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Investigation of fatty acid and cholesterol synthesis using stable isotopes in type 1 diabetes, liver failure, islet and liver transplant, and effect of dietary intervention

by

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This thesis is dedicated to my family - Bruce, Janice, Christina, and Ray

and

for Alessandro Vena

Abstract

Elevated plasma lipids are risk factors for cardiovascular disease (CVD). In certain conditions plasma lipids are normal yet individuals experience increased morbidity. Type 1 diabetes (T1D) is associated with elevated CVD despite normal lipids, while in liver failure low plasma lipids may indicate increasing hepatic damage. Plasma lipids can therefore belie underlying dysregulated lipid metabolism. Islet (ITx) or liver (LTx) transplants represent therapies for T1D and liver failure, respectively, but are associated with altered lipid metabolism attributed to immunosuppressive medications; however, causative mechanisms are unknown. Partial success of dietary therapy in post-transplant patients may be due to interventions limited in scope. Regulation of plasma lipids involve absorption, synthesis, and clearance. These studies examined lipogenesis and cholesterol synthesis using deuterium incorporation.

In brittle T1D lipogenesis and cholesterol synthesis were similar to healthy controls; however hepatic lipogenesis and cholesterol synthesis tended to be lower in T1D compared to matched control subjects.

Plasma cholesterol was lower and triglyceride similar in liver failure patients compared to controls. Lipogenesis was higher while cholesterol synthesis was lower in liver failure compared to controls. Disturbances in lipid synthesis may be influenced by underlying disease, such as hepatitis C.

In ITx and LTx lipogenesis was lower whereas cholesterol synthesis was similar compared to controls. Lipid synthesis is therefore unlikely to contribute to post-transplant hyperlipidemia, inviting investigation of other mechanisms.

Dietary intervention emphasizing fish oil, phytosterols, soy, fibers, and almonds lowered plasma lipids in controls but had mixed effects in transplant subjects. Reduction in plasma lipids occurred in transplant patients with higher baseline lipids, suggesting this intervention may be successful in hyperlipidemic patients; however the potential of this diet intervention requires further study in hyperlipidemic patients. Diet intervention lowered lipogenesis but did not significantly change 24h cholesterol synthesis in controls. Diet did not change 24h lipogenesis or cholesterol synthesis in transplant subjects. Plasma lipid response to dietary therapy was related to baseline cholesterol synthesis and to dietary compliance in transplant subjects. Further study is required to determine if cholesterol synthesis is predictive of response to diet.

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Abbreviations

- AA = arachidonic acid
- ABCAI = ATP-binding cassette sub-family A member 1
- ABCG5/G8 = ATP-binding cassette sub-family G member 5/8
- ACAT = acyl-CoA:cholesterol acyltransferase
- ACC = acetyl CoA carboxylase
- ADH = alcohol dehydrogenase
- AHA = American Heart Association
- ALA = alpha-linolenic acid
- APE = atom percent excess
- Apo = apolipoprotein
- ASR = absolute synthetic rate
- BMI = body mass index
- C = combustion
- CD36/FABP = fatty acid binding protein
- CE = cholesteryl ester
- CETP = cholesteryl ester transfer protein
- ChREBP = carbohydrate responsive element-binding protein
- CM = chylomicron
- CM-R = chylomicron remnants
- CRP = C-reactive protein
- CVD = cardiovascular disease
- DAG = diacylglycerol
- DASH = Dietary Approaches to Stop Hypertension
- DGAT = diacylglycerol acyltransferase
- DHA = docosahexanoic acid
- DNFA = de novo synthesized fatty acid
- $DNFA_{Total}$ = total de novo synthesized fatty

  acid
- DNL = de novo lipogenesis
- EPA = eicosapentanoic acid
- ER = endoplasmic reticulum
- FA = fatty acid
- FAS = fatty acid synthase
- FC = free cholesterol
- FKBP-12 = FK506 binding protein
- FSR = fractional synthetic rate
- GC = gas chromatography
- GC/MS = gas chromatography/ mass spectrometry
- HBV = hepatitis B virus
- HCC = hepatocellular carcinoma
- HCV = hepatitis C virus
- HDL = high-density lipoprotein
- HDL-c = high-density lipoprotein cholesterol
- HIV = human immunodeficiency virus
- HL = hepatic lipase
- HMGCoA = 3-hydroxy-3-methyl-glutaryl CoA
- HMGCoA-r = HMGCoA-reductase
- HSL = hormone-sensitive lipase

• IDL = intermediate-density lipoprotein

• IL-6 = interleukin 6

• IRMS = isotope ratio mass spectrometry

• ITx = islet transplant

• LA = linoleic acid

• LCAT = lecithin:cholesterol acyltransferase

• LDL = low-density lipoprotein

• LDL-c = low-density lipoprotein cholesterol

• LDL-r = LDL receptor

• LPL = lipoprotein lipase

• LTx = liver transplant

• LXR = liver X receptor

• MAG = monoacylglycerol

• MELD = model-for-end-stage liver disease

• MIDA = mass isotopomer distribution analysis

• MMF = mycophenolate mofetil

• mTOR = mammalian target of rapamycin

• MTTP = microsomal triglyceride transfer protein

• MUFA = monounsaturated fatty acids

• NAFLD = non-alcoholic fatty liver disease

• NASH = non-alcoholic steatohepatitis

• NCEP = National Cholesterol Education Program

• NHANES = National Health and Nutrition Examination Survey

• NPC1L1 = Niemann-pick cell-1 like-1 protein

• P = pyrolysis

• PL = phospholipid

• PLTP = phospholipid transfer protein

• PPAR = peroxisome proliferator-activated receptor

• PROVE IT-TIMI = Pravastatin or Atorvastatin Evaluation and Infection Therapy - Thrombolysis in Myocardial Infarction 22

• PUFA = polyunsaturated fatty acids

• SCD-1 = stearoyl-CoA desaturase-1

• SFA = saturated fatty acids

• SR-BI = scavenger receptor class B type I

• SREBP = sterol regulatory element-binding protein

• TC = total cholesterol

• TC-EA = thermal combustion elemental analyzer

• T1D = Type 1 diabetes

• TG = triglyceride

• TNF-α = tumour necrosis factor α

• TPL = total plasma phospholipids

• TTR = tracer-tracee ratio

• V-FC = VLDL free cholesterol

• VLDL = very low-density lipoprotein

1. Introduction

Lipids are a major influence on health and disease processes, and appear to be a central factor in development and worsening of a variety of conditions such as cardiovascular disease (CVD). CVD affects millions of people and is a major cause of death worldwide. Elevated plasma lipid levels have been implicated in the development and progression of CVD, yet there are segments of the population in whom plasma lipid levels are considered "normal" who still experience CVD-related morbidity and mortality. Presence of "normal" plasma lipids can therefore belie underlying dysregulated lipid metabolism. Diabetes is estimated to affect 11.6% of Canadians (*International Diabetes Federation 2009*), while type 1 diabetes makes up approximately 10% of this figure. Mortality in Type 1 diabetes is not frequently due to diabetes-related complications but rather primarily to CVD. Individuals with Type 1 diabetes often have normal lipid levels, which would normally confer protection against CVD. Diabetes, therefore, may induce changes in lipid metabolism contributing to CVD risk in Type 1 diabetes patients, however the mechanisms are not fully clear. Other conditions, such as liver failure, are associated with normal or low plasma lipids yet an increased morbidity. In liver failure low plasma lipids may indicate increasing hepatic damage, and may be mediated through disease-specific mechanisms. Individuals with Type 1 diabetes who receive an islet transplant enjoy freedom from exogenous insulin and issues with glucose tolerance. Similar to other organ transplant patients, such as liver transplant, long-term survival is hampered by morbidity and mortality related to CVD. Immunosuppressive medications may influence lipid metabolism and contribute to CVD in transplant recipients, though mechanisms are not clear. Dietary therapy in post-transplant patients has met with only partial success, and may be mediated by influence of immunosuppressive medications. Changes in

plasma lipid levels may be due to alterations in absorption, synthesis or clearance. Therefore, analysis of these processes may shed light on the metabolic pathways that are altered in diseases associated with normal plasma lipid levels and increased morbidity, allowing for development of appropriate treatment modalities.

