emulsification provided by the bile acids and micelle formation improves digestion and absorption of fat by increasing the total surface area for lipase action (31).

About 95% of dietary fat is absorbed by the intestine. Short chain fatty acids and medium chain fatty acids travel out of the absorptive cell (enterocyte) and through the portal vein to the liver. The long chain fatty acids are first re-formed into TG in the absorptive cell and are packaged into chylomicron, which is surrounded by a thin shell of PL, cholesterol and protein (31). Chylomicron enters

the lymphatic system and remnants are subsequently removed by the liver. Fatty acid from the remnant as well as endogenously synthesized fatty acid within liver are assembled into TG and incorporated into very low density lipoprotein (VLDL) and are released into peripheral circulation. Most of the fatty acids in circulation are hydrolyzed by muscle and adipose cells. TG are broken down into fatty acids and glycerol by an enzyme called lipoprotein lipase, located on the inside wall of the blood vessel. Cells can immediately use absorbed fatty acids for energy needs, or they can reform them into TG and store them.

1.6 Regulation of hepatic fat metabolism

The liver is a major site for fatty acid oxidation and synthesis. Several genes are involved in regulating liver fat metabolism (**Table 1-1**). TG accumulation can occur when synthesis and uptake increase or oxidation and export decrease. **Figure 1-2** shows the schematic diagram of the genes involved in fatty acid metabolism in liver. Two major transcriptional factors that regulate fatty acid synthesis and oxidation have been identified. Sterol regulator element binding protein (SREBP-1c) controls the expression of hepatic genes involved in fatty acid synthesis (32), whereas, peroxisome proliferator-activated receptor alpha (PPAR-α) regulates hepatic genes involved in fatty acid oxidation (33). Impaired balance between oxidation and synthesis may lead to dysregulation of fatty acid in liver.

Fatty acid metabolism is facilitated by two enzymes; acetyl-CoA caboxylase 1 (ACC1) and acetyl-CoA carboxylase 2 (ACC2); (34). ACC1-generated malonyl-CoA is utilized by fatty acid synthase (FAS) for the synthesis of fatty acid in the cytosol. Palmitic acid (C16:0) is the predominant fatty acid generated by FAS (35) and is the essential precursor for almost all the newly synthesized fatty acid including the formation of very long chain fatty acid (36). C16:0 is desaturated by stearoyl-CoA desaturase-1 (SCD-1) to palmitoleic acid (C16:1) or elongated to yield stearic acid (C18:0). SCD-1 catalyzes the conversion of stearoyl-CoA to oleoyl-CoA, which is a major substrate for TG synthesis (37).

Palmitoyl-CoA is elongated by type III FASs, known as elongates (ELOVLs; elongation of very long chain fatty acids); (38). Seven ELOVL enzymes (ELOVL1-7) are found in mammals, and they have different fatty acid substrate preferences for catalyzing the elongation reaction (39). ELOVL1 and ELOVL3 are found to have higher activity toward all of the saturated C_{20} to C_{26} CoAs, while ELOVL2 elongates C_{20} and C_{22} PUFA (40). Fatty acids with chain lengths longer than C_{26} are elongated by ELOVL4 (40)**.** ELOVL5 has been shown to be responsible for the elongation of C18 substrates and ELOVL6 participates in the elongation of C_{12} to C_{16} fatty acids (40). ELOVL7 found to have significant activities to C_{18}-CoAs and less to C16:0-CoA. ELOVL1 and ELOVL7 are expressed in many tissues, suggesting that ELOVL7 elongates C18:0-CoA to C20:0-CoA, which is then transferred to ELOVL1 (38-40).

The ACC2-generated malonyl-CoA is the C_2 donor in *de novo* fatty acid synthesis, and plays an important role as an inhibitor of the carnitine/palmitoyl shuttle system for fatty acid oxidation (41). The malonyl-CoA generated by ACC1 and ACC2 within the cell do not mix. It becomes possible to inter-relate fatty acid synthesis and oxidation and glucose oxidation through the active regulation of CPT-1 (Carnitine palmitoyltransferase-I) by malonyl-CoA (35). When cellular fuel supply is low, 5' adenosine monophosphate-activated protein kinase increases the flux of acyl-CoA into the pathway of β-oxidation while simultaneously inhibiting GPAT (glycerol-3-phosphate acyltransferase) activity and TG synthesis. Conversely SREBP-1c responds to high fuel supplies and when it is expressed at high level, the expression of genes such as FAS or GPAT increases (42).

UCP-2 (uncoupling protein-2), a mitochondrial inner membrane protein is emerging as a potential regulator of mitochondrial reactive oxygen species (ROS) production (43). It mediates a proton leak across the inner membrane and uncouples fuel oxidation from ATP synthesis (44). ROS formed during the process of fatty acid oxidation are also eliminated by other antioxidant enzymes such as superoxide dismutase and catalase, and by compounds such as GSR

(glutathione reductase), which is produced by glutathione synthetase. When cytosolic fatty acids accumulate due to impairment of oxidative capacity in mitochondria, alternative pathways in the peroxisomes (β-oxidation) and in microsomes (ω-oxidation) are activated (45). In peroxisomal β-oxidation, straight chain ACOX (acyl coenzyme A oxidase) and branched-chain ACOX are responsible for the initial oxidation of very-long-chain fatty acyl-CoAs (45).

The six-member family of FATP-1 (fatty acid transporter-1) through FATP-6 (fatty acid transporter-6) present as integral trans-membrane proteins facilitates the uptake of long chain fatty acids and very long chain fatty acids into cells (46-47). Hepatocytes contain cytoplasmic L-FABP-1 (liver fatty acid binding protein-1) and fatty acid translocase (FAT/CD36) are also known to be involved in the cellular uptake of fatty acids (48). Fatty acids in hepatocytes are transported from serum by ADRP (adipose differentiated related protein); (49).

The extracellular non-esterified fatty acids enter the liver from the plasma and are esterified by DGAT-1 (diglyceride acyltransferase-1) into TG for storage in cytosol (50). Cytosolic TG are then mobilized by the lipolytic action of aryl-acetamide deacyclase and TG hydrolase and re-esterified by DGAT-2 (51). Some TG products are recycled to the cytosol and some are channeled into a TG-rich VLDL precursor (51). The formation of this precursor is enhanced by MTP (microsomal triglyceride transfer protein) and inhibited by insulin (51). TG becomes associated with Apo-B (apolipo protein-B) in the VLDL assembly process (51-52).

1.7 Alterations in plasma fatty acid in cancer

There is limited literature on aberration of fatty acid metabolism in patients with advanced cancer including CRC. Available evidence suggests that there is abnormality of fat metabolism in cancer, including increased lipolysis (53-55) and oxidation of free fatty acids (56) and elevated circulating TG (57).

Table 1-2 summarizes previous reports on measures of fatty acid status in patients of different cancer types. The available reports suggest that majority of the cancer patients have depletion of n-6 fatty acids (58-62) and n-3 fatty acids

(58-61, 63-65) in different blood lipid fractions as plasma PL and cholesterol esters or red blood cell PL. It was also reported that plasma fatty acid composition varies among different cancer types. For example, reduced levels of n-3 fatty acids were found in pancreatic cancer, tended to be reduced in lung cancer and not altered in oesophageal cancer patients' plasma PL and cholesterol ester fractions (64). A recent study has reported plasma PL fatty acids as 35% lower in colorectal and lung cancer patients closest to death versus those surviving >8 months (66).

1.8. Essential fatty acid deficiency in cancer chemotherapy

Advanced cancer patients have alterations in lipid metabolism potentially due to nutritional status and/or chemotherapy (61, 66). Literature on the effect of cancer chemotherapy on fatty acid status in advanced cancer patients is limited. The first report by Pratt et al., (2002) revealed that plasma PL of breast cancer patients treated with 5-FU, adriamycin and cyclophosphamide had very low levels of long chain PUFA (both n-6 and n-3 fatty acids), indicating that these drugs may target PUFA and interfere with PUFA metabolism (61). One single report exists that describes cytotoxic agents interfere with PUFA metabolism (67), which may limit the endogenous syntheses of C20:5n-3 and C22:6n-3 from C18:3n-3 and C20:4n-6 from C18:2n-6. Whether such effects are specific to particular drug or how long the fatty acids remain depleted is not known, therefore it would be important to investigate the effects of chemotherapy on fat metabolism.

Usami et al., (2006) reported a significant decrease in C20:5n-3, C22:6n-3 and C20:5n-3/C20:4n-6 ratio in colonic mucosa of rats with CPT-11 (60 mg/kg) treatment, 3 days after 4 consecutive days of treatment (68). Another study on rats treated with CPT-11 (150 mg/kg) for 3 consecutive days found decreased amount of liver TG; however, 7 days later the amount of TG returned to levels comparable to those found in untreated animals (69). Therefore, it remains to be determined what happens to liver fatty acid status after additional cycles of the chemotherapy treatment, since the majority of patients are treated with three or

more cycles. Assessment of fatty acid status and lipid metabolism from the time of diagnosis and throughout the progression of different tumor types and characteristic therapies will provide valuable information regarding the timing of supplementation (62).

1.9 Effect of chemotherapy drug on liver

In advanced CRC, tumor typically spreads to the liver. Hence, surgery is required to remove the liver tumors. Clinically, it is common practice to administer chemotherapy before liver resection (70) due to theoretical advantages such as reducing tumor size, increasing the curative resection rates and converting some patients from having unresectable to resectable disease (71). Combination of surgery and modern chemotherapy has been associated with 5-year survival approaching 60% in selected patients (72-73). However, despite improved efficacy and outcomes, modern chemotherapy can exert adverse side effects on liver including steatosis (74), steatohepatitis (75) and sinusoidal injury (76), those increase the risk of perioperative morbidity and mortality (71,77-78) and may affect post operative outcome.

There are reports of severe histopathologic changes in the resected liver of patients treated with CPT-11 and 5-FU regimens (75-76) despite of response rates up to 56% (79). Clinically combined therapy of CPT-11 and 5-FU has been widely used in CRC patients due to increased efficacy; however, the combined treatment associated side effects due to hepatic toxicity have not been investigated in detail. It would be beneficial to explore the mechanism of pathogenicity of chemotherapy associated steatohepatitis to derive effective preventive and treatment strategies in CRC therapy.

1.10 Animal models for fat metabolism

The literature on the pathogenicity of chemotherapy associated steatohepatitis is limited. Several reports have been published on animal models of non alcoholic steatohepatitis to study the underlying mechanism of non alcoholic fatty liver disease (80-84). Non alcoholic fatty liver disease is frequently associated with altered fatty acid metabolism in obese patients (85-87). It is known that obesity is

a risk factor for CRC, and also in development of fatty liver following chemotherapy. Thus, the extent to which the mechanisms of chemotherapy associated steatohepatitis are similar to molecular causes of steatosis in other populations of non-alcoholic fatty liver disease is not known.

Fischer 344 rats bearing Ward colon tumor and treated with CPT-11 plus 5-FU (24) has been used to study chemotherapy associated side effects, such as diarrhea and intestinal toxicity (88), nutritional modulations (89-90) and alteration in fat metabolism (69). The dosage of those drugs have been established as having anti-tumor effect in the animal model with colon tumor, cause intestinal injury and diarrhea, which are well known side effects of this time of therapy in clinical patients. The drug schedule is same as provided to CRC patient clinically and has the similar toxic effects as seen in humans. In addition to this, the animal tissues are easily accessible for investigating mechanism of chemotherapy associated steatohepatitis and the genetic variation in liver after CPT-11 plus 5-FU treatment for CRC. Use of this model has enabled control in dietary intake and to follow relevant clinical or mechanistic measures in target organs as liver, at defined time points. Investigation of liver pathogenicity from this animal model will help to determine pathways altered in liver fat metabolism due to CPT-11 plus 5-FU therapy, which may direct clinical strategies for intervention. Clinically, it is complex to study on the patient's liver samples directly, due to lack of easy access to biopsy samples. Thus, an animal model is an important tool to understand the mechanism of chemotherapy associated steatohepatitis.

1.11 Summary

Adult populations of western countries are at increased risk of developing CRC, despite the development of various diagnostic tools and treatment therapies. Chemotherapy is one of the most commonly preferred regimens due to its efficacy on killing cancer cells by arresting cell cycle. Currently, treatment of CPT-11 plus 5-FU had been commonly used in patients with CRC. However, the previous reports have revealed that the drugs used to treat CRC are involved in inducing chemotherapy associated steatohepatitis, which reduces the survival rate in

clinical patients (71). To date, there is paucity of information on understanding mechanism of chemotherapy associated steatohepatitis in CRC.

The liver is the major site for synthesis, packaging and distribution of fatty acids and energy regulator and is also the major organ to metabolize the toxic drugs. Circulating drugs used for CRC treatment may interfere with fatty acid synthesis, packaging and distribution in liver by generating ROS or oxidative stress in mitochondria (91). Impaired balance between oxidation and synthesis may lead to increased TG accumulation in liver. Measures of particular gene expression related to fat metabolism in liver would help in determining the ability of liver to metabolize fat after chemotherapy that would identify underlying causes of chemotherapy associated steatohepatitis. Pathways identified from the animal model would help in identifying the impediments in pathways of fat metabolism in CRC patients with CPT-11 plus 5-FU treatment.

1.12 Tables and figures

Table 1-1 Hepatic genes involved in lipid metabolism with functional roles

Genes	Roles in liver
ACC 1 (Acetyl coenzyme A Carboxylase 1)	Catalyzes the carboxylation of acetyl-CoA to malonyl-CoA (the rate-limiting step in fatty acid synthesis).
ACAT -1 (acetyl-CoA acetyltransferas- 1)	Role in breaking down proteins and fats from the diet. Also involved in processing ketones during fasting.
ACLY (ATP citrate lyase)	Synthesis of cytosolic acetyl-CoA. It catalyzes the formation of acetyl-CoA and oxaloacetate from citrate and CoA with a concomitant hydrolysis of ATP to ADP and phosphate.
ACOX-1 (acyl-coenzyme A oxidase 1)	First enzyme of the fatty acid β-oxidation pathway and catalyzes the desaturation of acyl-CoAs to 2-trans-enoyl-CoAs.
ADRP (adipose differentiated related protein)	Associated with the surface of lipid droplets and function in the intracellular mobilization and storage of neutral lipids such as TG.
APOA1 (Apolipoprotein A-I)	Promotes cholesterol efflux from tissues to the liver for excretion. It is a cofactor for lecithin cholesterol acyl transferase which is responsible for the formation of most plasma cholesterol esters.
APOB (Apolipoprotein B)	It is an essential component for the assembly and secretion of VLDL.
CD 36 (Cluster of Differentiation 36)	Mediates uptake of free fatty acid transport in plasma membrane of hepatocytes. It binds to long chain fatty acids and may function in the transport and/or as a regulator of fatty acid transport.
CD68 (Cluster of differentiation 68)	A glycoprotein which binds to low density lipoprotein and is a macrophage marker.
CHKA (Choline kinase α)	The major pathway for the biosynthesis of phosphatidylcholine occurs via the CDP-choline pathway. The protein encoded by this gene is the initial enzyme in the sequence and may play a regulatory role.
CHKB (Choline kinase β)	Choline kinase and ethanolamine kinase catalyze the phosphorylationof choline/ethanolamine to phosphocholine/phosphoethanolamine. This is the first enzyme in the biosynthesis of Phosphatidylcholine/ phosphoethanolamine in all animal cells.
DGAT1 (diacylglycerol O-acyltransferase homolog 1)	Catalyzes the terminal and only committed step in TG synthesis by using a diglycerides and fatty acyl CoA as substrates. May be involved in VLDL assembly.
FABP (fatty acid binding protein 2)	Uptake, intracellular metabolism and/or transport of long-chain fatty acids.