1.1. Lipid Metabolism

Lipids are a very diverse class of molecules and comprise a variety of structures. Lipids have a wide variety of functions including cell membrane and structural support, detergents, hormones, and energy substrates.

1.1.1. Digestion and absorption of dietary lipids

Lipases hydrolyze up to 30% of dietary triglyceride (TG) into diacylglycerol and fatty acids (FA) before entry into the intestine (*Wilson et al. 1994*; *Hui et al. 2002*). Two sources of cholesterol are present in the intestine: exogenous cholesterol from dietary sources represents approximately a quarter of cholesterol present, while endogenous cholesterol from bile comprises the rest (*Grundy 1983*; *Gylling 2004*; *Wang 2007*). Pancreatic lipases facilitate lipid breakdown in the intestinal lumen, which hydrolyze all lipids into FA, glycerol, monoacylglycerol, and free cholesterol (FC) (*Wilson et al. 1994*; *Hui et al. 2005*; *Wang 2007*). Bile salts released from the liver emulsify dietary cholesterol, TG, FA and other lipids, forming micelles which transport lipids across the unstirred water layer to the brush border membrane (*Grundy 1983*; *Wilson et al. 1994*; *Hui et al. 2002*; *Gylling 2004*).

Absorption and metabolism of cholesterol and FA are highly regulated processes (**Figure 1-1**). FA are reported to be taken up by fatty acid binding proteins, and once inside the enterocyte FA are re-esterified into TG for incorporation and transport in CM through the lymphatic system (*Hui et al.*

2002). Transfer of FC from micelles into enterocytes occurs via mediated and non-mediated transport mechanisms. The Niemann-pick cell-1 like-1 protein (NPC1L1) transporter expressed on the brush border membrane is responsible for the majority of cholesterol absorption by the intestine (*Altmann et al. 2004*; *Wang 2007*), however cholesterol can also be taken up by simple passive diffusion down a concentration gradient (*Turley et al. 2003*). ATP-binding cassette G5 and G8 proteins function as sterol efflux pumps, promoting efflux of cholesterol and plant sterols from the enterocyte back into the intestinal lumen for excretion (*Levy et al. 2007*; *Wang 2007*). Bile acids are absorbed into enterocytes and return to the liver via the enterohepatic circulation (*Thomson et al. 2001*). Once absorbed, the majority of dietary-derived cholesterol is esterified by acyl-CoA:cholesterol acyltransferase to cholesteryl ester (CE) for incorporation into chylomicrons (CM) (*Thomson et al. 2001*; *Hui et al. 2005*; *Wang 2007*). Evidence also suggests that the intestine synthesizes cholesterol which contributes to cholesterol secreted in CM particles (*Wilson et al. 1968*; *Stange et al. 1985*), fecal neutral sterol excretion (*Wilson et al. 1968*; *Kruit et al. 2006*) and may contribute to HDL particles (*Stange 1987*). There may also be intestinal secretion of cholesterol from lipoprotein particles via transintestinal cholesterol transport, however this field is relatively novel and requires further investigation (*Vrins 2010*).

1.1.2. Postprandial lipid metabolism

Pre-CM are synthesized in the rough endoplasmic reticulum with addition of apolipoprotein (Apo) ApoB48, which is required for assembly of CM particles (*Hui et al. 2005*; *Chahil et al. 2006*). Microsomal triglyceride transfer protein (MTTP) incorporates TG, FC, and CE into CM particles, along with phospholipids (PL) and ApoB48 (*Hui et al. 2005*; *Zawaideh et al. 2006*). Mature CM migrate to the basolaterol membrane where they fuse with the plasma membrane and are released into the intestinal lymph (*Hui et al. 2002*;

Hui et al. 2005). CM travel through the lymphatic system acquiring other apolipoproteins such as ApoCI, CII, CIII, and E, and enter the bloodstream through the thoracic duct (*Grundy 1983*; *Chahil et al. 2006*). As CM travel in the bloodstream, lipoprotein lipase (LPL) located on the surface of capillary endothelial cells hydrolyzes TG to release FA which are used for energy via β-oxidation by muscle cells, or stored as TG in adipose tissue (*Grundy 1983*; *Hui et al. 2002*; *Chahil et al. 2006*). Hepatic lipase (HL) also contributes to hydrolysis of lipoprotein-associated TG (*Chahil et al. 2006*). CM-remnants (CM-R) are cleared by the liver via the LDL-receptor (LDL-r) as well as the LDL receptor-related protein (*Grundy 1983*; *Hui et al. 2002*; *Groen et al. 2004*; *Chahil et al. 2006*). ApoE acts as a ligand for hepatic lipoprotein receptors to facilitate hepatic uptake of lipoprotein particles (*Chahil et al. 2006*).

The liver plays a central role in regulating plasma lipoproteins and therefore is often considered the body's cholesterol homeostasis organ (*Groen et al. 2004*). The liver is also important for cholesterol homeostasis by the ability to up- or down-regulate cholesterol synthesis as well as lipoprotein uptake in response to stimuli, including fasting and CM delivery of absorbed cholesterol (*Dietschy et al. 1993*). Dietary and biliary cholesterol delivered by CM-R and other lipoproteins are primarily esterified to CE for storage or secretion into plasma with very low-density lipoprotein (VLDL) but can also contribute to biliary cholesterol (*Grundy 1983*; *Stange 1987*; *Dietschy et al. 1993*). The exception is HDL-derived cholesterol which may preferentially contribute to biliary cholesterol and bile acid formation (*Schwartz et al. 1978*; *Stange 1987*). Newly-synthesized cholesterol appears to be preferentially used for synthesis of bile acids and contribution to biliary cholesterol as opposed to secretion into plasma with VLDL (*Stange 1987*; *Bilhartz et al. 1989*; *Empen et al. 1997*). Hepatic stores of TG destined for packaging in VLDL particles can come from FA released from adipose tissue and taken up by the liver, FA coming from CM or other lipoprotein remnants,

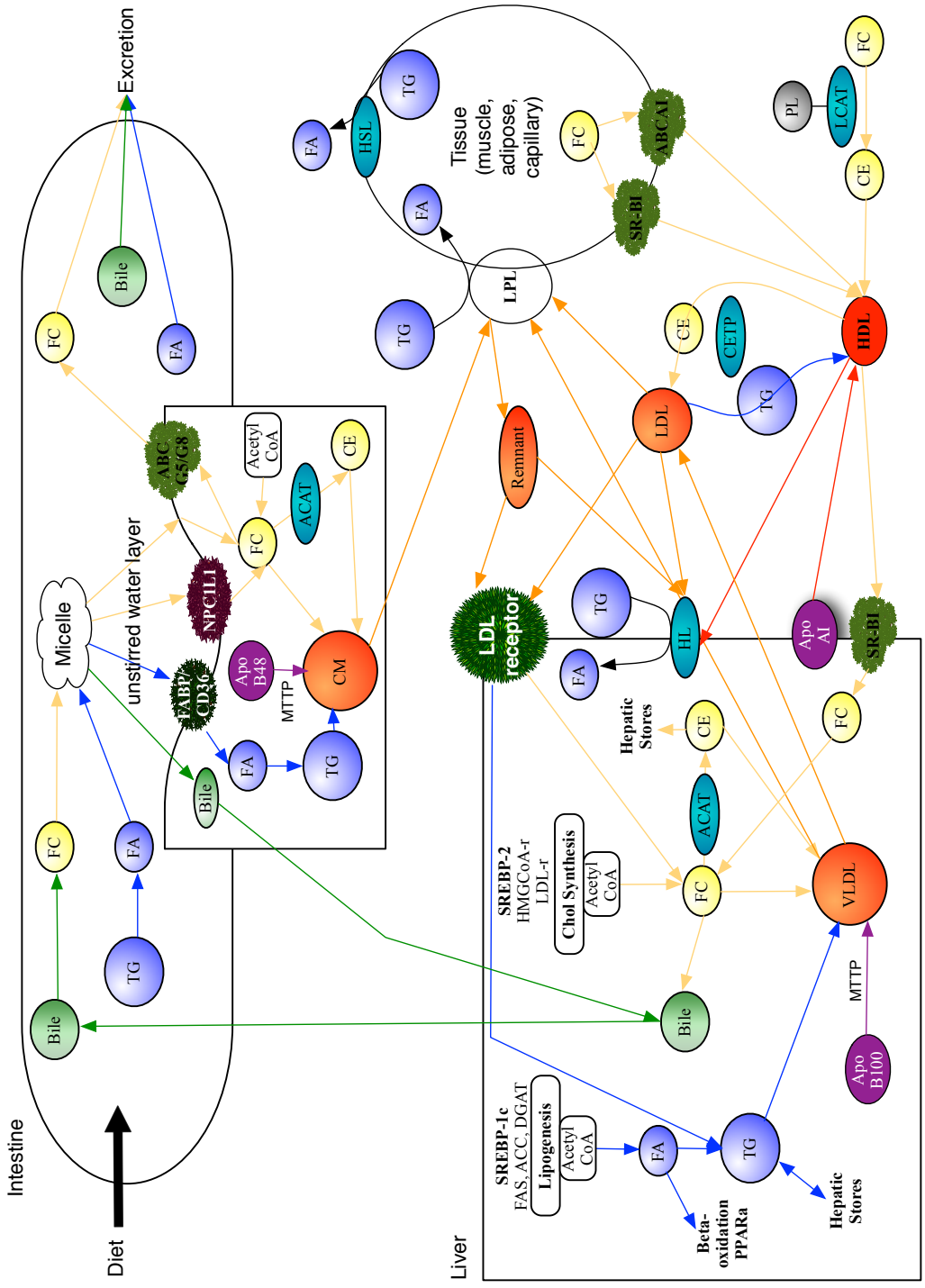
or from de novo lipogenesis within the liver (*Chahil et al. 2006*). VLDL is synthesized with addition of ApoB100 and assembly of lipids including TG, FC, CE, and PL, mediated by MTTP (*Chahil et al. 2006*). ApoB100 is a ligand for LDL-r, and plays a role in lipoprotein uptake and clearance from the circulation (*Chahil et al. 2006*).

VLDL circulates in the plasma and is hydrolyzed by LPL on the surface of endothelial cells to release TG as well as FC and PL to tissues, and in the process is converted to intermediate-density lipoprotein (IDL) or VLDL-remnants (*Chahil et al. 2006*). Amounts of ApoCII and ApoCIII in VLDL particles may influence LPL hydrolysis as ApoCII activates while ApoCIII inhibits LPL activity (*Chahil et al. 2006*). VLDL can acquire CE via exchange of TG with high- (HDL) or low-density (LDL) lipoprotein particles by cholesteryl ester transfer protein (CETP) (*Grundy 1983*; *Chahil et al. 2006*). VLDL-remnants can either be removed by the liver or further hydrolyzed by HL to form LDL (*Chahil et al. 2006*), and much of the cholesterol content of VLDL is transferred to LDL (*Grundy 1983*). LDL particles are primarily composed of CE, and deliver cholesterol to peripheral tissues (*Grundy 1983*; *Chahil et al. 2006*). CETP can modify TG content of LDL particles by increasing transfer of TG from VLDL and CM to LDL in exchange for CE, creating TG-rich LDL particles (*Chapman et al. 2010b*). HL then hydrolyzes TG in LDL particles creating small dense LDL (*Chapman et al. 2010b*). Cholesterol concentrations in extrahepatic tissues are influenced by the balance of de novo synthesis, influx from LDL delivery and efflux by HDL uptake (*Grundy 1983*; *Groen et al. 2004*). LDL can be taken up by peripheral tissues or the liver by the LDL-r (*Groen et al. 2004*).

Extrahepatic tissues are capable of making cholesterol and therefore do not require exogenous cholesterol from lipoproteins (*Groen et al. 2004*), however making cholesterol is an energetically expensive process. Since excess cholesterol cannot be degraded in peripheral tissues it must be

returned to the liver for degradation and excretion, known as "reverse cholesterol transport" in which HDL is the primary player (*Grundy 1983*; *Groen et al. 2004*). ApoAI synthesized by the liver acquires cholesterol and PL effluxed from tissues, such as hepatocytes and intestinal cells, by ABCAI, thereby forming nascent HDL (*Groen et al. 2004*; *Ohashi et al. 2005*). Scavenger receptor class B type 1 protein may facilitate binding of HDL to cells and for lipid reorganization in the plasma membrane (*Lewis et al. 2005*; *Ohashi et al. 2005*). Nascent HDL further acquires cholesterol and PL via transfer of surface lipids of CM and VLDL during lipolysis by LPL (*Lewis 2006*). FC in the outer core of HDL is esterified by lecithin:cholesterol acyltransferase, prompting CE to move towards the HDL core and increasing particle size (*Grundy 1983*; *Lewis et al. 2005*; *Ohashi et al. 2005*). Phospholipid transfer protein also modulates the transfer of PL from TG-rich lipoproteins to HDL during lipolysis by LPL (*Lewis et al. 2005*; *Ohashi et al. 2005*). Exchange of HDL-CE for TG from ApoB-containing lipoproteins is mediated by CETP, which is an alternate delivery method for HDL-c to reach the liver (*Lewis et al. 2005*; *Ohashi et al. 2005*; *Rader et al. 2009*). TG-rich HDL particles can undergo lipolysis by HL, creating small HDL particles which do not bind efficiently to ApoAI and therefore are cleared from plasma reducing HDL particle number (*Chahil et al. 2006*). Mature HDL particles then deliver cholesterol to the liver through SR-BI for removal (*Chahil et al. 2006*). HDL catabolism takes place primarily in the kidney, liver, and steroidogenic tissues (*Lewis et al. 2005*).