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Genes	Roles in liver
FABP1 (fatty acid binding protein 1)	Binds long-chain fatty acids and other hydrophobic ligands. Roles include fatty acid uptake, transport, and metabolism.
FAD2 (fatty acid desaturase 2)	The enzyme introduces double bonds into fatty acyl chains. Major enzyme responsible for the synthesis of C18:2n-6
FAS (fatty acid synthase)	Catalyzes synthesis of C16:0 from acetyl-CoA and malonyl-CoA, in the presence of NADPH, into long chain fatty acids
FATP4 (fatty acid transport protein 4)	Enhances the uptake of long chain fatty acids and very long chain fatty acids into cells
GPAT (Glycerol-3-phosphate acyltransferase 1)	Catalyzes the initial and committing step in glycerolipid biosynthesis, is predicted to play a pivotal role in the regulation of cellular TG and PL levels.
GPX1 (Glutathione peroxidase 1)	Functions in the detoxification of hydrogen peroxide
GSR (Glutathione reductase)	Reduces glutathione disulfide to the sulfhydryl form glutathione, which is an important cellular antioxidant.
HMGCR (hydroxymethylglutaryl-CoA reductase)	The rate-limiting enzyme for cholesterol synthesis.
HSPA5 (heat shock protein 5)	Involved in the assembly of secreted and membrane-bound proteins
INSIG 1 (insulin induced gene 1)	Important role in the sterol regulatory element binding protein-mediated regulation of cholesterol biosynthesis: by binding to the sterol-sensing domain of sterol regulatory element binding protein cleavage activating protein.
LCAD (long-chain-acyl-CoA dehydrogenase)	Role in metabolizing long chain fatty acids and the long-chain 3-hydroxy acyl-coenzyme A dehydrogenase activity is specific for compounds of C12-C16 chain length.
LDLR (Low-Density Lipoprotein Receptor)	Binds low density lipoproteins (primary carriers of cholesterol in the blood). The number of LDLR on the surface of liver cells determines how quickly cholesterol (in the form of low density lipoprotein) is removed from the bloodstream.
Lipin 1	It acts as a transcriptional co-activator in liver, and stimulates gene expression of mitochondrial enzymes.
MCAD (medium chain acyl-Coenzyme a dehydrogenase)	It is essential for fatty acid oxidation, which is the multistep process that breaks down (metabolizes) fats and converts them to energy.
ME1 (Maleic enzyme 1)	Is a part of the tricarboxylate shuttle that provides NADPH and acetyl-CoA required in fatty acid biosynthesis
MGAT2 (Alpha-1,6-mannosyl-glycoprotein 2-beta-N-acetylglucosaminyltransferase)	Catalyzes the synthesis of diglyceride, a precursor of TG.

To be continued on next page………

Genes	Roles in liver
MTP (Microsomal triglyceride transfer protein)	Helps to produce beta-lipoproteins that consist of proteins combned with cholesterol, PL and TG. Beta-lipoproteins contain the protein apolipoprotein (Apo), which is critical for the creation of chylomicrons. Other beta-lipoproteins containing ApoB, particularly low density lipoprotein and VLDL, are created by MTP in the liver. These lipoproteins transport fats, cholesterol, and fat-soluble vitamins throughout the body in the bloodstream.
PCK1 (Phosphoenolpyruvate carboxykinase 1)	Regulation of gluconeogenesis. The enzyme catalyzes the formation of phosphoenolpyruvate from oxaloacetate, with the release of carbon dioxide and Guanosine diphosphate.
PPAR-α (Peroxisome proliferator-activated receptor alpha)	Regulation of energy homeostasis in a fatty acid β-oxidation.
SCD1 (The stearoyl-CoA desaturase 1)	It is the rate limiting enzyme necessary for biosynthesis of monounsaturated fatty acids (MUFA)
SLC27A5 solute carrier family 27 (FA transporter) , member 5	It is capable of activating very long chain fatty acids containing 24- and 26-carbons. Its primary role is in fatty acid elongation or complex lipid synthesis rather than in degradation.
SREBF 1 (sterol regulatory element binding transcription factor 1)	Regulates transcription of the low density lipoprotein receptor gene as well as the fatty acid and to a lesser degree the cholesterol synthesis pathway.
SREBF 2 (sterol regulatory element binding transcription factor	Involved in cholesterol biosynthesis and uptake
TNF-α (tumor necrosis factor-α)	Mediator of hepato-toxicity and also contributes to the restoration of functional liver mass by driving hepatocyte proliferation and liver regeneration
UCP2 (Uncoupling protein 2)	Control of mitochondria-derived ROS.
VLCAD (Very long chain acyl-Coenzyme A dehydrogenase)	Break down (metabolize) very long chain fatty acids.

Table 1-2 Summary of studies reporting essential fatty acid deficiency in cancer

Study	Study population	Tissue lipid	Fatty acid status
Mosconi et al., 1989	Untreated patients (n=8; men and n=4; women; mean age=55yrs) affected by tumors of the gastrointestinal tract (n=7, esophagus; n=3, stomach; n=2, pancreas) and healthy controls (n=8; age and sex matched)	Plasma PL, cholesterol esters & red blood cell PL	Cancer patients had: ➢ Lower level of C18:2n-6 in plasma PL and cholesterol esters and in red blood cell PL. ➢ Levels of C16:0 and C18:1n-9 were higher in plasma PL and cholesterol esters and in red blood cell PL. ➢ Level of C20:4n-6 was reduced in plasma cholesterol esters but not in PL.
McClinton et al., 1991	Bladder cancer patients (n=98) those with active disease (n= 55) and with no recurrence (n=43) vs control (n=477)	Plasma PL	Cancer patients had: ➢ Lower levels of C18:2n-6, C20:4n-6 and C22:4n-6. ➢ Lower levels of C20:5n-3, C22:5n-3 and C22:6n-3
Skorepa et al., 1991	Plasma levels of EFA in patients with carcinoma	Plasma lipid	Cancer patients had: ➢ lower levels of C20:4n6, C20:5n3, C22:6n3
Baro et al., 1998	Untreated CRC patients (n=17) vs age matched controls (n=12) with no malignant disease and with same geographical area. Tumours were classified as Dukes' A (n=2), Dukes' B (n=4), Dukes' C (n=9) and Dukes' D (n=2).	Plasma PL, TG & cholesterol esters and red blood cell PL	CRC patients had: ➢ Higher concentration of C18:1n-9 and lower C18:2n-6 in total plasma fatty acids. ➢ Proportionate amount of SFA were higher and C18:2n-6 and total n-6 fatty acids were lower in plasma PL. ➢ Proportionate amount of C18:2n-6, C18:3n-3, C20:3n-6 and n-6 fatty acids were lower and SFA and MUFA were higher in plasma TG. ➢ C16:0, C18:1n-9, SFA and MUFA were higher and C20:3n-6 and the n-6 fatty acids were lower in plasma cholesterol esters. ➢ Higher proportionate amount of C18:0 in red blood cell PL.
Zuidgeest-van et al,et al., 2002	Newly diagnosed, untreated cancer patients (n=71) of three tumour types: oesophageal or cardia cancer (n=35), non-small cell lung cancer (n=22) and pancreatic cancer (n=15) vs healthy subjects (n=45)	Plasma PL and cholesterol esters	➢ Pancreatic cancer patients had reduced level of n-3 fatty acids both in PL and in cholesterol esters ➢ n-3 fatty acids in lung cancer tended to be reduced but was not statistically significant. ➢ In oesophageal cancer, n-3 fatty acids concentrations were comparable to those in healthy subjects.

To be continued on next page………..

Study	Study population	Tissue lipid	Fatty acid status
Pratt et al., 2002	Advanced cancer patients (n=15; burn injury, n=12 and women with high risk of stage II and III breast cancer undergoing chemotherapy treatment, n=3) vs control (n=6)	Plasma PL and neutrophil PL	Cancer patients had: ➢ Total plasma PL and most individual fatty acids including EFA (18:2n-6 and 18:3n-3) were lower and the ratio of 20:3n-9 to 20:4n-6, a measure of EFA deficiency was elevated. ➢ Chemotherapy induction had very low levels of long chain PUFA including 18:2n-6 and 20:3n-6, where C22:6n-3 was undetectable and C20:5n-3 levels fell to about 7% of control values in plasma PL. ➢ elevated 20:4n-6 content in neutrophil PL.
Mikirova et al., 2004	Cancer patients (n=255; breast, prostate, liver, pancreas, colon, and lung) vs non-cancer patients (n=2800) vs control healthy volunteers (n=24).	Red blood cell fatty acid	Cancer patients had: ➢ a lower level of C18:0 and an increased level of C18:1 than non-cancer patients and control
Castro et al., 2006	Patients with advanced (Stages III–IV) non-small cell lung cancer (n=50), vs health controls (n=50)	Red blood cell and plasma platelet total lipid	Cancer patients had: ➢ Higher C18:0 and C18:1 and lower C18:2n-6 and C20:4n-6 in red blood cell lipids. ➢ Lower C20:4n-6 and a parallel increase in C16:0 in the platelet lipid.
Murphy et al., 2010	Seventy two colorectal and lung cancer patients divided into two groups as >238 days before death (n=36) and <238 days before death(n=36)	Plasma PL	Cancer patients had ➢ 35% lower fatty acid in plasma PL on those closest to death versus those surviving >8 months.
Murphy et al., 2010	Non small cell lung cancer patients (n=41)	Plasma PL	Cancer patients with sarcopenia had ➢ depleted levels of n-3 FAs compared to non-sarcopenia

Table 1-3 Hepatic Triglyceride fatty acid composition at Day 1 and Day 7 after first cycle of chemotherapy vs tumor-bearing rats

Fatty acid (%)	Tumor (n=6)	D1 (n=3)	D7 (n=5)	P-value
C16:0	32.5 ± 1.1	30.8 ± 0.8	31.0 ± 2.2	0.214
C18:0	12.2 ± 4.6	6.7 ± 0.8	10.9 ± 1.2	0.073
C18:1n-9	27.3 ± 3.5	26.7 ± 1.4	28.8 ± 1.7	0.478
C18:2n-6	18.6 ± 2.6	20.8 ± 2.3	18.3 ± 1.8	0.304
C18:3n-6	0.5 ± 0.1	0.6 ± 0.1	0.6 ± 0.2	0.461
C18:3n-3	0.2 ± 0.0	0.3 ± 0.1	0.3 ± 0.0*	0.004
C20:4n-6	1.9 ± 0.9	4.1 ± 0.7*	2.7 ± 1.0	0.019
C20:5n-3	0.2 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.594
C22:6n-3	1.1 ± 0.9	3.1 ± 0.8*	1.2 ± 0.5#	0.004
∑SFA	45.1 ± 4.7	38.2 ± 1.2*	42.3 ± 1.6	0.032
∑MUFA	31.0 ± 4.2	30.7 ± 1.8	32.7 ± 2.1	0.554
∑PUFA	23.8 ± 4.5	31.1 ± 2.7	25.0 ± 3.5	0.054
∑n-6	22.3 ± 3.6	27.4 ± 2.0	23.2 ± 2.9	0.097
∑n-3	1.5 ± 1.0	3.7 ± 0.9*	1.8 ± 0.6#	0.008
Total (µg/g)	2555.1 ± 859.7	1402.8 ± 103.3	2172.0 ± 447.3	0.069

*Significantly different from Tumor ($p<0.05$)
#significantly different from D1 ($p<0.05$)

Amount of fatty acid in liver triglyceride of tumor-bearing rats and those treated with chemotherapy (CPT-11; 150 mg/kg for 3 consecutive days). Data are expressed as means ± SD. Significant differences ($p<0.05$) were determined using a one-way ANOVA. Abbreviations: CPT-11, irinotecan; TG, Triglyceride; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids. Groups: D1 and D7 indicate 1 and 7 days after the chemotherapy treatment.

(**Source**: modified from Pawlowicz, 2008)

Table 1-4 Hepatic Phospholipid fatty acid composition at Day 1 and Day 7 after first cycle of chemotherapy vs tumor-bearing rats

Fatty acid (%)	Tumor (n=6)	D1 (n=3)	D7 (n=5)	P-value
C16:0	12.9 ± 1.8	15.7 ± 1.3	11.7 ± 0.1[#]	0.010
C18:0	41.4 ± 9.4	34.2 ± 1.4	37.7 ± 4.6	0.353
C18:1n-9	3.6 ± 1.3	4.0 ± 0.9	3.7 ± 0.5	0.863
C18:2n-6	9.9 ± 2.2	10.7 ± 2.0	9.0 ± 0.9	0.450
C18:3n-3	0.2 ± 0.1	0.2 ± 0.0	0.3 ± 0.0	0.512
C20:4n-6	19.9 ± 3.3	21.2 ± 1.8	24.1 ± 3.0	0.103
C20:5n-3	0.6 ± 0.2	0.5 ± 0.1	0.5 ± 0.2	0.546
C22:6n-3	7.6 ± 1.2	9.8 ± 2.0	9.0 ± 1.5	0.130
∑SFA	55.2 ± 7.7	50.6 ± 1.8	50.1 ± 4.9	0.357
∑MUFA	4.9 ± 1.6	5.6 ± 0.7	5.0 ± 0.5	0.716
∑PUFA	39.9 ± 6.5	43.9 ± 2.1	44.9 ± 4.8	0.305
∑n-6	31.4 ± 5.7	33.4 ± 0.4	35.1 ± 3.6	0.421
∑n-3	8.5 ± 1.1	10.5 ± 2.1	9.8 ± 1.5	0.159
Total (µg/g)	13406.9 ± 1508.4	13602.3 ± 619.1	15036.6 ± 1962.4	0.248

[#]significantly different from D1 (p<0.05)

Amount of fatty acid in liver phospholipid of tumor-bearing rats and those treated with chemotherapy (CPT-11; 150 mg/kg for 3 consecutive days). Data are expressed as means ± SD. Significant differences (p<0.05) were determined using a one-way ANOVA. Abbreviations: CPT-11, irinotecan; TG, Triglyceride; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids. Groups: D1 and D7 indicate 1 and 7 days after the chemotherapy treatment.

(**Table Source**: modified from Pawlowicz, 2008)

Figure 1-1 Desaturation and chain elongation

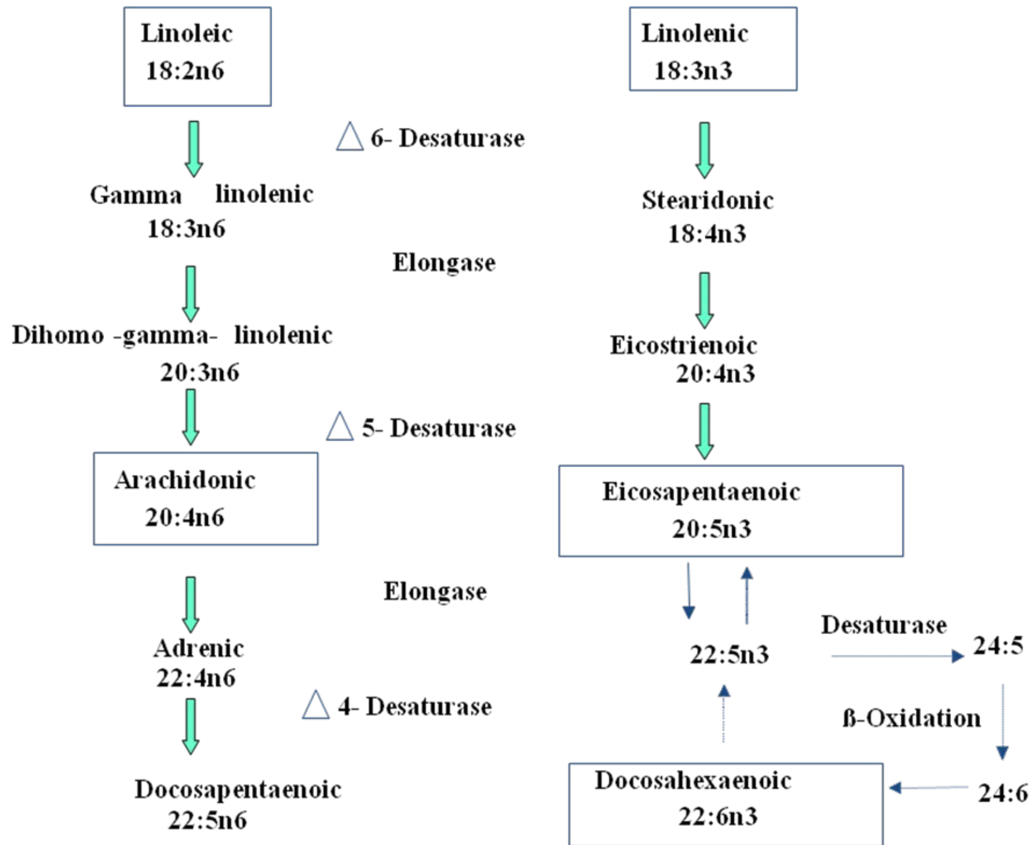

Figure 1-2 Hepatic genes involved in pathways of fat metabolism

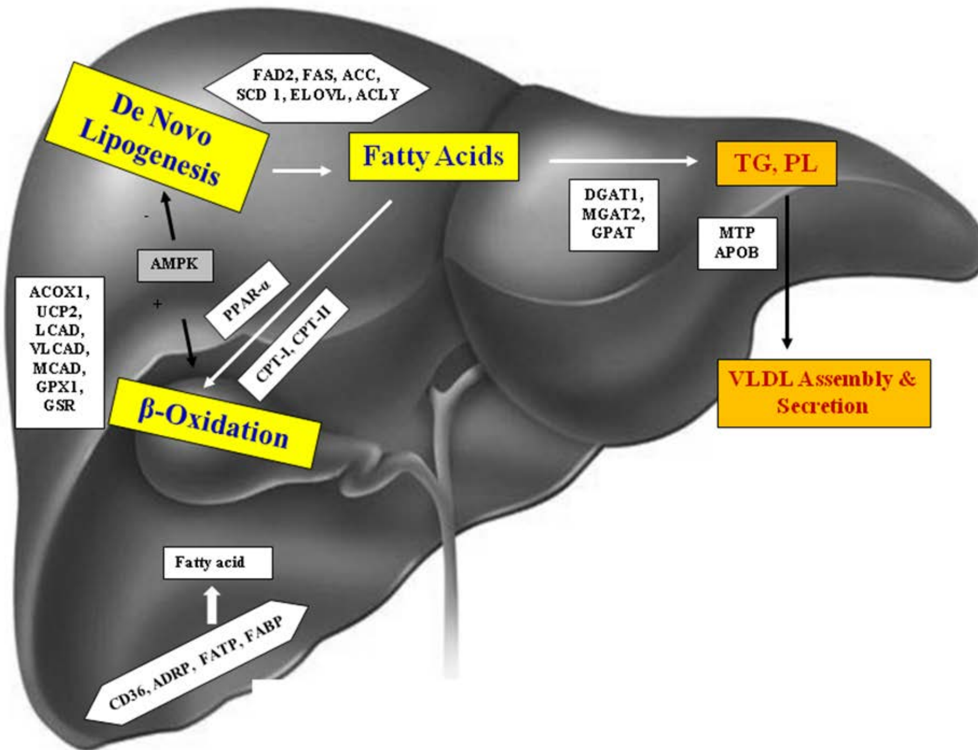

Figure represents brief schematic overview of fatty acid metabolism in liver. Abbreviations: ACOX1, acetyl coenzyme A oxidase 1; UCP2, uncoupling protein 2; LCAD, long chain acyl coenzyme A dehydrogenase; VLCAD, very long chain acyl coenzyme A dehydrogenase; MCAD, medium chain acyl coenzyme A dehydrogenase; GPX1, glutathione peroxidase 1; GSR, glutathione reductase; ACLY, ATP citrate lyase; FADS2; fatty acid desaturase 2; FAS, fatty acid synthase; ACC, acetyl coenzyme A carboxylase; SCD 1, stearoyl coenzyme A desaturase 1; ELOVL, elongation of very long chain fatty acid; CD 36, cluster of differentiation 36; ADRP, adipose differentiated related protein, FATP, fatty acid transport protein; FABP, fatty acid binding protein; DGAT1, diacylglycerol acyltransferase; MGAT2, alpha 1,6-mannosylglycoprotein 2-beta-N-acetylglucosaminyltransferase; GPAT; glycerol-3 phosphate acyl transferase; APOB, apolipoprotein B; MTP, microsomal triglyceride transfer protein; PPAR-α, peroxisome proliferator activated receptor alpha; CPT-1; carnitine palmitoyl transferase; AMPK, 5' adenosine monophosphate-activated protein kinase; TG, triglyceride; PL, phospholipid; VLDL, very low density lipoprotein

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Chapter 2: Research plan

2.1 Rationale

In advanced stages of CRC, the tumor typically spreads to the liver causing colorectal liver metastases requiring surgery. Chemotherapy is commonly administered prior to resection to shrink the tumor. However, there are reports indicating chemotherapy associated liver injury as steatosis (1), steatohepatitis (2) and sinusoidal injury (3). CPT-11 and 5-FU have been commonly used in CRC treatment. However, it was reported that preoperative chemotherapy with CPT-11 caused sinusoidal dilation (4.3%), steatosis (10.6%) and steatohepatitis (20.2%) and 5-FU caused steatosis (16.6%) and steatohepatitis (4.8%); (4), which provide evidence for alterations in fat metabolism that have a negative impact on clinical outcome. Chemotherapy associated steatohepatitis is problematic because liver function can be severely compromised when tumor tissue is removed and chance of post-operative mortality after hepatic surgery is increased (4). Drug therapies may potentially contribute to dysregulation of fatty acid synthesis, oxidation, packaging and/or distribution to the periphery. Genes involved in fat metabolism which are naturally found in the liver may be affected by the toxicity of the drug or due to additional strain on the liver to efficiently metabolize the drugs.

Although literature on alteration in hepatic fat metabolism with chemotherapy treatment is not well defined, previous report has found reduced amount of plasma long chain PUFA (n-6 and n-3 fatty acids) in cancer patient undergoing different chemotherapy regimens (5), indicating that the drugs may interfere PUFA metabolism (5). Another study has reported a significant decrease in C20:5n-3, C22:6n-3 and C20:5n-3/C20:4n-6 ratio in colonic mucosa of rats treated with 60 mg/kg of CPT-11 (6). A study from our lab has previously determined differences in the type and amount of fatty acids in plasma and liver of rats undergoing CPT-11 treatment (150 mg/kg) for three consecutive days for CRC and reported that the chemotherapy may have short term deleterious effects on hepatic lipids, but by 7 days they recover (7). Alterations of fatty acid amount and composition in liver may lead to deficits observed in the periphery. A recent

report from our lab group provided evidence that a depletion of plasma PL likely indicates a deficit of EFA in the periphery which may contribute to loss of adipose and lean tissue (8). It remains to be determined the time coursed changes in liver fatty acids and the effect of single vs multiple cycles.

The researchers from our study groups have developed a rodent model of CRC with combined treatment of CPT-11 plus 5-FU, which parallels clinical treatment, as the dose level has established as having anti-tumor effect, cause intestinal injury and diarrhea and the drug schedule is similar with toxic effects in clinical patients. Clinically, it is complex to study patient's liver samples directly, due to lack of access to biopsy samples, which are small. Use of this animal model has enabled control in dietary intake and to follow relevant clinical or mechanistic measures in the liver, at defined time points. The animal tissues are easily accessible for investigation mechanism of altered fat metabolism and the genetic variation after chemotherapy treatment. Study of liver pathogenicity from the animal model will help to determine pathways altered in liver fat metabolism due to CPT-11 plus 5-FU therapy, which will direct clinical strategies for intervention.

2.2 Research objectives and hypothesis

The overall goal of this study is to determine differences in hepatic fatty acid status in rats given one and two cycles of CPT-11 plus 5-FU compared to control tumor-bearing animals not treated with chemotherapy. It is expected that second cycle of CPT-11 plus 5-FU treatment will have a more deleterious effect on fatty acid status than the first cycle.

Objective: The current study has overall two objectives. They are as follows:

1. determine changes in fatty acid amount and composition from time of CPT-11 plus 5-FU delivery, during the 7 days period and then 2 days after the second cycle.
2. determine relative expression of hepatic genes regulating pathways of fat metabolism with two cycles of CPT-11 plus 5-FU.

Hypothesis: It is hypothesized that:

1) Compared to tumor-bearing animals, the liver of rats treated with two cycles of CPT-11 plus 5-FU will have increased TG and decreased EFA (n-6 and n-3 fatty acids).
2) Compared to tumor-bearing animals, the liver of rats treated with two cycles of CPT-11 plus 5-FU will have up-regulation of genes involved in fatty acid packaging and synthesis and down-regulation of genes involved in fatty acid oxidation and export

This work represents a first step in exploring pathways of lipid metabolism that may be altered following chemotherapy. The aim is to define interventions that circumvent development of fatty liver in patients treated for CRC.

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Chapter 3: Alterations in hepatic fatty acids following one cycle of irinotecan plus 5-fluorouracil treatment

3.1. Introduction

Chemotherapy is one of the effective treatment options for cancer; however, toxicity related side effects are common. Changes in fatty acid status during cancer chemotherapy in advanced cancer patients have not been well defined. Drugs treatment may be a contributing factor for alterations in fat metabolism characterized largely by depletion of long chain PUFA (n-6 and n-3 fatty acids) in patients with advanced cancer (1, 2). Cytotoxic agents have been reported to interfere with PUFA metabolism (3), which may limit the endogenous syntheses of C20:5n-3 and C22:6n-3 from C18:3n-3 and C20:4n-6 from C18:2n-6. However, there is a paucity of information on whether effects are specific to particular drugs or how long the depletion of PUFA remains following cancer treatment. Detailed indices of fatty acid status and the relationship to toxic side effects of therapies are also of interest to define nutritional strategies to reduce adverse effects of cancer chemotherapy.

5-FU has been used to treat cancers of colorectal, breast and aero-digestive tract (4) and is associated with multiple toxicities such as nausea, vomiting, cytopenias and cardio-toxicities (4-5). CPT-11 is used to treat colorectal, esophageal, gastric, non small cell and small cell lung cancer (6) and is associated with gut toxicity and diarrhea (7). Recently, a combination of CPT-11 plus 5-FU has been applied to CRC treatment due to increased efficacy over other modalities. Several *in vitro* and *in vivo* studies have been published on efficacy and toxicity effects with CPT-11 plus 5-FU treatment against tumor cells (8-11). However, the toxic effect of the combined drugs on lipid status has not been explored.

CPT-11 (60 mg/kg) treatment for 4 consecutive days has been reported to interfere with intestinal fatty acid composition by reducing C20:5n-3, C22:6n-3 and C20:5n-3/C20:4n-6 ratio in colonic mucosa of rats (12). Another report by Pawlowicz (2008) on rats treated with CPT-11 (150 mg/kg) for 3 consecutive

days found lower liver TG immediately following treatment; however, 7 days later TG returned to levels comparable to those found in untreated rats (13). While these two reports suggest important alterations in fatty acid status with CPT-11 treatment, it remains to be determined the effect of combined treatment of CPT-11 plus 5-FU in the period of time after delivery of the drugs.

This study was designed to analyze hepatic fatty acid composition for 7 days following one cycle of CPT-11 plus 5-FU. It was hypothesized that rats treated with one cycle of CPT-11 plus 5-FU would have deficits in EFA including long chain PUFA and elevated liver TG and PL. Fatty acid composition in PL may be used to detect aberrations in EFA metabolism because majority of EFA are found in PL.

3.2 Materials and supplies

Animal use was reviewed and approved by University of Alberta Animal Care Committee and conducted in accordance with the Guidelines of the Canadian Council on Animal Care**.** Female Fischer 344 rats (body weight; 150-180 g and age; 11-12 weeks) were obtained from Charles River (QC, Canada). The rats were housed 2 per cage in a room with controlled temperature (22°C) and 12h light/dark cycles. Water and food were provided *ad libitum* under aseptic conditions that included positive-air-pressured room and filter tops cages. Handling of rats was carried out under a laminar flow hood. The rats were adapted for one week prior to the start of the experiment. After adaptation, rats were fed a semi-purified diet based on AIN-76 basal diet (**Table 3-1**), with a modified fat component similar to a North American dietary pattern (40% of energy, polyunsaturated: saturated fat ratio of 0.35).

The Ward colorectal carcinoma was provided by Dr. Y Rustum, Roswell Park Institute (9). After two weeks on diet, all rats (n=24) underwent tumour implantation. The tumour pieces (0.05g) were transplanted subcutaneously into the left flank of the rats via trocar under slight isoflurane anesthesia. Calipers were used to measure the tumors in three dimensions as length (L), width (W),

and height (H). Tumor volume was estimated as (cm^3) = 0.5 x L x W x H. When the tumor size reached $2cm^3$, chemotherapy treatment was initiated.

The rats were separated into individual housing one week before the treatment. Atropine was provided prior to administration of the treatment. CPT-11 (50 mg/kg) was administered on “D/-1” followed by 5-FU (50 mg/kg) on second day marked as “D/0”, the combination of which represents one cycle of chemotherapy treatment. The rats marked as D1 (1 day post chemotherapy group; n=6), D5 (5 days post chemotherapy group; n=6) and D7 (7 day post chemotherapy group; n=6) were killed 1, 5 and 7 days after 5-FU was administered. The remaining tumor-bearing rats (n=6) not treated with CPT-11 plus 5-FU served as “Tumor control” group for comparison. Rats were killed with carbon-dioxide asphyxiation, the liver was removed, weighed, cut into equal pieces, immediately snap frozen in liquid nitrogen and stored at -80°C until analysis.

3.3 Methods

3.3.1 Fatty acid analysis

3.3.1.1 Lipid extraction

Rat liver (0.05g) was homogenized with calcium chloride solution (0.025%) using a sonicator to obtain a uniform mixture. A modified Folch method was used for extraction of total lipids from liver (14). Chloroform-methanol (C:M; 2:1) solution was added to the mixture and vortexed and stored at 4 ^{o}C overnight to separate layers. The bottom layer containing the lipid was dried under Nitrogen gas.

3.3.1.2 Thin layer chromatography

Thin layer chromatography plates (TLC, Silica Gel G, 20x20cm, 250 microns, Analtech Inc, Newark, DE) were heated for one hour at 110 ^{0}C in an oven prior to spotting the samples in duplicate columns. Solvent tanks were lined with filter paper (Fisher, Whitby, ON) and a solvent mixture of petroleum ether + ethyl ether + glacial acetic acid (80:20:1) were saturated in the tanks for one hour before adding the plates. When the sample reached the top of the plate (approx, 30 min), the TLC plates were dried and 0.1% 8-anilino-1-naphthalene-sulphonic acid was

sprayed to visualize the PL and TG bands. Ultraviolet light was used to identify the PL and TG bands which were subsequently scraped and added to clean methylation tubes. C15:0 (100 μg/mL) and C17:0 (100 μg/mL) standards were added to the TG and PL bands, respectively.

3.3.1.3 Methylation

PL samples were methylated with 2 mL hexane and 1mL boron trifluoride. The samples were boiled on a dry bath for an hour at 110 ^{0}C then left to cool at room temperature. The TG samples were methylated with 1 mL potassium hydroxide in methanol and were boiled at 110 ^{0}C on the dry bath. After one hour, 2 mL of hexane plus 1 mL of boron trifluoride solutions were added and boiling resumed for an additional hour at 110 ^{0}C. Once the PL and TG samples were cooled, 1 mL of distilled water was added to each tube. The tubes were vortexed and allowed to separate in the fridge. Once layers were separated, the top layer was removed and added to a gas chromatography vial. The vial was dried under dry nitrogen gas and 200 μL of pure hexane was added and mixed carefully. The hexane was pipetted into a glass insert which was returned to the GC vial and the sample was stored -20 ^{0}C until analysis.

3.3.1.4 Gas liquid chromatography

The automated gas liquid chromatography Varian model 3400x equipped with a Star Chromatography Workstation data system and a Varian 8200 auto-sampler (Varian Instrument Company, Gorgetown Ontario) separated fatty acid methyl esters. The system used a bonded phase silica capillary column, BP20:30mx0.22ODSGE product. Helium was used as the carrier gas at a flow rate of 30 ml/min using a split injector (28:1). The gas liquid chromatography oven temperature of 90 ^{0}C was increased to 170 ^{0}C at 20 ^{0}C/min and held for 23 minutes followed by a second stage temperature increase to 230 ^{0}C at a 4/minute for a total analysis time of 54 minutes. These conditions separated SFA, MUFA and PUFA between 6 and 24 carbon chain lengths, which were identified using a standard of known composition (Supelco, Bellefonte, Penn, division of Sigma

Chemical Company, St. Louis. MO). Fatty acids were expressed as a proportion and absolute amount of total TG and/or PL respectively.

3.3.2 Statistical Analysis

Data are reported as means ± SEM. To identify significant differences between multiple samples, results were evaluated with a one-way analysis of variance (ANOVA). When significant overall difference was observed, a post-Hoc analysis was performed using the Bonferroni model. Statistical significance was determined at a p value was <0.05. All statistical analyses were conducted using SPSS 18.0 (Chicago, Illinois) for Windows.

3.4 Results

3.4.1 Triglyceride

Average liver weight of the rats was about 5.6 ± 0.3g. Total amount of fatty acid within the liver TG were not significantly altered with chemotherapy treatment. Total SFA were comparable between groups; however, C18:0 was lower at D1 than D5 and D7. Total MUFA were transiently lower at D1 due largely to lower C18:1n-9 (**Table 3-2**), as values were restored to the values observed in the tumor group at D5 and D7. Total n-6 fatty acids were lower at D5 and D7 compared to amounts observed in the D1 due largely to lower C18:2n-6. C20:4n-6 transiently higher at D1 and then was lower at D7 compared to D1. Total n-3 fatty acids were transiently lower at D5 compared to D1 and tumor groups, which were restored at D7. C18:3n-3 was lower at D5 than at D7. C20:5n-3 was transiently higher at D1 but returned to control level tumor group at D5 and D7. The amounts of C22:6n-3 were not detected at D5 and D7, but were similar between D1 and the tumor group.

3.4.2 Phospholipid

In general, chemotherapy did not alter hepatic PL containing most fatty acids (**Table 3-3**). However, the total amounts of PL were increased in all three chemotherapy treatment groups compared to the tumor groups. Total SFA were highest at D1 but were normalized at D5 and D7. Total MUFA were higher at D7 than D1.

3.5 Discussion

There is a paucity of information on the effect of chemotherapy on hepatic fat composition and content. Literature available to date suggests alterations such as fatty liver with certain drug therapies used to treat CRC (15). Only unpublished data from our lab detailed the amounts and types of liver fatty acid composition following treatment with 150 mg/kg of CPT-11 delivered over 3 days in the same tumor model used here (13). Unlike this study, we investigated a single dose of 50 mg/kg of CPT-11 plus 5-FU given on consecutive days in the one week period following the treatment.