Figure 1-1: General overview of postprandial lipid metabolism



Lipid metabolism therefore involves multiple points of regulation and compensation by enzyme and transporter protein activities, and genetic control of gene expression for synthesis and absorption (**Figure 1-1**). Elevation in plasma lipid levels can occur due to imbalances related to synthesis, absorption, and clearance.

1.2. Cardiovascular Disease

CVD is the leading cause of death in the U.S., Canada, and other developed countries, accounting for ~30% of all deaths (*Rosamond et al. 2008*; *Kriekard et al. 2009*). Growing awareness and treatment of traditional risk factors such as smoking, blood pressure, and total cholesterol (TC) has led to a reduction in CVD mortality over the past few decades (*Cooney et al. 2009*), however this effort has been hampered by the increase in prevalence of obesity and diabetes (*Cannon 2007*).

1.2.1. Cardiovascular Disease - Pathogenesis

The clinical impact of CVD is development of atherosclerosis, in which arteries of the heart, brain and periphery become hardened and narrowed with plaque. Atherosclerosis is the gradual development from a "fatty streak" to advanced lesion, causing reduction in blood flow and eventually occlusion. Main processes in lesion development include accumulation of lipoproteins, inflammatory and smooth muscle cells, and formation of connective tissue such as collagen, elastin, and proteoglycans (**Figure 1-2**) (*Mallika et al. 2007*). These processes lead to endothelial cell death and formation of plaque.

Endothelial damage causes production of thromboxane A2 and prostaglandins to induce vasoconstriction, and also triggers platelet aggregation and adherence at the site of damage (*Mallika et al. 2007*). Endothelial damage also causes the endothelium to be leaky, which allows molecules and lipoprotein particles to enter, be retained, and modified (*Falk*

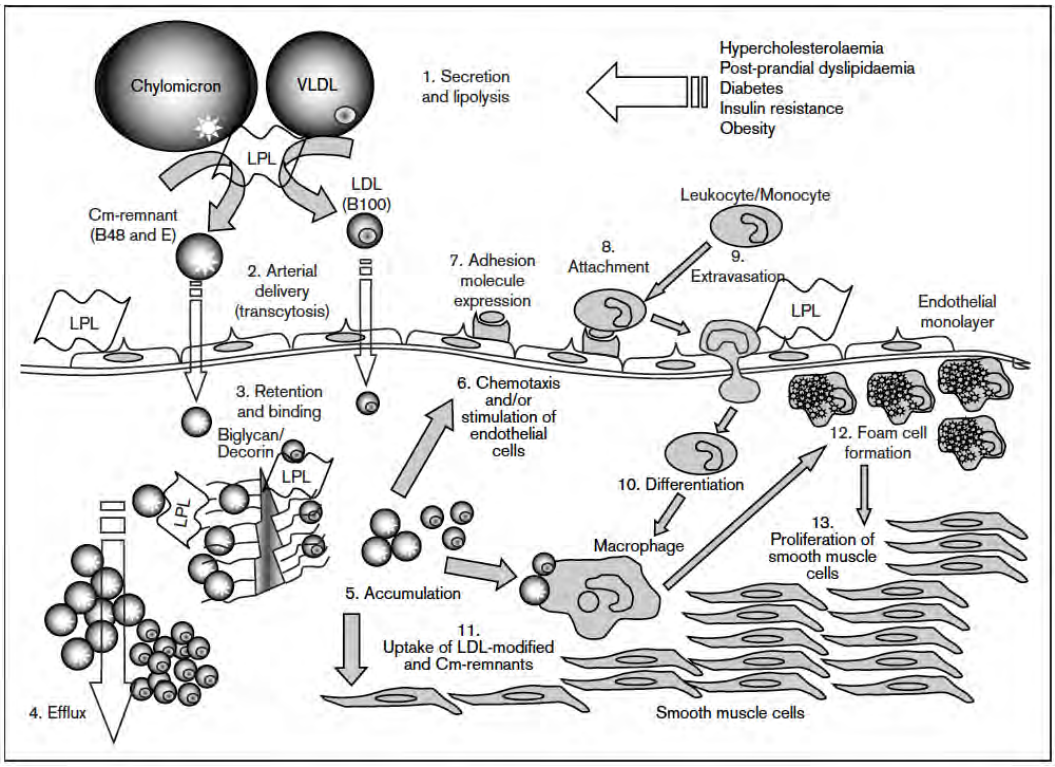
2006). Platelet aggregation stimulates monocytes and T-cells to enter and proliferate near the damaged site causing the arterial wall to bulge, initiate coagulation cascades, and cause pro-inflammatory cytokine secretion (*Falk 2006*; *Mallika et al. 2007*; *Galkina et al. 2009*). Bulging of the arterial wall and coagulation progressively cause the arterial lumen to narrow, constricting blood flow (*Mallika et al. 2007*). Occlusion in blood flow can stimulate smooth muscle cell proliferation, as well as trigger platelet growth factors which further stimulate smooth muscle cell proliferation and collagen synthesis contributing to plaque formation (*Mallika et al. 2007*).

Free radicals or reactive oxygen species and lipid peroxidation products generated within the cell can disrupt plasma membranes by altering fatty acid structure, as well as cause chronic activation of the endothelium and immune system (*Mallika et al. 2007*; *Galkina et al. 2009*). Reactive oxygen species that overwhelm the body's natural antioxidant mechanisms, such as superoxide dismutase and glutathione, induce oxidative stress contributing to endothelial and mitochondrial dysfunction, inflammation, and damage to cellular lipids, proteins, and DNA (*Higashi et al. 2009*; *Puddu et al. 2009*). Oxidative stress can cause oxidation of polyunsaturated fatty acids (PUFA) in LDL particles (*Itabe 2009*), altering the particle structure through ApoB modification which impairs the ability of LDL to bind to the LDL-r (*Mallika et al. 2007*).

In the arterial intima monocytes differentiate into macrophages and readily take up oxidized LDL and other lipoproteins, creating foam cells (*Falk 2006*; *Mallika et al. 2007*; *Galkina et al. 2009*). Smooth muscle cells secrete extracellular matrix factors such as collagen which increase retention of lipoproteins (*Rader et al. 2008*). Macrophages continue to accumulate lipid-rich lipoproteins in an unregulated fashion until apoptosis, contributing to development of a lipid-rich unstable plaque core, also called a "fatty streak" (*Falk 2006*; *Rader et al. 2008*).

As atherosclerosis progresses, endothelial, foam, and smooth muscle cells die by apoptosis or necrosis, possibly due to inflammatory mediators, activated T-cells, or hypoxia, releasing lipid-rich contents and contributing to unstable plaque (*Falk 2006*; *Mallika et al. 2007*; *Rader et al. 2008*; *Moreno et al. 2009*). Inflammatory mediators may cause the fibrous cap of the atheroma to weaken and become unstable by secretion of metalloproteinases from foam cells which degrade the cap, allowing it to become infiltrated with macrophages and T-cells (*Mallika et al. 2007*; *Ambrose et al. 2010*). As plaque builds in the arterial wall the arterial lumen progressively narrows, leading to reduced blood flow that can affect the heart and brain. Unstable plaque may, alternatively, rupture, leading to blood clot, myocardial infarction, or stroke (*Falk 2006*; *Mallika et al. 2007*; *Rader et al. 2008*).