There was no alteration in total TG after chemotherapy. However, the food intake was significantly reduced at 1day after the treatment compared to tumor group. Previously, Pawlowicz (2008) reported a 54% decrease in total TG with CPT-11, 1 day following 3 consecutive days of treatment (13). The author also found reduced amount of food intake in rats treated with CPT-11, which might have lowered liver TG (13). Cole et al (2010) reported a 72% increase in liver TG with tamoxifen, following 5 consecutive days of treatment (16). These two studies suggest rapid, but perhaps transient alterations that may be attributed, in part, to reductions in food intake. A pair-feeding study would clarify the role of the chemotherapy versus food intake on TG amount and composition. Usami et al., (2006) reported a significant decrease in total plasma TG in rats treated with 60 mg/kg of CPT-11, 3 days following 4 consecutive days of treatment (12); however, hepatic lipids were not assessed. Differences in the results of these studies could be due to the type of drug and the dose used as well as the length over time that the drug is administered and the point in time that the assessments are made. Collectively, these data suggests TG synthesis and/ or export are altered following delivery of therapeutic drugs.

Total SFA within the liver TG were virtually the same between treated and untreated rats, although they did increase by almost 12% at the 7 day time point. This is similar to Pawlowicz (2008), who reported no difference in fatty acids by 7 days after the treatment (13). We observed C18:0 was decreased to almost 28%

of tumor group level at 1 day after treatment and were restored at 5 and 7 days post treatment. The possible reason for this effect could be due to alterations in enzymes that govern fatty acid synthesis, such as elongases and desaturases or changes in food intake. However, we did not assess enzymatic activity in this study. The pair feeding study is required to determine if reduced food intake is accountable for the alteration in fatty acid levels.

Total MUFA within TG were lowest at 1 day after treatment, contributed largely by C18:1n-9. Pawlowicz (2008) also reported MUFA tend to be lower 1 day after the treatment but were restored by 7 days after the treatment (13). Collectively, the lower 18:0 and lower 18:1n-9 may suggest that the drugs interfered with *de novo* fatty acid synthesis very rapidly but that these pathways recover. It would be interest to assess the enzymatic activities, which are involved in elongation of C16:0 to synthesize C18:0 and desaturation of C18:0 for synthesis of C18:1n-9, so that we could determine the rate of C18:0 and C18:1n-9 conversion. However, the current study reveals that the treatment may have short term deleterious effects on MUFA, because by 5 and 7 days, the MUFAs are replenished either by *de novo* biosynthesis in the liver or are obtained from other sources, such as adipose tissue.

C18:2n-6 is a substrate for synthesis of C20:4n-6. Result of our study indicates the transitional increase of C18:2n-6 may have contributed to elevated C20:4n-6 at 1 day post treatment. Interestingly C22:6n-3 was undetectable at 5 and 7 days post treatment. Pratt et al (2002) reported that C22:6n-3 became undetectable in plasma PL of breast cancer patients undergoing 5-FU, adriamycin and cyclophosphamide treatment (1). The depletion of 22:6n-3 indicates that the cytotoxic agents such as CPT-11 plus 5-FU may interfere with PUFA metabolism. Chemotherapy induced hepatotoxicity generates ROS, resulting oxidative stress, which might have played a role in oxidation of C22:6n-3. The same effect was not observed with 20:5n-3, which increased at 1 day post treatment, but restored at 5 and 7 days. These effects on the PUFA were not reported in the Pawlowicz (2008) study (13).

Total PL fatty acids increased following the treatment, with a transient increase in total SFA at 1 day post treatment. However, Pawlowicz (2008) reported SFA to be comparable between 1 and 7 days post-treatment and tumor-bearing rats (13). The treatment did little to alter fatty acids within PL compared to the TG, where most of the alterations were observed. This may be due to TG comprising a larger pool within the liver and also having a greater capacity for fluctuations in fatty acid composition. Also, we cannot differentiate PL within the liver cell membranes from those available for export to the periphery in VLDL. Further work could identify VLDL-TG and PL which would help to make this distinction.

3.6 Tables and figures

Table 3-1 Composition of the diet

	Ingredient *(g/100g of diet)*	**Diet**
Modified basal mix *70g/100g*	Casein	25.2
	Methionine	0.25
	Maize starch	23.7
	Glucose	13.95
	Vitamins AIN-76	1
	Minerals AIN-76	5
	Inositol	0.6
	Choline	0.3
Lipids *20g/100g*	Canola stearine	11.4
	Flaxseed oil	0.8
	Sunflower oil	7.8
Fibers *10g/100g*	Cellulose	10
Total		100

All diets contained 262 g protein and 15.48 MJ of energy per kg. The diet consisted of pre-mixed modified basal ingredients (Harlan Teklad, Madison, WI) using AIN-76 rodent diet. Formulated fat elements were as supplied: canola stearine (ICN Biomedicals Inc., Cleveland, OH), flax seed oil (Canadian Superstore, President's Choice, AB) and sunflower oil (Planet Organic, Gold Top, AB).

Table 3-2 Hepatic Triglyceride fatty acid composition at different time points after one cycle vs tumor bearing rats

Fatty acid (%)	Tumor (n=5)	D1 (n=6)	D5 (n=6)	D7 (n=6)	P-value
C16:0	26.1 ± 0.3	28.4 ± 0.6	27.4 ± 0.5	28.7 ± 2.5	0.572
C18:0	7.5 ± 0.4	5.5 ± 0.7	8.4 ± 0.5#	8.3 ± 0.4#	0.002
C18:1n-9	30.2 ± 0.7	24.6 ± 0.8*	30.8 ± 0.8#	28.0 ± 0.9#	p<0.001
C18:2n-6	26.7 ± 0.4	29.9 ± 0.6	25.0 ± 0.7#	25.5 ± 1.2#	0.002
C18:3n-3	0.5 ± 0.1	0.4 ± 0.2	0.1 ± 0.1	0.6 ± 0.1≠	0.018
C20:4n-6	3.2 ± 0.3	4.7 ± 0.3*	3.6 ± 0.4	2.9 ± 0.3#	0.005
C20:5n-3	0.1 ± 0.1	0.4 ± 0.0*	0.2 ± 0.1	0.2 ± 0.0	0.037
C22:6n-3	0.8 ± 0.1	0.7 ± 0.4	ND	ND	0.008
∑SFA	34.3 ± 0.1	35.6 ± 1.2	37.3 ± 0.8	38.5 ± 2.2	0.176
∑MUFA	31.3 ± 0.7	25.5 ± 0.8*	31.8 ± 0.9#	29.1 ± 0.7	p<0.001
∑PUFA	34.4 ± 0.9	39.0 ± 1.2	30.9 ± 1.0#	32.4 ± 1.7#	0.001
∑n-6	32.6 ± 0.7	36.9 ± 0.8	30.4 ± 0.9#	31.2 ± 1.6#	0.002
∑n-3	1.8 ± 0.2	2.1 ± 0.4	0.6 ± 0.2*#	1.2 ± 0.1	0.004
Total (µg/g)	3895.6±90.7	4022.4±357.5	4409.5±479.9	5629.5±881.2	0.146

*Significantly different from Tumor (p<0.05)
#Significantly different from D1 (p<0.05)
≠Significantly different from D5 (p<0.05)

Fatty acids within liver TG were determined by gas chromatography. Total amount of liver TG (µg/g) were calculated using 25µl of C15:0 standard (0.1 µg/µL). Individual fatty acids were determined as proportionate amount (%) of total TG. Data are expressed as means ± SEM. Abbreviations: TG, triglyceride; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; ND, not detected. Groups: D1, D5 and D7, indicate 1, 5 and 7 days after the chemotherapy treatment respectively.

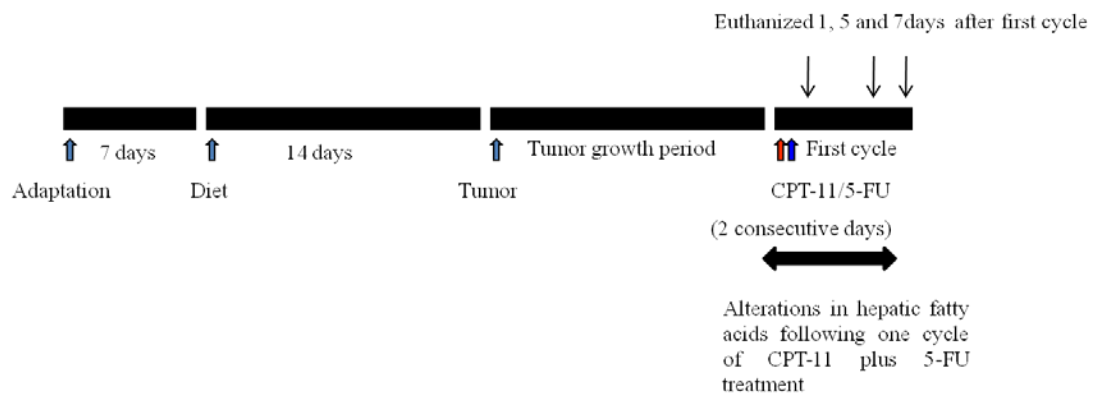
Table 3-3 Hepatic phospholipid fatty acid composition at different time points after one cycle vs tumor-bearing rats

Fatty acid (%)	Tumor (n=6)	Day 1 (n=6)	Day 5 (n=6)	Day 7 (n=6)	P-value
C16:0	11.2 ± 0.1	13.4 ± 0.6	12.7 ± 0.3	12.2 ± 0.8	0.063
C18:0	41.0 ± 0.2	40.6 ± 0.7	39.9 ± 0.5	39.4 ± 0.3	0.098
C18:1n-9	3.0 ± 0.2	2.6 ± 0.1	3.1 ± 0.2	3.6 ± 0.4	0.063
C18:2n-6	9.9 ± 0.3	9.0 ± 0.4	9.5 ± 0.4	10.1 ± 0.4	0.180
C20:4n-6	24.8 ± 0.2	24.3 ± 0.5	24.8 ± 0.4	24.2 ± 0.8	0.808
C20:5n-3	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.088
C22:6n-3	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.172
∑SFA	60.4 ± 0.2	62.5 ± 0.6*	60.6 ± 0.3#	59.7 ± 0.6#	0.002
∑MUFA	3.0 ± 0.2	2.6 ± 0.1	3.3 ± 0.2	3.9 ± 0.5#	0.025
∑PUFA	36.5 ± 0.1	34.9 ± 0.7	36.1 ± 0.5	36.3 ± 0.9	0.271
∑n-6 FA	35.8 ± 0.1	34.1 ± 0.7	35.3 ± 0.4	35.5 ± 0.9	0.257
∑n-3 FA	0.7 ± 0.0	0.8 ± 0.0	0.7 ± 0.1	0.9 ± 0.1	0.108
Total (µg/g)	13204.4±332.2	20909.1±270.4*	19445.4±663.5*	20418.7±570.9*	P<0.001

*Significantly different from Tumor (p<0.05)
#Significantly different from D1 (p<0.05)

Amount of fatty acids in liver PL were determined by gas chromatography. Total liver PL were calculated using 50 µg of C17:0 standard (0.1 µg/µL) to determine the µg of total fatty acids. Individual fatty acids were determined as proportionate amount (%) of total TG. Data are expressed as means ± SEM. Abbreviations: PL, phospholipid; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids. Groups: D1, D5 and D7, indicate 1, 5 and 7 days after the chemotherapy treatment respectively.

Figure 3-1 Experimental design



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Chapter 4: Aberrations in liver fat status following two cycles of irinotecan plus 5-fluorouracil treatment

4.1. Introduction

In advanced CRC, the tumor typically spreads to the liver causing colorectal liver metastases. Hence, surgery is required to remove the tumors from the liver. Resection of liver metastases is associated with the best long-term survival recently approaching 50-60 % in CRC (1-4). It is common practice to administer chemotherapy before liver resection due to theoretical advantages (5). However, despite improved efficacy and outcomes, chemotherapy drugs such as CPT-11 and 5-FU can exert adverse side effects on the liver such as steatosis, steatohepatitis and sinusoidal injury (5-8). Chemotherapy-associated steatosis/steatohepatitis is problematic because liver function can be severely compromised when tumor tissue is removed increasing risk of post-operative mortality after hepatic surgery (5).

Patients with advanced cancer of mixed tumor types exhibit a deficit of 30-50% of plasma PL compared to healthy subjects (9) and the depletion of amount of PL in blood is associated with shorter survival (10). Recently, it has been reported that liver size increases during the cancer treatment trajectory (11). However, it remains to be determined if the increase in liver size correlates with liver fat content and or chemotherapy treatment. It is of interest that the timeline of enlarged liver parallels observations of PL and TG depletion in the plasma of cancer patients who are receiving chemotherapy treatment of various types (10). Collectively, this work suggests that a reduction in hepatic lipid secretion may lead to fatty liver (steatosis) and in turn contribute to the apparent peripheral deficiency of EFA that contributes to fat and muscle tissue wasting in cancer patients (12). However, causes of liver fatty acid abnormalities in CRC patients treated with CPT-11 plus 5-FU have not been clearly defined.

We have refined an animal model that parallels the clinical treatment of CRC in several ways: First the chemotherapy associated side effects such as diarrhea and intestinal toxicity are similar to that experienced by humans (13); Secondly,

the dosage of the drugs and drug schedule for anti-tumor effect in the animal model with CRC (14) is similar to what is given in the clinic. Use of this model has enabled control in dietary intake and to follow relevant clinical or mechanistic measures in the liver, at defined time points to study hepatic fatty acid status with CPT-11 plus 5-FU treatment.

The liver is the major site for oxidation, synthesis, packaging and/or distribution of fatty acids as well as overall homeostasis and is also the major organ that metabolizes the toxic drugs. Circulating drugs used for CRC treatment may interfere with synthesis, packaging and distribution of fats. The impaired balance between oxidation and synthesis may lead to increased TG accumulation in liver. Important pathways involved may be revealed by gene analysis, to delineate defects in the ability of the liver to synthesize, package, oxidate and/or export fatty acids into the periphery. Compared to rats bearing tumor alone, it is hypothesized that tumor-bearing rats receiving CPT-11 plus 5-FU treatment will have higher TG, lower n-3 and n-6 fatty acids and up-regulation of expression of genes involved in fatty acid packaging and synthesis and down-regulation of expression of genes involved in fatty acid oxidation and export and the effect will be more pronounced after the second cycle compared to the first cycle.

4.2 Materials and supplies

The experimental design is explained in chapter 3 (**section 3.2 in page 33**). Some of the additional procedures are explained here in detail. The rats marked as D2 (2 days post chemotherapy group; n=6) were killed 2 days after the first cycle and D9 (9 days post chemotherapy group; n=6) were killed 2 days after second cycle (2nd dose of 5-FU) of the chemotherapy treatment and the livers were collected, respectively. The remaining tumor-bearing rats (n=6) without CPT-11 plus 5-FU treatment served as “Tumor” control group for comparison with the chemotherapy treated groups after each cycles. The rats were killed with carbon-dioxide asphyxiation, the liver was removed, weighed, cut into equal pieces, immediately snap frozen in liquid nitrogen and stored at -80°C until analysis.

4.3 Methods

4.3.1 Fatty acid analysis

Methods of analysis of liver TG and PL fatty acid compositions are explained in detail in chapter 3 (**Section 3.2 pages 34-36**).

4.3.2 RNA preparation

Liver (30 mg) was homogenized with mortar and pestle on dry ice. The liver homogenate was disrupted with homogenizer and RNA was extracted using the Rneasy Mini Kit (Qiagen). RNA samples were treated with RNase free Dnase (Qiagen). RNA concentration and purity (in relationship to protein) were measured spectro-photometrically using a Nano drop (NanoDrop Technologies). Purity was measured by dividing absorbance at 260 nm (RNA) by absorbance at 280 nm (protein). All ratios were above 2.0. Agilent 2100 Bioanalyzer (Agilent Technologies) was used to confirm the RNA was of high-quality.

4.3.3 Reverse transcriptase polymerase chain reaction

A high-throughput real-time PCR (rt-PCR) procedure was used to quantify gene expression using Biomark® (Fluidigm) qPCR system based on micro-fluidic arrays, which permits the simultaneous measurement of 48 genes in 48 samples (2304 reactions) using a Universal Probe Library (Roche). The complete list of genes that were measured is listed in **Table 4-1.** All samples were normalized to B-actin and the results were expressed as fold changes of Ct value relative to tumor controls using the $\Delta\Delta^{ct}$ formula.

4.4 Statistical analysis

Data are reported as means ± SEM. To identify significant differences between multiple samples, data were evaluated using a one-way ANOVA. A *p* value with <0.05 was considered a significantly different. When significant overall differences were observed, a post-Hoc analysis was done using the Bonferonni model. The genes those were significantly altered or not altered were divided into **Table 4-4 and Table 4-5** respectively. Pearson correlations were performed to correlate between fatty acid composition within TG or PL and the gene

expressions (**Appendix I-8**). All statistical analyses were conducted using SPSS 18.0 (Chicago, Illinois).