Figure 1-2: Development and progression of atherosclerotic lesion



From: Proctor, Vine and Mamo (2002) *Curr Opin Lipidol* 13:461-470; Atherosclerotic lesion development and progression. Lipoprotein lipolysis and delivery to arterial endothelium (stage 1 and 2), retention within arterial intima and efflux (stage 3 and 4). Lipids accumulate (stage 5) and stimulate proliferation of endothelial cells (stage 6) and

expression of adhesion molecules (stage 7), which attract inflammatory cells to infiltrate the endothelium (stage 8 and 9). Monocytes differentiate into macrophages (stage 10), which can take up modified LDL and CM remnants (stage 11), forming foam cells (stage 12) and stimulating proliferation of smooth muscle cells (stage 13).

1.2.2. Cardiovascular Disease - Traditional and Novel Risk Factors

Traditional risk factors associated with CVD include non-modifiable factors such as age and male gender, and modifiable risk factors such as TC, LDL-cholesterol (LDL-c), systolic blood pressure, diabetes, and smoking (*Wilson et al. 1998*; *Cannon 2007*). These factors have been shown to be predictive of CVD events such as myocardial infarction, stroke, and death (*Pencina et al. 2009*).

Among US adults >65 years of age participating in the National Health and Nutrition Examination Survey (NHANES) 1999-2004 datasets, ~70% have hypertension, 60% have dyslipidemia, and 20% have diabetes (*McDonald et al. 2009*). These prevalence rates may be higher than those observed in other developed countries (*Panagiotakos et al. 2009*). Nonetheless, it is evident that prevalence of these risk factors represents a significant proportion of the population in developed countries, and rates may be increasing along with obesity and reductions in physical activity (*Panagiotakos et al. 2009*).

Obesity is becoming increasingly prevalent, with one third of the population being overweight and another one quarter considered obese (*Rosamond et al. 2008*). Central obesity in particular, as measured by waist circumference and waist-to-hip ratio but not body mass index (BMI), has been found to be a significant predictor of CVD death, even when corrected for traditional Framingham risk factors, and may help to identify those at risk of CVD death in those who have a lower Framingham score (*Dhaliwal et al. 2009*). Central obesity is growing increasingly prevalent (*Preis et al. 2009*), and may be present in 25% of the population even in those who do not have diabetes or CVD (*Knuiman et al. 2009*). Obesity is positively associated with

number of metabolic syndrome components, with those having more risk factors having higher waist circumference (*Knuiman et al. 2009*). However, obesity itself can be considered an independent risk factor which may be mediated through the co-existance of factors such as insulin resistance and inflammation that often accompanies presence of obesity (*Dhaliwal et al. 2009*).

C-reactive protein (CRP) is a relatively newer risk factor for CVD and is implicated by the inflammatory nature of atherosclerotic development (*Nordestgaard et al. 2009*). CRP is an acute-phase protein that has been consistently reported to be associated with CVD, particularly acute and fatal CVD as opposed to stable and non-fatal CVD (*Abdelmouttaleb et al. 1999*; *Sattar et al. 2009*; *Nordestgaard et al. 2009*). CRP and other inflammatory mediators such as interleukins (IL) may promote atherosclerosis by inducing adhesion molecules in endothelial cells as well as activating the complement system which can increase inflammation within plaques (*Bisoendial et al. 2007*). There is currently controversy regarding the role of CRP in CVD, as it is not clear whether CRP is a marker of CVD or an active player in CVD development and progression (*Nordestgaard et al. 2009*).

As inflammatory markers such as CRP and other cytokines are increased in states of inflammation and infection, it has been questioned whether CVD may the result of unresolved infection or past exposure to common infection (*Elkind et al. 2010*). Infections are proposed to contribute to CVD by direct injury, remote systemic induction of inflammatory cells, cross-reactivity with self-antigens causing an autoimmune reaction, and dysregulation of cellular activity (*Fong 2000*; *Elkind et al. 2010*). Common infections such as Chlamydia pneumoniae, Helicobacter pylori, and cytomegalovirus in particular have been implicated. "Infectious burden" of prior infection with common viruses has been shown to be significantly associated with carotid plaque thickness, atherosclerosis, myocardial

infarction, and stroke (*Espinola-Klein et al. 2000*; *Smieja et al. 2003*; *Elkind et al. 2006*; *Elkind et al. 2010*).

It has been shown previously that risk factors tend to cluster in certain populations such as those with diabetes or obesity (*Acharjee et al. 2010*). This appears to be particularly true for metabolic syndrome, which is a combination of any three of the following factors: elevated waist circumference, plasma TG, blood pressure, and fasting glucose and low plasma HDL-c (*Knuiman et al. 2009*). Recent analyses have suggested that it is the sum of individual factors as well as the number of risk factors present may be more informative as to CVD risk than diagnosis of the metabolic syndrome per se (*Knuiman et al. 2009*; *Baldassarre et al. 2010*). Indeed, in the recent PROVE IT-TIMI 22 trial, a greater number of risk factors correlated strongly with increased rate of death, myocardial infarction, and stroke (*Acharjee et al. 2010*). Risk factors included in this study were age >65 years, male, family history of premature CVD, elevated LDL-c, low HDL-c, elevated systolic blood pressure, diabetes, smoking, elevated CRP, elevated TG, pre-diabetes, and obesity defined as BMI $\geq$30 kg/m^2 (*Acharjee et al. 2010*).

1.2.3. Cardiovascular Disease - Role of Lipids

LDL-c is considered a classic risk factor for CVD. LDL-c is implicated in the development and progression of atherosclerosis via deposition of cholesterol by oxidized LDL particles into damaged endothelium (**Figure 1-2**), contributing to plaque formation (*Mallika et al. 2007*). LDL-c has been a major focus of treatment and prevention trials in the past few decades. Elevated LDL-c levels are present in 25% of the population (*Rosamond et al. 2008*). LDL-c has been shown to have a positive association with CVD risk in a number of large epidemiological studies (*Castelli et al. 1992*; *Wilson et al. 1998*), and reducing LDL-c has been shown to reduce CVD risk (*LaRosa et al. 2005*; *Preiss et al. 2009*). Definitions of LDL-c concentrations have largely

been determined through results from epidemiological studies of cholesterol concentrations and incidence of cardiovascular events, as well as studies that suggested that lowering plasma cholesterol levels reduces cardiovascular risk; these theories have sometimes been criticized for overgeneralization of findings, patient selection criteria, and translation from animal studies (*NCEP 1988*; *Stehbens 1993*; *Stehbens 2001*). The literature suggests that of those individuals receiving treatment for elevated LDL-c levels, only half achieve goal lipid levels (*McDonald et al. 2009*). Despite the prevalent use of statin therapy and the documented ability of statins to reduce LDL-c as well as CVD risk, a significant portion of individuals still experience cardiovascular events despite attainment of "normal" or low LDL-c levels (*Cannon 2007*; *Acharjee et al. 2010*). In addition, the definition of "normal" LDL-c levels for the general population may not translate to levels appropriate for an individual or for a specific disease group. While the general relationships between LDL-c and CVD risk may be similar across groups, there may be quantitative differences. For example, it has been suggested that at a given concentration LDL-c may be less atherogenic in women compared to men, and this relationship may qualitatively change as women become post-menopausal and the amount of small, dense LDL increases; such an increase in small LDL particles would not necessarily be reflected in higher LDL-c concentrations, leading LDL-c concentration to be a relatively poor predictor in women (*LaRosa 1992*; *Bass et al. 1993*). Despite tailoring of recommendations in some specific groups (*Antonopoulos 2002*), there is still a significant risk of CVD mortality in these groups. Therefore, risk factors other than LDL-c concentration must play an equal or greater role in CVD development and progression.