4.5 Results

4.5.1 Triglyceride

Average liver weight of the rats was 5.7±0.2g (3.6 % of body weight). Total amount of TG fatty acids were significantly lower at D9 compared to D2 and tumor group. C16:0 was lower at D9 than at D2. C18:1n-9 was significantly higher at D9 compared to D2 and contributed to total MUFA being highest at this time point (**Table 4-2**). Total n-6 fatty acids were lower at D9 compared to D2 due largely to lower C18:2n-6. C18:3n-3 was lower at D9 compared to the tumor group. Undetectable C22:6n-3 was found at both D2 and D9. However, C20:5n-3 was transiently increased at D2. For both time points the n6/n3 fatty acid ratio was significantly higher after chemotherapy treatment.

4.5.2 Phospholipid

Total amount of PL were transiently increased at D2 but were comparable at D9 to the tumor group. C16:0 was higher at D2 and D9 than the tumor group. Total MUFA were significantly higher at D9 than other groups primarily due to higher C18:1n-9 (**Table 4-3**). C20:4n-6 was lower and C22:6n-3 was higher at D9 than the tumor group.

4.5.3 Gene regulation

Out of 44 genes analyzed, 13 genes were altered with treatment (**Table 4-4**). Compared to tumor group, expression of VLCAD (oxidation) and DGAT1 (synthesis) remained higher after both cycles of the treatment. Expression of LCAD (oxidation), CHKA (synthesis), MTP (transport), ADRP (transport), APOA1 (transport) and FATP5 (transport) were only transiently elevated at D2, as by D9, the values were similar to the tumor group. Expression of I-FABP (transport), ELOVL2 (synthesis) and FADS2 (synthesis) were lowest at D9 compared to others. **Table 4.5** represents the genes not altered with the treatment.

The genes altered after the treatments are shown in the schematic diagram from **Figure 4.1-4.3**.

4.6 Discussion

Capacity to metabolize fat in patients with cancer and those following chemotherapy treatments is poorly understood. Literature available to date suggest chemotherapy treatment induces fatty liver in the majority of CRC patients (5), which suggests that key impediments exist at the hepatic level. However, none of the literature to date has determined the type and amount of fatty acid that characterize fat accumulation in the liver following chemotherapy. The single previous report on liver fatty acid composition following chemotherapy treatment was limited to one cycle of high dose CPT-11 only (15). This is the first report to examine impediments in hepatic fatty acid and gene expression following two cycles of chemotherapy. Our aim was to fill the gap in literature by determining types and amounts of fatty acid and the related gene expression altered in pathways of fat metabolism with two cycles of CPT-11 plus 5-FU treatment and use these results for therapeutic applications.

Hepatic gene expression and cell signaling pathways are sensitive to change in hepatic lipid content (16). DGAT1 encodes for the enzyme that catalyzes the final step in TG synthesis, which was found to be up-regulated after both cycles of the treatment. Higher amounts of this enzyme would be expected to increase TG, however there was no such increase. Other genes DGAT2, MGAT2 and GPAT involved in TG synthesis were not altered with the treatment. Further work is needed to determine if differences in the protein are evident as we did not assess the protein amount.

Expression of VLCAD as higher after both cycles may indicate an increased rate of long chain fatty acid oxidation after the therapy. This might be one of the causes for the depletion of C22:6n-3 after both treatment cycles due to increased long chain fatty acid oxidation. Expression of UCP2 was higher after second cycle. From previous *in vivo* study, it was reported that UCP2 is over-expressed in hepatocytes during progression of non alcoholic steatohepatitis, which causes a

proton leak and avoids a progressive increase in the rate of mitochondrial H_2O_2 production. This mechanism permits mitochondria to elevate substrate oxidation and reduce redox pressure during fat accumulation, acting as a protective mechanism against damage (17). Despite these favourable effects, the chronic lack of ATP exposes the hepatocytes to increased susceptibility to harmful stimuli when the hepatic energy requirement increases, which emphasises the key role of the hepatic environment in NASH progression (17).

FADS2 (Δ6 desaturase) encodes for the enzyme regulating the initial step of PUFA synthesis through desaturation of C18:2n-6 and C18:3n-3 to form C18:3n-6 and C18:4n-3 respectively (18). Lower expression of this gene was observed after the second cycle, which suggests impairment in the initial step of desaturation after the second cycle of the treatment. ELOVL2 encodes for the enzyme regulating conversion of C20:4n-6 and C20:5n-3 to C22:4n-6 and C22:5n-3 respectively, and also conversion from C22:5n-3 to C24:5n-3 (19). We found ELOVL2 as down-regulated after the second cycle, which may suggest impairment in the EFA metabolism pathway. Amount of C18:3n-3 was also depleted within TG and C20:4n-6 was depleted within PL after second cycle, which indicates that after the second cycle substrates and EFA products may be altered within this pathway. Undetectable C22:6n-3 following either treatment cycles suggests that the chemotherapy has severe effect in alteration of PUFA. The implications of this are currently not known. Undetectable C22:6n-3 in plasma PL of breast cancer patients with different chemotherapy regimens (9) was previously reported in the literature. Cytotoxic agents have been reported to interfere with PUFA metabolism (20). From our findings, C18:3n-3 seemed to contribute to major synthesis of C20:5n-3 than C22:6n-3 after the treatment. Depletion of n-3 fatty acids has been reported to promote hepatic steatosis by increasing TG content (21) however, we did not find an increase in TG despite of depleted n-3 fatty acid after the treatment. It may take longer for fatty liver to develop and our study was limited to two cycle of the treatment only. On the other hand, hepatic n6/n3 fatty acid ratio within the liver TG was found to be higher

after the treatment in both cycles. The increased n6/n3 ratio has been associated with chronic diseases such as obesity and inflammation (22-23), which over longer period of time may be manifest as steatosis.

Impaired liver PL biosynthesis has been shown to reduce VLDL secretion and induce steatosis (24-26). However, we did not find such alteration in major genes involved in PL biosynthesis except for CK-α, which was transiently increased after first cycle. Total PL were transiently increased after first cycle only. C16:0 was increased after both cycles however, C18:0, a product of C16:0 elongation was not altered with the treatment. ELOVL6 encodes the enzyme that elongates C14:0 and C16:0 to C18:0 (27) and was not altered with the treatment.

Total MUFA within liver TG and PL were increased after the second cycle, which were contributed by C18:1n-9. Earlier findings in human and in mice (21) have indicated increased accumulation of C18:1n-9 in hepatic steatosis. However, SCD-1, the gene encoding for a rate limiting enzyme for MUFA synthesis was not altered after either cycles. Another gene ELOVL5 encodes for the enzyme, which may involve in long chain MUFA synthesis (28) was also not altered with the treatment. Thus, our gene data does not adequately explain altered C18:1n-9.

Chemotherapy treatment altered some of the genes involved in lipid transport pathway. FATP-5 was up-regulated after the first cycle and up-regulation of FATP-5 has been associated with liver damage and fibrosis in obese patients (29). ADRP was up-regulated after first cycle and it was reported to be up-regulated in steatotic livers of ob/ob mice (30). These findings are supported by the literature that fatty acid transport may be altered in promoting fatty liver following chemotherapy.

Overall, some of our findings support development of fatty liver after chemotherapy treatment however, our lack of observing increased TG is problematic in applying these findings clinically. It would be of interest to determine total TG and to grade the livers for presence of steatosis after several cycles of the treatment, as chemotherapy is provided at least three or more cycles in clinical patients.

4.7 Tables and figures

Table 4-1 List of the genes selected for RT-PCR

List of Genes	Gene Name	NCBI accession
ACAT-1	sterol O-acyltransferase 1	NM_031118.1
ACC	acetyl-coenzyme A carboxylase alpha	NM_022193.1
ACOX	acyl-Coenzyme A oxidase 1, palmitoyl	NM_017340.2
ADRP	adipose differentiation related protein	NM_001007144.1
APO B	apolipoprotein B (including Ag(x))	NM_019287.2
APO A-1	apolipoprotein A-1	NM_012738.1
CD36	CD36 molecule (thrombospondin receptor)	NM_031561.2
CD68	Cd68 molcule	NM_001031638.1
CK-α	Choline kinase alpha	NM_017127
ACL	ATP citrate lyase	NM_016987.2
DGAT1	diacylglycerol O-acyltransferase homolog 1	NM_053437.1
FADS2	fatty acid desaturase 2	NM_031344.2
FAS	Fatty acid synthase	NM_017332
FATP4	solute carrier family 27 (fatty acid transporter), member 4	XM_231115.4
FATP5	solute carrier family 27 (fatty acid transporter), member 5	NM_024143.1
GPX	glutathione peroxidase 1	NM_030826.3
GSR	glutathione reductase	NM_053906.1
GPAT1	glycerol-3-phosphate acyltransferase	NM_017274.1
GRP78	heat shock protein 5	NM_013083.1
HMGCR	3-hydroxy-3-methylglutaryl-Coenzyme A reductase	NM_013134.2
IFABP	fatty acid binding protein 2, intestinal	NM_013068.1
INSIG-1	insulin induced gene 1	NM_022392.1
LCAD	acyl-Coenzyme A dehydrogenase, long chain	NM_012819.1
LDLR	low density lipoprotein receptor	NM_175762.2
L-FABP	fatty acid binding protein 1, liver	NM_012556
L-1	Lipin 1	NM_001012111.1
ME-1	malic enzyme 1, NADP(+)-dependent, cytosolic	NM_012600.2
MCAD	acyl-Coenzyme A dehydrogenase (C-4 to C-12)	NM_016986.1
MGAT2	monoacylglycerol O-acyltransferase 2	NM_001109436.2
MTP	microsomal triglyceride transfer protein	NM_001107727.1
PEPCK	phosphoenolpyruvate carboxykinase 1 (soluble)	NM_198780.3
PPAR-α	Peroxisome proliferator-activated receptor alpha	NM_013196.1
SCD-1	stearoyl-Coenzyme A desaturase 1	NM_139192.2
SREBP-1α	sterol regulatory element binding transcription factor 1	XM_213329.4
SREBP-2	sterol regulatory element binding transcription factor 2	NM_001033694.1
TNF	tumor necrosis factor (TNF superfamily, member 2)	NM_012675.2
UCP-2	uncoupling protein 2 (mitochondrial, proton carrier)	NM_019354.2
VLCAD	acyl-Coenzyme A dehydrogenase, very long chain	NM_012891.1
CHK-β	Choline kinase beta	NM_017177

Table 4-2 Hepatic Triglyceride fatty acid composition at Day 2 and Day 9 (2 days after first and second cycle respectively) vs tumor-bearing rats

Fatty acid (%)	Tumor (n=5)	D2 (n=6)	D9 (n=5)	P-value
C16:0	26.1 ± 0.3	27.2 ± 0.7	23.8 ± 1.0#	0.017
C18:0	7.5 ± 0.4	6.0 ± 0.7	7.5 ± 1.0	0.267
C18:1n-9	30.2 ± 0.7	27.2 ± 1.2	33.6 ± 0.6#	0.001
C18:2n-6	26.7 ± 0.4	29.3 ± 0.4*	25.6 ± 0.8#	0.001
C18:3n-3	0.5 ± 0.1	0.3 ± 0.1	0.1 ± 0.1*	0.018
C20:4n-6	3.2 ± 0.3	3.9 ± 0.3	3.6 ± 0.6	0.501
C20:5n-3	0.1 ± 0.1	0.4 ± 0.0*	0.3 ± 0.0	0.004
C22:6n-3	0.8 ± 0.1	ND	ND	P<0.001
∑SFA	34.3 ± 0.1	34.8 ± 0.5	32.7 ± 0.7#	0.026
∑MUFA	31.3 ± 0.7	28.5 ± 1.1	35.4 ± 0.7*#	0.001
∑PUFA	34.4 ± 0.9	36.7 ± 1.2	31.9 ± 0.9#	0.018
∑n-6 FA	32.6 ± 0.7	35.6 ± 1.1	31.2 ± 0.9#	0.014
∑n-3 FA	1.8 ± 0.2	1.1 ± 0.2*	0.7 ± 0.1*	0.001
n6/n3	19.0 ± 1.4	35.8 ± 4.9*	46.4 ± 4.9*	0.002
Total (µg/g)	3895.6±90.7	4894.6±352.6	2481.7±233.3*#	P<0.001

*Significantly different from Tumor (p<0.05)
#Significantly different from D2 (p<0.05)

Fatty acids within liver TG were determined by gas chromatography. Total amount of liver TG (µg/g) were calculated using 25µl of C15:0 standard (0.1 µg/µL). Individual fatty acids were determined as proportionate amount (%) of total TG. Data are expressed as means ± SEM. Abbreviations: TG, triglyceride; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; ND, not detected. Groups: D2 and D9 indicate 2 days after first and second cycle of the chemotherapy treatment respectively.

Table 4-3 Hepatic Phospholipid fatty acid composition at Day 2 and Day 9 (2 days after first and second cycle respectively) vs tumor-bearing rats

Fatty acid (%)	Tumor (n=6)	D2(n=6)	D9(n=6)	P-value
C16:0	11.2 ± 0.1	13.7 ± 0.3*	14.2 ± 0.8*	0.001
C18:0	41.0 ± 0.2	40.1 ± 1.0	40.7 ± 1.5	0.803
C18:1n-9	3.0 ± 0.2	3.5 ± 0.3	4.9 ± 0.4*#	0.004
C18:2n-6	9.9 ± 0.3	9.3 ± 0.5	9.6 ± 0.5	0.581
C20:4n-6	24.8 ± 0.2	23.9 ± 0.7	22.3 ± 0.7*	0.037
C20:5n-3	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.129
C22:6n-3	0.08 ± 0.02	0.15 ± 0.01	0.20 ± 0.02*	0.001
∑SFA	60.4 ± 0.2	61.5 ± 0.8	61.2 ± 1.3	0.675
∑MUFA	3.0 ± 0.2	3.6 ± 0.3	5.1 ± 0.4*#	0.001
∑PUFA	36.5 ± 0.1	34.8 ± 0.7	33.6 ± 1.2	0.062
∑n-6 FA	35.8 ± 0.1	34.1 ± 0.7	32.9 ± 1.1	0.050
∑n-3 FA	0.7 ± 0.0	0.7 ± 0.0	0.7 ± 0.0	0.562
Total (µg/g)	13204.4±332.2	20306.0±801.6*	11423.8±461.3#	P<0.001

*Significantly different from Tumor (p<0.05)
#Significantly different from D2 (p<0.05)

Amount of fatty acids in liver PL were determined by gas chromatography. Total liver PL were calculated using 50 µg of C17:0 standard (0.1 µg/µL) to determine the µg of total fatty acids. Individual fatty acids were determined as proportionate amount (%) of total PL. Data are expressed as means ± SEM. Abbreviations: PL, phospholipid; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids. Groups: D2 and D9 indicate 2 days after first and second cycle of the chemotherapy treatment respectively.