Particle size and composition may be important factors, influencing how particles interact with receptors, transfer proteins, and arterial vessels. Smaller LDL particle size may be associated with increased occurrence of CVD events, as well as increased particle concentration (*Campos et al. 1992*;

Blake et al. 2002). Prevalence of small dense LDL particles may be due to elevated plasma TG levels, as TG levels show strong correlation with small LDL particles (*Miller et al. 2008*). Small, dense LDL particles are considered more atherogenic because they more readily penetrate the arterial intima and may be more prone to oxidation, causing endothelial dysregulation and lipid accumulation leading to formation of foam cells and plaque (*Proctor et al. 2002*; *Chapman et al. 2010b*).

Abnormal HDL-c and TG concentrations are cornerstones of the metabolic syndrome, and are also now considered significant risk factors for CVD (*Knuiman et al. 2009*; *Evangelista et al. 2009*; *Sharma et al. 2009*). Low HDL-c and high TG levels may be prevalent even in individuals without diabetes or CVD (*Knuiman et al. 2009*). A significant portion of individuals who have achieved target goals for TC and LDL-c may have low HDL-c and high TG, indicating a residual dyslipidemia and therefore metabolic risk; this is likely because statin therapy has only modest effects on non-LDL-c (*Belsey et al. 2008*; *Chapman et al. 2010b*). Low HDL-c is negatively associated and high TG levels positively associated with an increasing number of risk factors (*Knuiman et al. 2009*; *Acharjee et al. 2010*). The protective effect of HDL-c is associated with the reverse cholesterol transport pathway responsible for cholesterol efflux from tissues back to the liver for excretion (*Rader et al. 2008*; *Ferns et al. 2008*). HDL particles may also be protective by increasing bioavailability of nitric oxide, or exhibit anti-inflammatory and anti-oxidative effects (*Rader et al. 2008*; *Chapman et al. 2010b*). Elevated TG is considered to be a marker for atherogenic lipoproteins and remnants, such as CM and VLDL, and increased formation of small dense LDL particles (*Alagona 2009*; *Chapman et al. 2010b*). This is particularly evident in individuals with elevated TG levels, as non-HDL cholesterol is often found to be a better predictor of atherogenic risk than LDL-c (*Alagona 2009*). Low HDL-c is often observed with elevated TG concentrations and is suggested to be due to

enhanced transfer of TG from TG-rich lipoproteins such as VLDL to HDL particles in exchange for CE, mediated by cholesteryl ester transfer protein; such exchange of TG to HDL can result in reduction of HDL particle size and increased renal catabolism leading to reduced HDL-c (*Chapman et al. 2010a*).

ApoB-carrying lipoproteins, comprising CM, VLDL, and LDL particles, represent TG-rich lipoproteins and non-HDL-associated cholesterol. Non-HDL cholesterol is positively correlated with ApoB concentration, and may be elevated due to abnormal secretion or catabolism of ApoB-containing particles (*Miller et al. 2008*). ApoB concentration has been shown to predict CVD risk, and sometimes better than TC or LDL-c (*Miller et al. 2008*; *McQueen et al. 2008*; *Contois et al. 2009*). ApoB concentration may be of particular use in predicting risk in individuals who have normal LDL-c levels or treated with statins (*Blake et al. 2002*; *Kastelein et al. 2008*; *Brown et al. 2008*; *Kampoli et al. 2009*).

Measurement of lipids for clinical diagnostic purposes are typically taken in the fasting state. However, most of the waking hours are spent in the fed or postprandial state. Therefore it has been proposed that elevations in postprandial lipid levels, or rather the way in which the body handles lipids following a meal, may contribute to atherosclerosis. Fasting levels of LDL-c and TG have been found to be correlated with non-fasting LDL-c and TG levels, however the relationships of fasting plasma lipid values with postprandial total change in plasma cholesterol or TG (incremental and/or area under the curve) show varying sensitivities which may be influenced by subjects studied, duration of testing, and test meal composition (*Cohn et al. 1988*; *Pirro et al. 2001*; *Chung et al. 2004*; *Mora et al. 2009*). Fasting plasma TG has been reported to be significantly related to total and incremental change in postprandial TG levels, however postprandial TG levels have a greater association with remnant lipoprotein levels (*Cohn et al. 1988*; *van Wijk et al. 2003*; *Nakajima et al. 2009*; *Rector et al. 2009*). Some reports suggest a

greater association between postprandial TG levels and fasting HDL-c levels (*Patsch et al. 2000*). In contrast, there are differing reports on the relationship between fasting plasma total or LDL cholesterol and change in postprandial cholesterol levels (*Cohn et al. 1988*; *Langsted et al. 2008*). Postprandial hyperlipidemia may arise by enhanced production of lipoprotein particles by the intestine and liver, impaired clearance from the circulation by enzymatic and receptor-mediated mechanisms, or both. CM, produced by the intestine, and VLDL, produced by the liver, compete for catabolism and clearance; if there are more such particles in the circulation, these processes can be saturated, resulting in accumulation of remnant CM and VLDL particles (*Alipour et al. 2008*). Postprandial hyperlipidemia may contribute to atherosclerosis by increasing the time for lipoprotein and remnant particles to associate with endothelial and inflammatory cells (*Alipour et al. 2008*). Reduced clearance of lipoprotein and remnant particles allows longer time for association with arterial endothelial cells for uptake, as well as interaction with inflammatory cells, promoting formation of foam cells (*Alipour et al. 2008*; *Su et al. 2009b*). CM and VLDL particles and remnants have been shown to also be readily taken up by endothelium and stimulate smooth muscle cell proliferation, and may be exported less readily from arterial tissue than LDL particles (*Proctor et al. 1998*; *Proctor et al. 2003*; *Proctor et al. 2004*; *Chapman et al. 2010b*). Concentration of cholesterol, ApoB, and TG in VLDL particles have been shown to predict recurrent coronary events as well as progression of atherosclerotic lesions, and may be inversely correlated with LDL-c (*Alaupovic et al. 1997*; *Sacks et al. 2000*). This finding indicates that atherogenic particles may be present even in those with normal or low LDL-c, and would represent a portion of the population that experience coronary events in the absence of elevated LDL-c. If LDL-r are overloaded, VLDL particles may spend more time in the circulation allowing for greater lipolysis via LPL, resulting in production of small, dense LDL particles which are more atherogenic (*Alipour et al. 2008*).