Table 4-4 Hepatic gene expression altered at Day 2 and Day 9 (2 days after first and second cycle respectively) vs tumor-bearing rats

Genes	Pathway	Tumor (n=3)	D2 (n=5)	D9 (n=5)	P-value
Up-regulated after the treatment					
LCAD	Oxidation	1.0 ± 0.1	1.9 ± 0.2*	1.2 ± 0.1$^{\#}$	0.002
VLCAD	Oxidation	1.0 ± 0.1	1.8 ± 0.1*	1.5 ± 0.1*	0.003
UCP2	Oxidation	1.1 ± 0.4	2.4 ± 0.3	3.2 ± 0.4*	0.014
DGAT1	TG Synthesis	1.0 ± 0.1	1.4 ± 0.1*	1.4 ± 0.1*	0.022
ACAT1	CE Synthesis	1.2 ± 0.5	4.1 ± 0.9	7.8 ± 2.0$^{\#}$	0.035
CHKA	PC Synthesis	1.1 ± 0.4	2.6 ± 0.2*	1.9 ± 0.1	0.007
APOA1	Transport	1.0 ± 0.0	1.3 ± 0.2*	0.8 ± 0.0	0.019
FATP5	Transport	1.0 ± 0.0	1.9 ± 0.1*	1.5 ± 0.3	0.034
MTP	Transport	1.0 ± 0.1	1.5 ± 0.1*	1.3 ± 0.0	0.004
ADRP	Transport	1.0 ± 0.2	3.0 ± 0.5*	1.6 ± 0.3	0.015
Down-regulated after the treatment					
FADS2	Syntheses	1.0 ± 0.1	0.6 ± 0.1	0.4 ± 0.1*	0.021
ELOVL2	Synthesis	1.0 ± 0.0	1.3 ± 0.1	0.5 ± 0.1$^{*\#}$	0.000
I-FABP	Transport	1.0 ± 0.1	1.3 ± 0.2	0.5 ± 0.0$^{*\#}$	0.001

*Significantly different from Tumor ($p<0.05$)
$^{\#}$Significantly different from D2 ($p<0.05$)

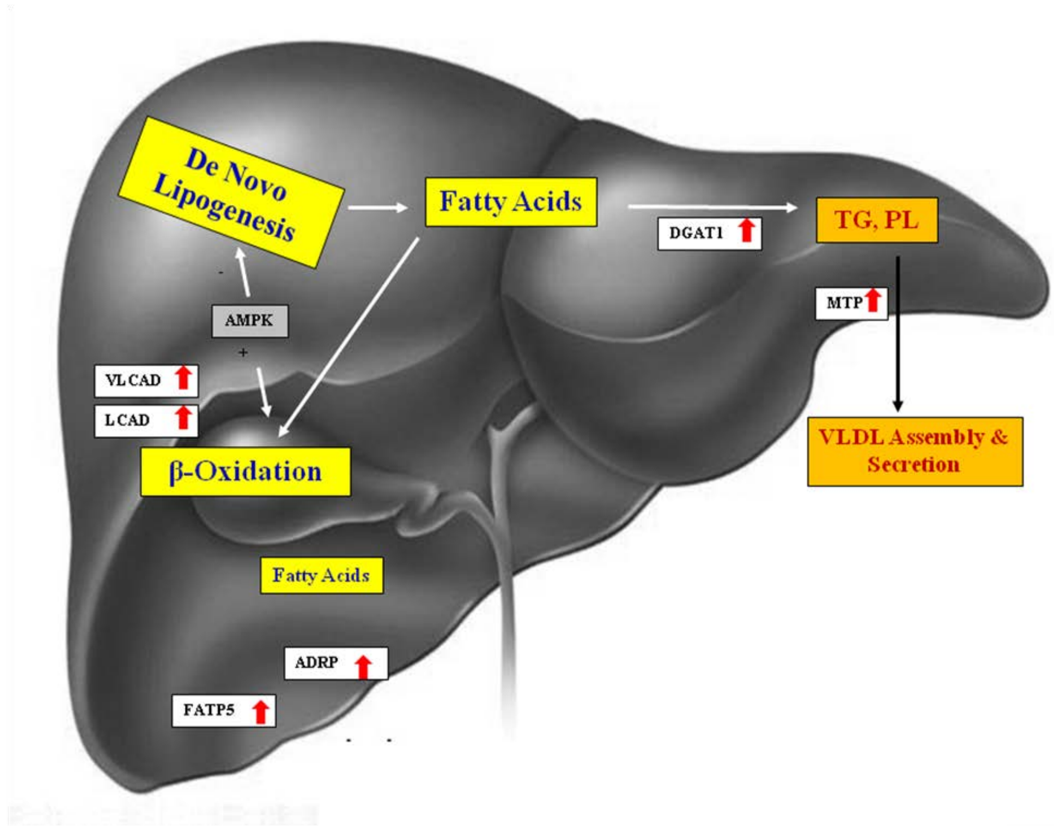
Relative gene expression was performed using RT-PCR. Gene regulation was determined as fold change. Data are expressed as means ± SEM. Significant differences ($p<0.05$) were determined using a one-way ANOVA. Groups: D2 and D9 indicate 2 days after first and second cycle of the chemotherapy treatment respectively.

Table 5-5 Hepatic gene expression not altered at Day 2 and Day 9 (2 days after first and second cycle respectively) vs tumor-bearing rats

Genes	Pathway	Tumor (n=3)	D2 (n=5)	D9 (n=5)	P-value
HMGCR	CHO regulation	1.2 ± 0.5	2.6 ± 0.6	3.5 ± 0.6	0.075
INSIG-1	CHO regulation	1.1 ± 0.3	1.3 ± 0.2	1.4 ± 0.2	0.774
LDLR	CHO regulation	1.0 ± 0.2	1.7 ± 0.2	1.6 ± 0.2	0.079
SREBP1-α	CHO regulation	1.3 ± 0.6	1.5 ± 0.2	1.2 ± 0.3	0.776
SREBP2	CHO regulation	1.3 ± 0.6	2.1 ± 0.7	1.6 ± 0.2	0.605
CD68	Anti-oxidation	1.0 ± 0.1	1.3 ± 0.1	1.9 ± 0.3	0.049
GPX	Anti-oxidation	1.0 ± 0.1	0.8 ± 0.1	0.7 ± 0.1	0.235
GSR	Anti-oxidation	1.1 ± 0.2	2.0 ± 0.5	2.5 ± 0.3	0.098
TNF	Macrophage	9.3 ± 7.3	10.4 ± 2.7	11.78 ± 5.7	0.946
ACOX	FA oxidation	1.0 ± 0.2	2.1 ± 0.3	1.8 ± 0.2	0.070
LIPIN 1	FA oxidation	1.1 ± 0.3	1.3 ± 0.1	1.2 ± 0.2	0.711
MCAD	FA oxidation	1.0 ± 0.1	1.1 ± 0.1	0.8 ± 0.1	0.162
ME1	FA oxidation	1.2 ± 0.6	1.6 ± 0.1	1.6 ± 0.4	0.776
PEPCK	FA oxidation	1.1 ± 0.3	2.7 ± 0.6	2.1 ± 0.5	0.175
PPAR-α	FA oxidation	1.1 ± 0.3	3.9 ± 1.3	2.0 ± 0.2	0.149
PPAR-γ	FA storage	1.8 ± 1.3	11.6 ± 6.3	12.3 ± 8.9	0.625
SCD1	FA synthesis	1.0 ± 0.3	0.2 ± 0.0	0.5 ± 0.3	0.084
FADS1	FA synthesis	1.0 ± 0.1	1.7 ± 0.2	1.1 ± 0.2	0.054
ELOVL5	FA synthesis	1.0 ± 0.0	0.9 ± 0.1	0.6 ± 0.1	0.043
ELOVL6	FA synthesis	1.0 ± 0.7	0.4 ± 0.1	0.4 ± 0.2	0.396
ACC	FA synthesis	1.3 ± 0.7	1.6 ± 0.1	2.2 ± 0.2	0.185
ACLY	FA synthesis	1.4 ± 0.9	1.5 ± 0.2	1.7 ± 0.4	0.919
FASN	FA synthesis	2.0 ± 1.5	0.9 ± 0.2	1.2 ± 0.2	0.542
CHKB	PC synthesis	1.0 ± 0.1	1.0 ± 0.1	1.1 ± 0.1	0.619
DGAT2	TG synthesis	1.0 ± 0.2	1.4 ± 0.2	1.2 ± 0.2	0.560
MGAT1	TG synthesis	2.4 ± 2.0	1.5 ± 0.5	2.6 ± 0.7	0.691
GPAT1	TG synthesis	1.1 ± 0.4	1.9 ± 0.4	1.8 ± 0.4	0.433
APOB	FA transport	1.3 ± 0.6	1.9 ± 0.9	1.1 ± 0.2	0.639
CD36	FA transport	1.0 ± 0.0	2.0 ± 0.5	2.4 ± 0.1	0.081
FATP4	FA transport	1.0 ± 0.1	1.1 ± 0.0	1.2 ± 0.0	0.118
L-FABP	FA transport	1.0 ± 0.1	1.0 ± 0.1	0.6 ± 0.1	0.049

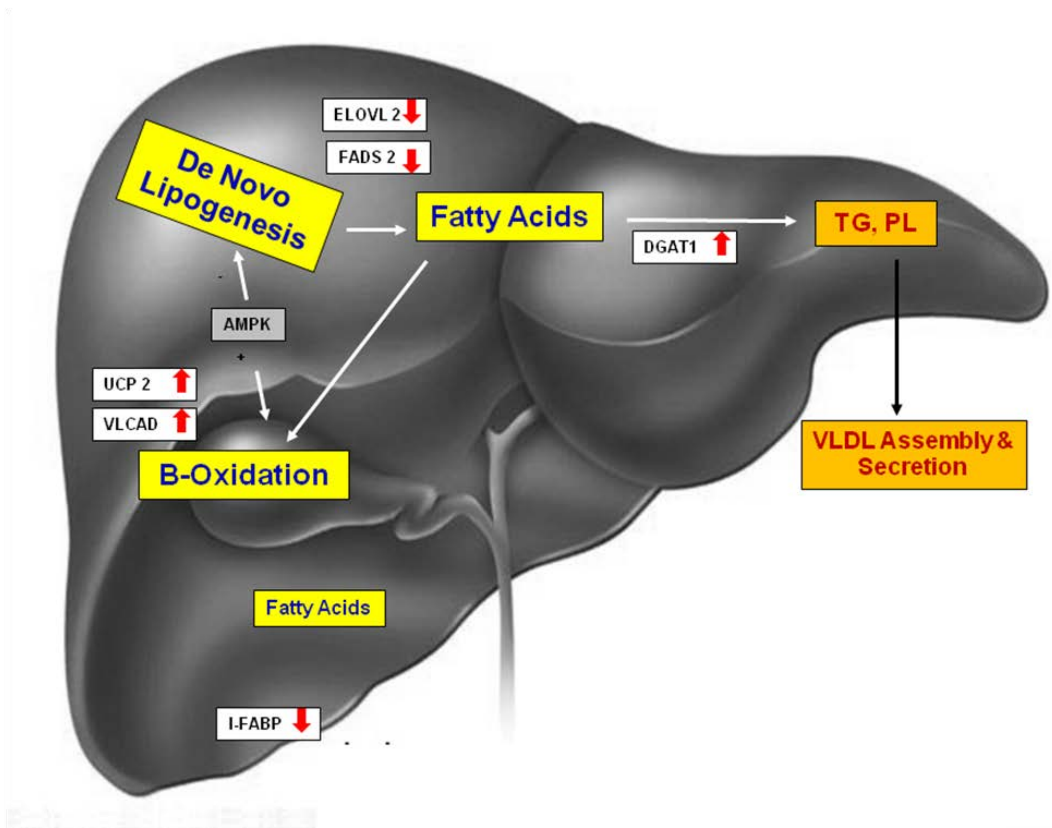
Relative gene expression was determined using RT-PCR. Gene regulation was determined as fold change. Data are expressed as means ± SEM. Significant differences ($p<0.05$) were determined using a one-way ANOVA. Groups: Day 2 and Day 9 indicate 2 days after first and second cycle of the chemotherapy treatment respectively.

Figure 4-1 Significantly altered hepatic gene expressions at Day 2 (2 days after the first cycle of chemotherapy) vs tumor-bearing rats



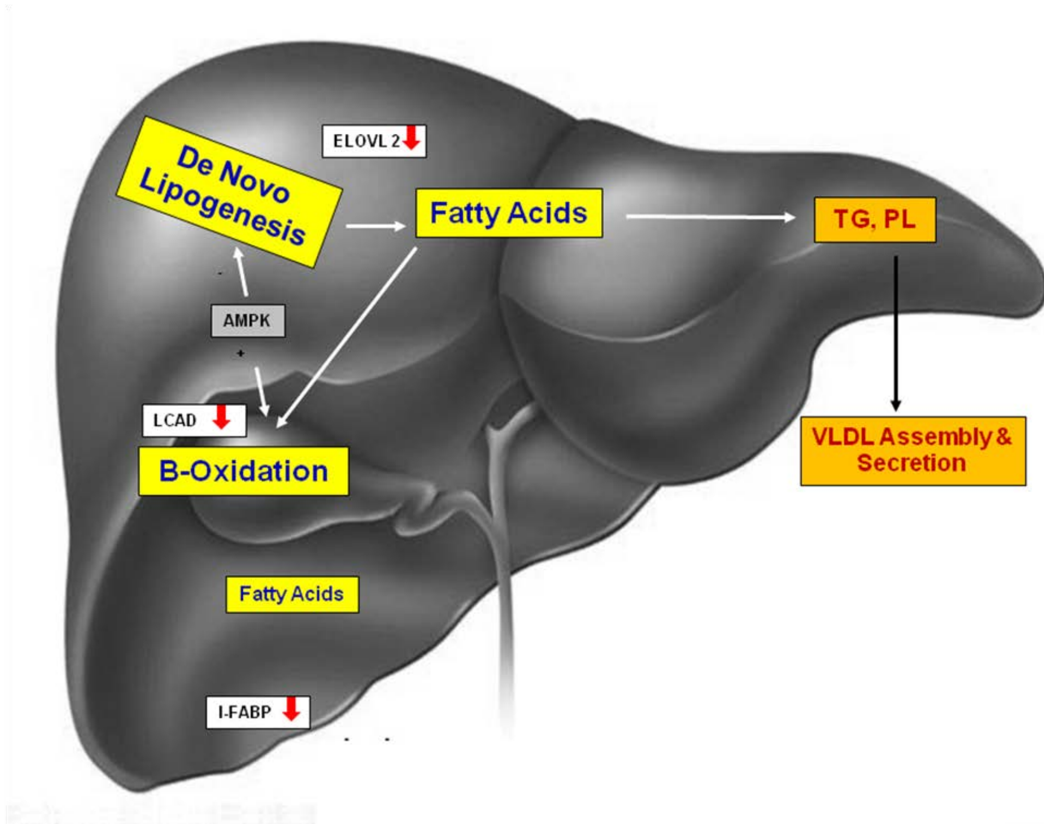
Where, ↑ indicates up-regulation of the genes at 2 days after the first cycle of the chemotherapy treatment versus tumor group

Figure 4-2 Significantly altered hepatic gene expressions at Day 9 (2 days after the second cycle of chemotherapy treatment) vs tumor-bearing rats



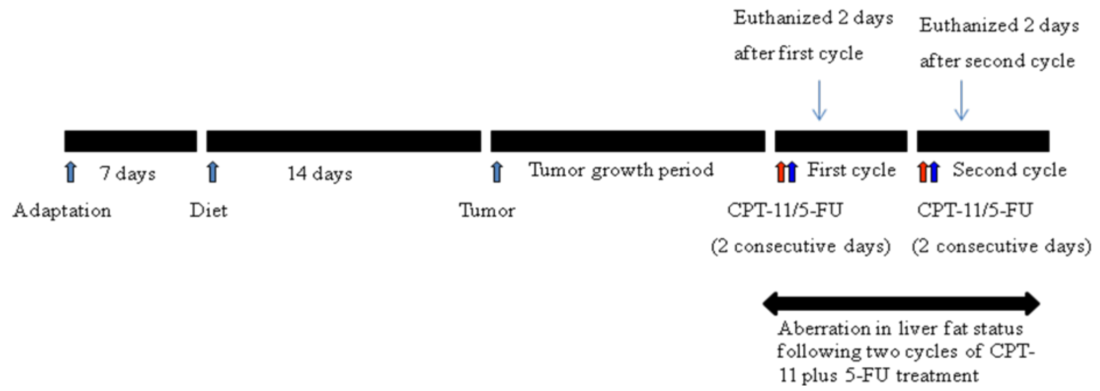
Where, ⬆ indicates the genes up-regulated and ⬇ indicates the genes down-regulated at 2 days after second cycle versus tumor group

Figure 4-3 Significantly altered hepatic gene expressions at Day 9 vs at Day 2 (2 days after the second cycle vs the first cycle)



Where, ⬆ indicates the genes up-regulated and ⬇ indicates the genes down-regulated at 2 days after the second cycle verses the first cycle

Figure 4-4 Experimental design



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Chapter 5

5.1 Conclusion

The first objective of our study was to determine changes in liver fatty acid amount and composition during the 7 day period following delivery of CPT-11 plus 5-FU, then 2 days after an additional cycle. It was hypothesized that compared to tumor-bearing animals, the liver of rats treated with two cycles of CPT-11 plus 5-FU would have more TG with lower amounts of n-3 and n-6 PUFA. Our study revealed that there was no alteration in amount of liver TG during the initial 7 day period, however 2 days after the second cycle TG levels were significantly reduced compared to tumor-bearing rats. This was not in line of our hypothesis where we expected increased TG accumulation following the chemotherapy treatment.

On the other hand, our study supports the hypothesis that PUFA would be depleted after treatment. C22:6n-3 became undetectable at 5 and 7 days following the first cycle and at 2 days after the second cycle. Perhaps this is the most important finding for our study, because of the implications of this for nutritional intervention with fish oil, which contains C22:6n-3, an intensive area of investigation, currently. Similarly, C18:3n-3 was also found to be lower and n6/n3 fatty acid ratio were elevated following the treatment. Reduced n-3 fatty acids following the treatment suggests that the drugs may have marked effects in alteration of PUFA, potentially more on n-3 versus n-6 fatty acids, which may indicate that other drugs interfere with synthesis of long chain n-3 fatty acid or result in increase in use or oxidation.

The second objective of our study was to measure hepatic genes that are involved in fat metabolism following two cycles of the treatment and relates these changes to fat amount and composition. It was hypothesized that compared to tumor-bearing animals, the liver of rats treated with two cycles of CPT-11 plus 5-FU will have an up-regulation of genes involved in fatty acid packaging and/or synthesis and down-regulation of genes involved in fatty acid oxidation and export. The overall findings were 1) not all genes within a general pathway are

affected in the same manner and 2) alterations in gene expression did not necessarily parallel the observations in fatty acids in all cases. For example, DGAT1 encodes for an enzyme involved in TG synthesis and was up-regulated following the each treatment cycles; however, total TG amounts were not increased. In this case, it may take time after change in gene is observed to observe a change in product.