Pharmacotherapy is typically used to target a specific risk factor, such as LDL-c, although many drugs have other unexpected effects; for example, statin therapy may also have anti-inflammatory effects (*Ridker et al. 2008*). However, literature suggests that of individuals receiving treatment for elevated LDL-c levels, only half of individuals achieve goal lipid levels (*McDonald et al. 2009*). In addition, low HDL-c and high TG levels are also significant risk factors, and are present in individuals with normal and abnormal LDL-c levels. Combined dyslipidemia may also pose a greater CVD risk than elevated LDL-c alone (*Dall et al. 2009*). There is increasing awareness of the need for comprehensive lipid management instead of aggressive management of LDL-c in monotherapy fashion (*Knopp et al. 2008*; *Dall et al. 2009*). The only treatment currently available that could potentially target multiple risk factors and aid in both treatment and prevention of CVD morbidity and mortality, though requiring longer treatment time and greater support to increase adherence to show benefit, is frequently the most underused, unpromoted, and unsupported in the health care system: lifestyle modification. Physical inactivity has been shown to be positively associated with CVD independently of common risk factors such as age, gender, smoking, blood pressure, LDL-c, as well as HDL-c, TG, and fasting glucose (*McGuire et al. 2009*). Physical activity aids in achieving and maintaining a healthy weight and improvement in blood pressure, lipid profiles, and glycemic control (*Ross et al. 2000*; *Ratner et al. 2005*; *Lichtenstein et al. 2006*). Participation in regular physical activity may also be associated with other positive lifestyle-related factors such as adoption and maintenance of dietary changes and not smoking (*Pitsavos et al. 2005*; *Aggarwal et al. 2010*). Therefore, physical activity may be protective against CVD even in the presence of known risk factors, and accounting for physical activity in risk appraisal may also aid in identifying those who may have an increased CVD risk despite normal levels of common risk factors. Lifestyle interventions

such as diet, exercise, or the combination can reduce progression to diabetes and CVD risk (*de Lorgeril et al. 1999*; *Knoops et al. 2004*; *Horton 2009*). Primary prevention of disease by diet is substantially more effective at reducing mortality than secondary prevention via medication (*Knoops et al. 2004*; *Unal et al. 2005*), and while dietary modification typically has greater adherence issues than pharmacotherapy, changes in diet may also be effective as therapy as prevention in those with established CVD (*de Lorgeril et al. 1999*).

Focused and multi-factorial diet interventions are still underutilized in CVD prevention and treatment. Perhaps some of this is due to lack of evidence for appropriate and effective dietary strategies for specific populations, as a "one size fits all" approach may not be appropriate for all disease groups.

1.2.4. Treatment for Cardiovascular Disease

1.2.4.1. Pharmacotherapy for Hyperlipidemia and Cardiovascular Disease

There are a variety of drugs available to lower plasma levels of cholesterol and TG. These lipid-lowering drugs act on different aspects of lipid metabolism.

Statins

The main pharmacological intervention currently prescribed for cholesterol reduction is a class of drugs referred to as statins. Statins inhibit 3-hydroxy-3-methyl-glutaryl CoA (HMG-CoA) reductase (HMGCoA-r), which is the rate-limiting enzyme in cholesterol biosynthesis. Statins are structural analogues of cholesterol, and therefore compete with cholesterol for binding to the HMGCoA-r enzyme. Inhibition of cholesterol synthesis causes an increase in expression of LDL-r to maintain cellular levels of cholesterol

(*Reihnér et al. 1990*; *Raggatt et al. 2002*), and reduction in production of VLDL (*Drexel 2009*). This effect results in a lowering of plasma cholesterol, and may improve postprandial lipemia (*Tentolouris et al. 2009*).

Statins have shown to reduce levels of LDL-c by 20-50% across a range of individuals including diabetes, those with and without existing CVD, and in those with high or normal plasma LDL-c levels (*Downs et al. 1998*; *Koh et al. 2010*; *Ray et al. 2010*). Statin therapy may be used in prevention of CVD events and may prevent complications in those with established CVD (*Sever et al. 2003*; *Baigent et al. 2005*; *Ridker et al. 2008*; *Ray et al. 2010*). Statin therapy may also reduce all-cause mortality, though this effect may be specifically for those individuals at higher risk (*Baigent et al. 2005*; *Ray et al. 2010*). Reduction of LDL-c may be increased if statins are used in conjunction with other lipid-lowering agents such as ezetimibe (*Robinson et al. 2009*; *Chirinos et al. 2010*). Statins have also been shown to reduce ApoB levels potentially via reduction in ApoB secretion in the liver (*Tentolouris et al. 2009*), and may or may not have effects on serum TG levels (*Robinson et al. 2009*; *Koh et al. 2010*). Statins are gaining interest as agents that may have CVD-reducing effects beyond lipid-lowering, such as improvement of endothelial function, reduction in smooth muscle cell proliferation, and anti-inflammatory effects such as reduction in CRP (*Jasińska et al. 2007*; *Ridker et al. 2008*; *Koh et al. 2010*). Reduction in CRP levels by statins appears to be independent of effects on LDL-c concentrations (*Albert et al. 2001*; *Everett et al. 2010*).

Statins are generally regarded as very safe in adults. However statins are also associated with side-effects, though quite rare, such as myopathy and elevation in liver enzymes (*Corsini 2003*), and some have been suggested to reduce insulin sensitivity leading to an increased risk for developing diabetes (*Sattar et al. 2010*; *Koh et al. 2010*). These side-effects may depend on differences between individual statin formulations and patient populations.

There is also potential for drug-interactions in patients on multiple drugs, as the hepatic CYP3A4 isoenzyme is responsible for metabolizing most statins as well as more than 30% of drugs currently used in clinical practice (*Corsini 2003*).

Ezetimibe

Ezetimibe inhibits intestinal absorption of dietary and biliary cholesterol across the enterocyte brush border membrane by binding to the NPC1L1 transporter in the intestine (*Wang 2007*; *Chapman et al. 2010b*). Ezetimibe does not affect the intestinal bile acid pool, pancreatic lipolytic enzymes, or expression of intestinal absorption proteins, and does not affect absorption of TG, FA, bile acids, or fat-soluble vitamins (*Wang 2007*). After ingestion, ezetimibe undergoes glucuronidation in the enterocyte, after which both the drug and its glucuronide are recycled enterohepatically and therefore delivered repeatedly back to the intestine (*Wang 2007*). This leads to multiple peaks in drug activity and to a long half-life (~22 hours). Blocking intestinal cholesterol absorption causes a decrease in CM cholesterol, leading to less intestinal cholesterol delivery to the liver; this reduction of hepatic cholesterol causes upregulation of the LDL-r and consequently increased clearance of circulating lipoproteins (*Chapman et al. 2010b*). Ezetimibe is estimated to block cholesterol absorption by ~50%, but there can be individual variation and cholesterol absorption has not always been correlated with response to ezetimibe (*Sudhop et al. 2002*; *Sudhop et al. 2009*; *Lakoski et al. 2010*). Most reports have either maintained subjects on a moderate cholesterol diet (200-350 mg/day) (*Sudhop et al. 2002*; *Dujovne et al. 2002*), or did not report dietary cholesterol intakes of subjects (*Lakoski et al. 2010*; *Chan et al. 2010*); therefore, it is unclear whether efficacy of ezetimibe or subject response is related to dietary cholesterol intake and also whether higher cholesterol intakes influence efficacy of ezetimibe.