On the other hand, our study revealed that at 2 days after the second cycle of the treatment, there is alteration of PUFA metabolism by altering the gene expression that encodes the enzymes for desaturation and elongation pathway. Down-regulation of FADS2 expression after second cycle suggests impairment in the initial step of desaturation pathway for PUFA metabolism. Parallel to this, down-regulation of ELOVL2 after the second cycle suggests impairment in elongation of PUFA. Alteration of these two gene expressions after chemotherapy might have played a role in reducing total n-3 fatty acid after the second cycle. Depletion of C18:3n-3 after the second cycle also indicates that substrates and PUFA within this pathway may be altered. It is not known if the alteration in pathway of PUFA metabolism is temporary, as one limitation of our study was that we did not assess the liver after subsequent cycles of the treatment.

UCP2 encodes for the enzyme that plays role in protection against ROS generated during mitochondrial oxidation, and has been reported to be up-regulated in liver steatosis. Our finding of up-regulation of this gene after the second cycle might be one of the indications that fatty liver may develop at a later time; however, we did not confirm this.

FATP-5 and ADRP were up-regulated after the first cycle, which would support fat accumulation in liver. However, because we did not observe elevated fatty acid it is difficult to determine whether these changes play a major role in fatty liver development. This finding was consistent with transport being significantly up-regulated, future work is needed to focus on these pathways.

The study has some limitations, as we did not visualize hepatic tissue to assess for steatosis. We did not assess other lipid classes besides TG and PL. We found

several genes altered following the treatment but did not assess protein levels, so the context to which gene alteration can be transient to change in protein need further investigation. We measured fatty acid following two cycles of the treatment only, which is a relatively short time period, because the chemotherapy is provided at least three or more cycles clinically. However, if we had obtained livers of rats after a longer period of time, we might have observed manifestations of lipid accumulation.

This study has several strengths. We used a tumor-bearing rat as the control comparison group because chemotherapy is provided to people with tumor, not to healthy people. Our study design parallels clinical treatment, in terms of having toxic anti-tumor effect, cause intestinal injury and diarrhea and the similar drug schedule. The aim was to enable translation of our findings to humans. It is complex to study the cancer patient's liver samples directly, due to lack of access to biopsy samples, which are in small quantities. Study of liver pathogenicity from the animal model will help to determine pathways altered in fat metabolism due to therapy, which will direct clinical strategies for intervention.

Overall, TG accumulation was not found in the liver following the two cycles of CPT-11 plus 5-FU treatment, even though some of the genes involved in pathways of fatty acid transport into the liver were up-regulated after the treatment. In addition, up-regulation of UCP2 after the treatment may indicate a risk for fatty liver. Interestingly, DHA was depleted and some of the genes involved in pathways of PUFA metabolism were down-regulated. Future exploration is required to assess liver capacity for fat metabolism after several chemotherapy cycles, as this is what is done clinically. Further investigation is needed to determine fatty acid status in different body depots such as plasma, adipose tissue, skeletal muscle to determine the overall fatty acid metabolic status following the drug treatment for CRC cancer.

Appendix 1 Person correlation between hepatic gene expression and fatty acid composition within TG of rats at 2 days after the first cycle of CPT-11 plus 5-FU treatment

Genes	C16:0	C18:0	C18:1n-9	C18:2n-6	C18:3n-3	C20:4n-6	C20:5n-3	SFA	MUFA	PUFA	n-3	n-6	Total
ACC	-0.004	0.226	-0.103	0.476	0.015	0.181	-0.324	0.100	-0.378	0.108	-0.268	0.178	-0.369
ACOX	0.775	-0.116	0.175	0.029	-0.435	-0.354	-0.397	0.511	0.105	-0.370	-0.448	-0.344	-0.477
ADRP	0.655	-0.442	-0.301	0.287	-0.205	-0.131	-0.142	0.169	-0.208	-0.012	-0.047	-0.005	-0.635
APO A1	0.628	-0.456	0.452	-.880*	0.087	-0.117	0.504	-0.021	0.617	-0.266	0.201	-0.349	0.780
APO B	0.118	-0.217	-0.340	0.411	0.455	0.564	0.178	-0.326	-0.534	0.448	0.198	0.483	-0.051
CD36	0.784	-0.040	0.304	-0.100	-0.616	-0.563	-0.491	0.658	0.286	-0.544	-0.553	-0.527	-0.494
CD68	0.265	-0.496	-0.229	-0.004	0.668	0.656	0.591	-0.529	-0.309	0.474	0.483	0.458	0.454
CHK-α	-0.249	-0.099	-0.792	.972**	0.195	0.391	-0.156	-0.263	-0.857	0.554	0.163	0.614	-0.807
CHK-β	0.848	-0.388	0.032	-0.060	-0.377	-0.358	-0.183	0.373	0.134	-0.297	-0.208	-0.305	-0.436
ACLY	-0.680	.879*	0.150	0.423	-0.375	-0.227	-0.720	0.376	-0.105	-0.190	-0.585	-0.107	-0.448
DGAT1	0.656	0.119	0.408	-0.167	-0.788	-0.751	-0.618	0.785	0.426	-0.688	-0.665	-0.673	-0.532
DGAT2	0.371	0.431	0.378	0.105	-0.814	-0.694	-0.853	0.838	0.248	-0.641	-0.837	-0.585	-0.710
FASN	-0.102	0.141	-0.260	0.570	0.190	0.358	-0.186	-0.084	-0.524	0.291	-0.098	0.358	-0.327
FATP4	0.690	-.910*	-0.548	0.175	0.414	0.413	0.515	-0.410	-0.396	0.438	0.543	0.405	-0.113
FATP5	0.609	-.994**	-0.484	-0.063	0.596	0.517	0.784	-0.590	-0.278	0.498	0.744	0.436	0.261
GPX	0.518	0.287	0.383	0.000	-0.818	-0.730	-0.765	0.829	0.319	-0.668	-0.772	-0.628	-0.663
GSR	-0.740	0.287	0.001	-0.040	0.450	0.408	0.293	-0.422	-0.094	0.309	0.272	0.307	0.566
GPAT1	-0.276	.925*	0.533	0.079	-0.840	-0.733	-.975**	0.834	0.342	-0.681	-.934*	-0.612	-0.530
HMGCR	-0.691	0.349	-0.336	0.493	-0.128	-0.056	-0.285	-0.032	-0.290	0.152	-0.025	0.182	-0.481
I-FABP	0.297	0.034	0.073	0.021	-0.610	-0.591	-0.442	0.495	0.216	-0.410	-0.357	-0.409	-0.583
INSIG-1	-0.476	0.592	0.096	0.214	-0.581	-0.525	-0.627	0.440	0.121	-0.332	-0.457	-0.298	-0.544
LCAD	0.785	-0.161	0.192	-0.081	-0.575	-0.539	-0.403	0.575	0.248	-0.475	-0.433	-0.470	-0.518

*Correlation is significant at the 0.05 level (2-tailed); **Correlation is significant at the 0.01 level (2-tailed)

To be continued on next page…………..

Appendix 2 Pearson correlation between hepatic gene expression and fatty acid composition within TG of rats at 2 days after the first cycle of CPT-11 plus 5-FU treatment

Gene	C16:0	C18:0	C18:1n-9	C18:2n-6	C18:3n-3	C20:4n-6	C20:5n-3	SFA	MUFA	PUFA	n-3	n-6	Total
LDLR	0.349	0.150	-0.071	0.463	-0.531	-0.364	-0.666	0.526	-0.169	-0.255	-0.532	-0.194	-.898*
L-FABP	0.528	-0.102	0.031	0.071	-0.575	-0.531	-0.426	0.501	0.142	-0.380	-0.359	-0.373	-0.647
LIPIN 1	0.317	-0.464	-0.262	0.114	0.586	0.612	0.463	-0.443	-0.372	0.448	0.386	0.447	0.274
MCAD	0.870	-0.356	0.103	-0.119	-0.416	-0.403	-0.206	0.421	0.198	-0.355	-0.253	-0.365	-0.402
ME 1	-0.058	0.840	0.605	-0.003	-0.874	-0.771	-.970**	.900*	0.406	-0.752	-.977**	-0.686	-0.512
MGAT1	0.701	-0.672	0.173	-0.681	0.118	-0.067	0.552	-0.115	0.428	-0.121	0.346	-0.208	0.535
MTP	0.702	0.032	0.268	-0.010	-0.662	-0.587	-0.573	0.687	0.240	-0.542	-0.597	-0.515	-0.599
PEPCK	0.493	-0.719	-0.645	0.472	0.505	0.591	0.382	-0.449	-0.652	0.579	0.461	0.585	-0.247
PPAR-α	0.295	-0.417	-0.119	-0.066	0.575	0.562	0.508	-0.423	-0.219	0.366	0.375	0.354	0.456
PPAR-γ	0.357	0.474	0.513	-0.084	-.935*	-0.864	-0.864	.925*	0.453	-0.789	-0.875	-0.749	-0.624
ACAT	0.438	-0.294	-0.282	0.391	0.207	0.331	0.008	-0.089	-0.439	0.255	0.028	0.292	-0.292
SREBP1- α	-0.389	.918*	0.453	0.162	-0.607	-0.484	-0.846	0.648	0.186	-0.493	-0.814	-0.416	-0.395
SREBP2	0.057	-0.225	-0.410	0.476	0.498	0.615	0.197	-0.380	-0.602	0.512	0.243	0.550	-0.077
TNF	0.171	-0.568	0.036	-0.611	0.561	0.358	0.849	-0.538	0.226	0.237	0.654	0.149	.919*
UCP2	0.796	-0.449	-0.098	0.102	0.007	0.073	0.011	0.097	-0.153	0.008	-0.046	0.018	-0.253
VLCAD	0.819	-0.206	0.128	0.003	-0.469	-0.407	-0.361	0.504	0.132	-0.378	-0.393	-0.364	-0.516
SCD1	-0.348	0.805	0.838	-0.562	-0.555	-0.626	-0.468	0.579	0.741	-0.701	-0.628	-0.695	0.321
FADS1	-0.006	-0.289	-0.343	0.267	0.659	0.711	0.426	-0.531	-0.499	0.561	0.406	0.575	0.255
FADS2	0.067	-0.339	-0.243	0.096	0.669	0.681	0.510	-0.532	-0.371	0.504	0.435	0.502	0.414
ELOVL2	0.279	-0.410	-0.611	0.666	0.368	0.522	0.101	-0.304	-0.725	0.520	0.234	0.561	-0.455
ELOVL5	0.492	-.886*	-0.348	-0.179	0.753	0.659	.882*	-0.677	-0.236	0.534	0.763	0.474	0.562
ELOVL6	-0.800	0.835	0.153	0.258	-0.438	-0.365	-0.625	0.343	0.058	-0.243	-0.469	-0.192	-0.364

*Correlation is significant at the 0.05 level (2-tailed); **Correlation is significant at the 0.01 level (2-tailed)

Appendix 3 Pearson correlation between hepatic gene expression and fatty acid composition within TG of rats at 2 days after the second cycle of CPT-11 plus 5-FU treatment

Gene	C16:0	C18:0	C18:1n-9	C18:2n-6	C18:3n-3	C20:4n-6	C20:5n-3	SFA	MUFA	PUFA	n-3	n-6	Total
ACC	-0.126	-0.358	0.120	0.331	0.693	-0.731	-0.192	-0.691	0.543	0.053	0.476	0.014	0.532
ACOX	-0.439	0.592	-0.545	0.186	0.243	0.012	0.529	0.197	-0.322	0.127	-0.426	0.160	0.118
ADRP	-0.565	0.588	-0.673	0.428	0.447	-0.035	0.386	-0.018	-0.402	0.355	-0.441	0.389	0.283
APO A1	-0.115	0.492	-0.693	0.053	-0.274	0.763	0.473	0.512	-.891*	0.376	-0.517	0.417	-0.060
APO B	0.157	-0.675	0.290	0.311	0.405	-0.396	-0.637	-0.753	0.388	0.231	0.584	0.183	0.415
CD36	0.140	-0.388	0.786	-0.101	-0.045	-0.465	-0.753	-0.355	0.694	-0.325	0.264	-0.345	-0.264
CD68	-0.036	-0.054	0.276	0.135	-0.141	0.069	-0.694	-0.182	0.002	0.134	-0.156	0.146	-0.266
CHK-α	-0.271	0.408	0.232	-0.228	0.048	-0.471	0.277	0.219	0.455	-0.550	-0.227	-0.529	-0.288
CHK-β	-0.439	0.472	-0.430	0.478	0.066	0.350	-0.330	-0.055	-0.619	0.567	-0.617	0.614	-0.073
ACLY	-0.677	0.505	-0.371	0.504	0.663	-0.480	0.093	-0.304	0.024	0.206	-0.341	0.232	0.278
DGAT1	-0.492	0.528	-0.513	0.289	0.402	-0.151	0.431	0.022	-0.210	0.162	-0.350	0.189	0.222
DGAT2	-0.596	0.469	-0.128	0.309	0.542	-0.593	0.089	-0.217	0.256	-0.056	-0.291	-0.032	0.113
FASN	-0.704	0.548	0.088	0.317	0.533	-0.758	-0.162	-0.276	0.440	-0.169	-0.404	-0.135	-0.072
FATP4	-0.578	-0.014	-0.188	.904*	0.875	-0.539	-0.750	-.957*	0.071	0.652	-0.042	0.652	0.516
FATP5	-0.470	.906*	-0.112	-0.100	-0.335	0.218	0.128	0.578	-0.289	-0.184	-.910*	-0.110	-0.655
GPX	-0.581	.882*	-0.256	0.044	0.000	-0.037	0.305	0.390	-0.182	-0.135	-0.771	-0.073	-0.356
GSR	-0.779	.909*	-0.207	0.265	0.251	-0.296	0.054	0.117	-0.041	-0.052	-0.803	0.013	-0.280
GPAT1	-0.443	0.502	0.007	0.012	0.253	-0.475	0.257	0.080	0.298	-0.313	-0.313	-0.286	-0.118
HMGCR	0.222	-0.620	0.643	0.017	0.174	-0.521	-0.658	-0.557	0.683	-0.166	0.538	-0.209	0.078
I-FABP	-0.780	0.604	-0.756	0.838	0.641	-0.019	-0.135	-0.369	-0.572	0.761	-0.593	0.806	0.363
INSIG-1	-0.190	0.017	0.543	-0.102	0.276	-0.825	-0.150	-0.228	0.839	-0.543	0.120	-0.551	-0.126
LCAD	-0.484	0.609	-0.801	0.388	0.290	0.241	0.466	0.130	-0.646	0.452	-0.503	0.491	0.250

*Correlation is significant at the 0.05 level (2-tailed); **Correlation is significant at the 0.01 level (2-tailed)

To be continued on next page…………..

Appendix 4 Pearson correlation between hepatic gene expression and fatty acid composition within TG of rats at 2 days after the second cycle of CPT-11 plus 5-FU treatment

Gene	C16:0	C18:0	C18:1n-9	C18:2n-6	C18:3n-3	C20:4n-6	C20:5n-3	SFA	MUFA	PUFA	n-3	n-6	Total
LDLR	0.155	-0.494	0.443	-0.060	0.352	-0.686	-0.117	-0.438	0.753	-0.314	0.604	-0.361	0.269
L-FABP	-0.365	0.794	-0.469	0.002	-0.271	0.483	0.364	0.574	-0.626	0.105	-0.786	0.168	-0.355
LIPIN 1	0.038	-0.314	0.007	0.079	0.476	-0.506	0.226	-0.355	0.406	-0.081	0.491	-0.121	0.484
MCAD	-0.575	.941*	-0.290	0.054	-0.136	0.157	0.209	0.467	-0.353	-0.047	-.900*	0.025	-0.465
ME 1	-0.504	0.697	-0.166	0.017	0.135	-0.243	0.356	0.259	0.037	-0.224	-0.529	-0.181	-0.207
MGAT1	0.632	-0.894	0.268	-0.089	0.502	-0.353	0.049	-0.505	0.464	-0.063	0.932	-0.155	0.670
MTP	-.961**	0.857	-0.095	0.539	0.515	-0.570	-0.365	-0.257	0.127	0.083	-0.799	0.147	-0.200
PEPCK	-0.087	-0.374	-0.677	0.694	0.762	-0.037	0.042	-0.692	-0.361	0.822	0.420	0.785	.989**
PPAR-α	-0.256	-0.320	-0.196	0.633	.884*	-0.602	-0.250	-0.858	0.241	0.433	0.406	0.399	0.764
PPAR-γ	0.431	-.887*	0.182	0.141	0.309	-0.219	-0.303	-0.620	0.297	0.209	0.842	0.140	0.575
ACAT	0.347	-0.738	0.332	0.124	0.149	-0.170	-0.559	-0.553	0.295	0.161	0.609	0.111	0.292
SREBP1- α	-0.531	0.458	-0.434	0.361	0.529	-0.320	0.311	-0.137	-0.068	0.160	-0.275	0.181	0.284
SREBP2	-0.129	-0.384	0.238	0.496	0.468	-0.425	-0.843	-0.792	0.298	0.336	0.252	0.315	0.281
TNF	0.417	-0.861	0.265	0.119	0.260	-0.235	-0.392	-0.608	0.341	0.163	0.793	0.099	0.481
UCP2	0.016	-0.098	0.131	0.169	-0.138	0.203	-0.602	-0.168	-0.153	0.255	-0.115	0.264	-0.158
VLCAD	-0.584	0.334	-0.664	0.670	0.764	-0.262	0.165	-0.419	-0.268	0.539	-0.194	0.553	0.585
SCD1	0.274	0.097	0.249	-0.679	-0.427	-0.005	0.662	0.624	0.285	-0.706	0.067	-0.709	-0.343
FADS1	-0.160	0.558	-0.558	-0.116	-0.175	0.425	0.746	0.578	-0.544	0.032	-0.435	0.067	-0.072
FADS2	0.088	0.337	-0.703	-0.139	-0.324	0.779	0.788	0.627	-0.826	0.236	-0.285	0.258	0.044
ELOVL2	0.004	0.407	-0.510	-0.238	-0.239	0.453	0.851	0.623	-0.492	-0.045	-0.268	-0.024	-0.032
ELOVL5	-0.304	0.600	-0.401	-0.062	-0.002	0.097	0.629	0.426	-0.263	-0.094	-0.434	-0.059	-0.085
ELOVL6	-0.215	0.415	0.080	-0.250	0.000	-0.308	0.443	0.316	0.287	-0.478	-0.223	-0.459	-0.227

*Correlation is significant at the 0.05 level (2-tailed); **Correlation is significant at the 0.01 level (2-tailed)

Appendix 5 Pearson correlation between hepatic gene expression and fatty acid composition within PL of rats at 2 days after the first cycle of CPT-11 plus 5-FU treatment

Gene	C16:0	C18:0	C18:1n-9	C18:2n-6	C20:4n-6	C20:5n-3	C22:6n-3	SFA	MUFA	PUFA	n-3	n-6	Total
ACC	-0.206	0.342	-0.639	-0.801	0.221	-0.213	0.069	0.372	-0.834	-0.259	-0.254	-0.309	-0.089
ACOX	0.724	0.569	0.239	-0.798	-0.399	0.676	0.298	0.802	-0.082	-0.809	-0.823	-0.218	-0.344
ADRP	0.829	0.279	0.741	-0.457	-0.38	0.651	0.145	0.514	0.467	-0.595	-0.612	0.032	-0.048
APO A1	0.196	-0.377	0.169	0.04	0.086	0.684	-0.391	-0.207	0.457	0.146	0.126	0.572	0.193
APO B	-0.359	-0.135	-0.632	-0.841	0.619	-0.097	-0.424	-0.022	-0.613	0.112	0.107	0.195	0.383
CD36	.882*	0.66	0.443	-0.587	-0.625	0.745	0.461	0.87	0.092	-.904*	-.916*	-0.307	-0.499
CD68	-0.435	-0.522	-0.572	-0.644	0.805	0.101	-0.756	-0.364	-0.315	0.419	0.405	0.602	0.636
CHK-α	0.026	0.109	0.14	-0.294	0.09	-0.317	0.045	0.11	-0.095	-0.099	-0.096	-0.147	0.181
CHK-β	.941*	0.36	0.733	-0.455	-0.507	0.857	0.214	0.621	0.48	-0.706	-0.726	0.014	-0.202
ACLY	-0.349	0.585	-0.556	0.04	-0.208	-0.744	0.603	0.333	-0.813	-0.223	-0.195	-0.825	-0.545
DGAT1	.948*	0.754	0.587	-0.305	-0.835	0.68	0.654	.898*	0.204	-.950*	-.957*	-0.453	-0.667
DGAT2	0.689	.937*	0.201	-0.454	-0.756	0.323	0.787	.994**	-0.269	-.979**	-.976**	-0.735	-0.783
FASN	-0.341	0.183	-0.679	-0.764	0.38	-0.314	-0.074	0.203	-0.814	-0.089	-0.084	-0.193	0.076
FATP4	0.436	-0.369	0.525	-0.504	0.244	0.657	-0.527	-0.068	0.572	-0.013	-0.041	0.658	0.57
FATP5	0.228	-0.659	0.448	-0.272	0.427	0.611	-0.747	-0.385	0.68	0.296	0.267	.887*	0.745
GPX	0.835	0.877	0.396	-0.407	-0.811	0.5	0.744	.980**	-0.051	-.996**	-.998**	-0.62	-0.743
GSR	-.958*	-0.447	-0.777	0.379	0.597	-0.763	-0.332	-0.67	-0.469	0.755	0.771	0.108	0.284
GPAT1	0.192	.920*	-0.165	0.051	-0.71	-0.283	.937*	0.743	-0.556	-0.679	-0.657	-.994**	-.925*
HMGCR	-0.07	0.129	0.304	0.652	-0.281	-0.575	0.379	-0.091	0.169	0.069	0.086	-0.378	-0.164
I-FABP	0.852	0.493	.898*	0.22	-0.816	0.428	0.588	0.543	0.591	-0.642	-0.646	-0.336	-0.474
INSIG-1	0.248	0.517	0.404	0.625	-0.693	-0.341	0.749	0.307	0.132	-0.334	-0.315	-0.675	-0.595
LCAD	.968**	0.567	0.668	-0.436	-0.665	0.792	0.426	0.784	0.336	-0.852	-0.867	-0.213	-0.423

*Correlation is significant at the 0.05 level (2-tailed); **Correlation is significant at the 0.01 level (2-tailed)

To be continued on next page………..

Appendix 6 Pearson correlation between hepatic gene expression and fatty acid composition within PL of rats at 2 days after the first cycle of CPT-11 plus 5-FU treatment

Gene	C16:0	C18:0	C18:1n-9	C18:2n-6	C20:4n-6	C20:5n-3	C22:6n-3	SFA	MUFA	PUFA	n-3	n-6	Total
LDLR	0.67	0.745	0.329	-0.563	-0.557	0.282	0.573	0.85	-0.135	-0.85	-0.852	-0.538	-0.48
L-FABP	.949*	0.531	0.86	-0.109	-0.753	0.601	0.512	0.67	0.515	-0.761	-0.77	-0.274	-0.422
LIPIN 1	-0.334	-0.382	-0.545	-0.78	0.726	0.131	-0.658	-0.205	-0.377	0.265	0.252	0.497	0.557
MCAD	.947*	0.388	0.705	-0.463	-0.529	0.878	0.237	0.649	0.452	-0.73	-0.75	-0.008	-0.243
ME 1	0.309	.963**	-0.159	-0.143	-0.722	-0.091	.911*	0.854	-0.567	-0.792	-0.774	-.950*	-.937*
MGAT1	0.438	-0.415	0.568	0.103	-0.018	0.8	-0.392	-0.2	0.799	0.089	0.064	0.656	0.287
MTP	.887*	0.724	0.465	-0.547	-0.68	0.669	0.543	.907*	0.071	-.939*	-.949*	-0.397	-0.552
PEPCK	0.143	-0.268	0.073	-0.807	0.456	0.354	-0.529	-0.009	0.063	0.001	-0.02	0.491	0.573
PPAR-α	-0.404	-0.432	-0.61	-0.672	0.739	0.126	-0.679	-0.277	-0.378	0.339	0.327	0.525	0.537
PPAR-γ	0.787	.939*	0.389	-0.178	-.925*	0.378	.882*	.967**	-0.058	-.981**	-.977**	-0.751	-.880*
ACAT	0.071	0.101	-0.3	-.987**	0.316	0.237	-0.233	0.302	-0.425	-0.248	-0.259	0.105	0.205
SREBP1- α	-0.123	0.773	-0.524	-0.111	-0.394	-0.463	0.73	0.583	-0.833	-0.476	-0.451	-.901*	-0.748
SREBP2	-0.398	-0.167	-0.63	-0.811	0.65	-0.155	-0.443	-0.067	-0.605	0.156	0.152	0.204	0.421
TNF	-0.289	-0.821	0.005	0.31	0.516	0.239	-0.735	-0.747	0.475	0.696	0.681	0.822	0.612
UCP2	0.47	0.169	0.082	-.940*	0.045	0.654	-0.152	0.458	-0.066	-0.46	-0.48	0.176	0.093
VLCAD	0.874	0.537	0.496	-0.649	-0.511	0.772	0.322	0.783	0.172	-0.826	-0.842	-0.168	-0.336
SCD1	-0.202	0.392	-0.386	0.471	-0.381	-0.29	0.528	0.171	-0.398	-0.118	-0.098	-0.559	-0.656
FADS1	-0.594	-0.411	-0.735	-0.645	0.825	-0.197	-0.639	-0.332	-0.551	0.42	0.415	0.398	0.571
FADS2	-0.588	-0.482	-0.723	-0.596	0.838	-0.109	-0.698	-0.388	-0.479	0.467	0.46	0.486	0.594
ELOVL2	0.033	-0.028	-0.147	-0.873	0.388	0.1	-0.309	0.154	-0.271	-0.118	-0.129	0.18	0.374
ELOVL5	-0.147	-0.775	-0.036	-0.385	0.72	0.43	-.916*	-0.538	0.313	0.505	0.48	.937*	0.828
ELOVL6	-0.219	0.452	-0.125	0.604	-0.416	-0.725	0.668	0.143	-0.317	-0.1	-0.072	-0.752	-0.556

*Correlation is significant at the 0.05 level (2-tailed); **Correlation is significant at the 0.01 level (2-tailed)

Appendix 7 Pearson correlation between hepatic gene expression and fatty acid composition within PL of rats at 2 days after the second cycle of CPT-11 plus 5-FU treatment

Gene	C16:0	C18:0	C18:1n-9	C18:2n-6	C20:4n-6	C20:5n-3	C22:6n-3	SFA	MUFA	PUFA	n-3	n-6	Total
ACC	-0.116	-0.44	0.618	0.462	0.084	0.656	0.189	-0.514	0.526	0.311	0.289	0.655	-0.243
ACOX	-0.731	0.767	-0.531	-0.668	0.147	-0.321	-.879*	0.435	-0.643	-0.173	-0.156	-0.451	-0.147
ADRP	-0.857	0.674	-0.6	-0.534	0.353	-0.106	-0.826	0.263	-0.706	0.04	0.056	-0.272	0.02
APO A1	-0.381	0.808	-.944*	-0.739	0.119	-0.515	-0.517	0.629	-.903*	-0.262	-0.236	-0.696	0.167
APO B	0.364	-.900*	0.629	.883*	0.153	0.84	0.854	-0.773	0.678	0.518	0.494	0.878	0.16
CD36	0.556	-.934*	0.71	0.859	0.021	0.4	0.694	-0.681	0.757	0.384	0.366	0.64	0.257
CD68	0.27	-0.569	-0.016	0.571	0.332	0.24	0.491	-0.47	0.092	0.461	0.461	0.356	0.665
CHK-α	-0.293	0.299	0.167	-0.304	-0.152	-0.359	-0.656	0.204	0.027	-0.231	-0.227	-0.25	-0.337
CHK-β	-0.406	0.109	-0.762	0.014	0.646	0.043	-0.162	-0.121	-0.694	0.446	0.466	-0.021	0.799
ACLY	-0.826	0.307	-0.231	-0.171	0.435	0.151	-0.707	-0.092	-0.374	0.269	0.276	0.083	0.059
DGAT1	-0.762	0.635	-0.412	-0.526	0.211	-0.153	-0.819	0.281	-0.538	-0.056	-0.044	-0.277	-0.137
DGAT2	-0.679	0.231	-0.03	-0.135	0.284	0.057	-0.685	-0.083	-0.184	0.173	0.177	0.063	-0.056
FASN	-0.647	0.028	0.06	0.068	0.393	0.096	-0.648	-0.272	-0.094	0.334	0.338	0.193	0.132
FATP4	-0.449	-0.603	0.028	0.748	0.815	.922*	0.228	-.884*	0.012	.940*	.933*	.890*	0.655
FATP5	-0.458	0.587	-0.703	-0.526	0.24	-0.695	-0.817	0.399	-0.732	-0.093	-0.064	-0.625	0.376
GPX	-0.693	0.692	-0.589	-0.602	0.235	-0.551	-.997**	0.391	-0.691	-0.104	-0.08	-0.54	0.126
GSR	-0.817	0.477	-0.504	-0.352	0.458	-0.303	-.965**	0.103	-0.625	0.174	0.196	-0.264	0.303
GPAT1	-0.523	0.365	-0.005	-0.317	0.048	-0.236	-0.753	0.147	-0.156	-0.087	-0.08	-0.188	-0.216
HMGCR	0.541	-.963**	0.832	.893*	-0.027	0.623	0.812	-0.727	0.866	0.384	0.358	0.782	0.069
I-FABP	-.932*	0.317	-0.774	-0.112	0.824	0.272	-0.552	-0.171	-0.818	0.554	0.573	0.106	0.61
INSIG-1	-0.04	-0.306	0.656	0.279	-0.091	0.126	-0.155	-0.301	0.531	0.081	0.068	0.305	-0.265
LCAD	-0.816	0.803	-0.803	-0.664	0.333	-0.224	-0.808	0.41	-0.871	-0.042	-0.021	-0.43	0.075

*Correlation is significant at the 0.05 level (2-tailed); **Correlation is significant at the 0.01 level (2-tailed)

To be continued on next page………..

Appendix 8 Pearson correlation between hepatic gene expression and fatty acid composition within PL of rats at 2 days after the second cycle of CPT-11 plus 5-FU treatment

Gene	C16:0	C18:0	C18:1n-9	C18:2n-6	C20:4n-6	C20:5n-3	C22:6n-3	SFA	MUFA	PUFA	n-3	n-6	Total
LDLR	0.236	-0.497	0.858	0.436	-0.247	0.431	0.318	-0.38	0.782	0.051	0.025	0.514	-0.454
L-FABP	-0.526	0.801	-.898*	-0.721	0.207	-0.654	-0.797	0.569	-.909*	-0.195	-0.165	-0.722	0.261
LIPIN 1	-0.098	-0.061	0.51	0.053	-0.197	0.321	-0.02	-0.104	0.404	-0.072	-0.09	0.27	-0.559
MCAD	-0.648	0.684	-0.765	-0.588	0.319	-0.605	-.933*	0.399	-0.822	-0.052	-0.023	-0.593	0.332
ME 1	-0.635	0.594	-0.312	-0.525	0.111	-0.421	-.922*	0.325	-0.446	-0.144	-0.128	-0.408	-0.106
MGAT1	0.949	-0.466	0.87	0.369	-0.488	0.562	0.733	-0.26	0.906	-0.088	-0.121	0.493	-0.603
MTP	-0.859	0.088	-0.365	0.072	0.747	0.064	-0.766	-0.33	-0.482	0.572	0.589	0.15	0.6
PEPCK	-0.345	-0.049	-0.079	0.151	0.304	0.735	0.224	-0.27	-0.089	0.329	0.318	0.466	-0.045
PPAR-α	-0.314	-0.394	0.356	0.476	0.333	0.835	0.197	-0.587	0.285	0.499	0.481	0.738	-0.034
PPAR-γ	0.498	-0.717	0.688	0.66	-0.141	0.745	.905*	-0.516	0.739	0.216	0.188	0.708	-0.199
ACAT	0.557	-0.849	0.589	0.794	0.001	0.677	.949*	-0.622	0.677	0.358	0.335	0.719	0.117
SREBP1- α	-0.758	0.477	-0.269	-0.364	0.26	0	-0.74	0.12	-0.406	0.057	0.065	-0.102	-0.121
SREBP2	0.146	-.904*	0.392	.940*	0.455	0.84	0.694	-.890*	0.442	0.75	0.734	.907*	0.513
TNF	0.538	-0.786	0.702	0.724	-0.116	0.733	.935*	-0.565	0.762	0.258	0.23	0.73	-0.121
UCP2	0.256	-0.481	-0.111	0.49	0.318	0.245	0.509	-0.394	0.011	0.416	0.418	0.305	0.633
VLCAD	-0.842	0.354	-0.37	-0.197	0.479	0.314	-0.552	-0.073	-0.478	0.295	0.301	0.129	0.061
SCD1	0.152	0.492	0.213	-0.594	-0.659	-0.672	-0.432	0.63	0.133	-0.734	-0.733	-0.61	-0.729
FADS1	-0.492	.970**	-0.682	-.922*	-0.104	-0.652	-0.829	0.767	-0.739	-0.484	-0.462	-0.796	-0.247
FADS2	-0.29	.966**	-0.791	-.938*	-0.179	-0.621	-0.551	0.847	-0.777	-0.553	-0.532	-0.839	-0.23
ELOVL2	-0.355	.969**	-0.556	-.954*	-0.275	-0.674	-0.739	0.837	-0.61	-0.618	-0.6	-0.826	-0.429
ELOVL5	-0.574	0.833	-0.485	-0.777	-0.043	-0.544	-.897*	0.594	-0.589	-0.367	-0.349	-0.629	-0.26
ELOVL6	-0.323	0.471	0.039	-0.473	-0.208	-0.445	-0.715	0.364	-0.095	-0.346	-0.34	-0.391	-0.413

*Correlation is significant at the 0.05 level (2-tailed); **Correlation is significant at the 0.01 level (2-tailed)