Ezetimibe is considered well-tolerated and safe for reduction of LDL-c by ~20% (*Dujovne et al. 2002*). Due to its inhibition of cholesterol absorption, there is a compensatory increase in hepatic cholesterol synthesis. Therefore, it is suggested that the combination of ezetimibe with statins may be a powerful approach with which to control plasma LDL-c levels, especially in those who do not show appreciable reductions with statin therapy alone, or in high-risk populations. Combination of statin and ezetimibe may produce larger reductions in plasma LDL-c than may be achievable with statin alone (*Chirinos et al. 2010*). Combination therapy is particularly advised in those who have not achieved sufficient cholesterol-lowering with statins alone or in whom statins are not well-tolerated (*Pandor et al. 2009*). There may be a subset of the population who are better cholesterol "absorbers" than "synthesizers"; in these individuals ezetimibe may be a more appropriate therapy for lipid-lowering than statins (*Ziajka et al. 2004*; *Chuang et al. 2007*).

Fibrates

Fibrates are ligands of peroxisome proliferator-activated receptor alpha (PPARα), which upregulates FA oxidation (*Jasińska et al. 2007*). Upregulation of FA oxidation causes an increase in LPL expression and in cellular FA uptake (*Chapman et al. 2010b*). Reduction of FA available for formation of TG leads to reductions of TG content in CM and VLDL, and increased clearance of CM, VLDL and remnant particles (*Jasińska et al. 2007*; *Drexel 2009*). Fibrates may also stimulate ApoAI and ApoAII synthesis and shift HDL particle size from small and lipid-poor to large and CE-rich, thereby increasing HDL-c levels (*Tentolouris et al. 2009*; *Chapman et al. 2010b*). Fibrates therefore reduce plasma TG and increase HDL-c levels (*Abourbih et al. 2009*; *Tentolouris et al. 2009*; *Belfort et al. 2010*). The effect of fibrates on LDL-c are more ambiguous with some reports suggesting an increase while other suggest a decrease in LDL-c levels; however it has been reported that

fibrates do cause a change in distribution of LDL particle size from small and dense to larger particles which may be less atherogenic (*Abourbih et al. 2009*; *Tentolouris et al. 2009*; *Chapman et al. 2010b*). Fibrates have also been reported to improve postprandial lipemia by reducing postprandial levels of TG and remnant lipoprotein particles including both CM and VLDL (*Tentolouris et al. 2009*; *Chapman et al. 2010b*). Fibrates appear to be most effective in patients who have elevations in plasma TG levels in both the fasting and postprandial state (*Chapman et al. 2010b*).

Fibrates may prevent nonfatal myocardial infarction but do not appear to affect cardiovascular or all-cause mortality (*Saha et al. 2007*; *Abourbih et al. 2009*). Fibrates are well-tolerated, however there are indications that they may increase homocysteine and creatinine levels indicating renal dysfunction, and fibrates may have drug interactions with statins, oral hypoglycemic medications, and cyclosporin (*Preiss et al. 2009*; *Chapman et al. 2010b*). Combination therapy of fibrates with statins may be useful in patients with combined hyperlipidemia, however there is a concern about increased risk of muscle damage (*Chapman et al. 2010b*).

Therefore, pharmacological agents are successful in reducing plasma cholesterol or TG levels to varying degrees. Such variation in individual response may reflect the dynamic and multifactorial nature of lipid metabolism and regulation, and that targeting one aspect of lipid metabolism may not be adequate to sufficiently lower plasma lipid levels in all at-risk individuals.

1.2.4.2. Dietary Therapy for Hyperlipidemia and Cardiovascular Disease

1.2.4.2.1. Lipid-lowering diets

Dietary therapy is a cornerstone of lipid and CVD management. There are a variety of recommendations for dietary intake for regulation and treatment of plasma lipids, weight, and blood pressure. These

recommendations focus on dietary patterns as opposed to specific nutrients, which will be discussed subsequently.

The Mediterranean diet has been proposed as a desirable dietary pattern for prevention and treatment of CVD (*Estruch et al. 2006*). The Mediterranean diet emphasizes plant foods, olive oil, minimally processed foods, low to moderate daily amounts of wine, lean meats, and minimization of red meat (*Katcher et al. 2009*). Subjects at high risk for CVD counselled to consume a Mediterranean-type diet showed reductions in TC and TG after 3 months, particularly after nut as opposed to olive oil consumption (*Estruch et al. 2006*).

The Dietary Approaches to Stop Hypertension (DASH) diet emphasizes whole grains, fruits, vegetables, and low-fat dairy products with the intent of minimizing dietary saturated and total fat as well as cholesterol (*Appel et al. 1997*; *Obarzanek et al. 2001*). A 2-month intervention using the DASH diet showed greater reductions in total (7%) and LDL-c (9%) compared to a control diet mimicking a typical American dietary intake (*Obarzanek et al. 2001*). The DASH diet was recently reported to reduce estimated 10-year CVD risk by 18% compared to a control diet in subjects with mild hypertension (*Chen et al. 2010*).

The American Heart Association put forward diet and lifestyle recommendations in 2006. These "lifestyle" recommendations emphasized dietary as well as physical activity components. The dietary messages were to consume a diet rich in fruits and vegetables, whole-grain high-fiber foods, oily fish, and prepared foods with little to no salt, while minimizing intake of beverages and foods with added sugar, saturated and trans fat as well as cholesterol, and alcohol in moderation (*Lichtenstein et al. 2006*). These messages are extensions of the dietary profiles discussed below, from the National Cholesterol Education Program (NCEP).

The NCEP developed Step I and Step II dietary protocols for reducing

CVD risk. The Step I diet emphasizes reducing dietary fat to ≤30% of energy, ≤10% energy from saturated fatty acids, and dietary cholesterol intake ≤300 mg/day (*Yu-Poth et al. 1999*). The the Step II diet is more restrictive, limiting saturated fat intake to ≤7% of energy and cholesterol to ≤200 mg/day (*Yu-Poth et al. 1999*). A meta-analysis reported reduction in TC by 10%, LDL-c by 12%, and TG by 8% with the Step I diet, and TC by 13%, LDL-c 16% and TG 8% with the Step II diet over 37 dietary interventions using these dietary programs (*Yu-Poth et al. 1999*).

The NCEP also released the Adult Treatment Panel Therapeutic Lifestyle Change diet with the goal of maximal dietary therapy. This set of recommendations emphasizes saturated fat <7%, polyunsaturated fat <10%, monounsaturated fat <20%, total fat 25-35%, carbohydrate 50-60% (to be derived primarily from complex carbohydrates including whole grains, fruits, and vegetables), and protein 15% of energy, as well as 20-30 g/day fiber including 5-10 g/day viscous soluble fiber, <200 mg/day cholesterol, trans fatty acids as low as possible, and 2-3 g/day plant sterols (*Van Horn et al. 2001*; *Antonopoulos 2002*). The Therapeutic Lifestyle Change diet has been shown to reduce plasma LDL-c 11% with minimal changes in plasma TG after a month in hyperlipidemic individuals, as compared to a typical Western diet (*Lichtenstein et al. 2002*). When combined with an exercise intervention, there may be a significant reduction (19%) in plasma TG (*Welty et al. 2002*).

Therefore a variety of suggestions exist for dietary patterns. As emphasized by the NCEP Therapeutic Lifestyle Change program, addition of specific nutrients or functional foods to existing healthy dietary patterns may contribute additional benefits. Incorporation of various specific dietary nutrients may confer benefits as these components act on different aspects of lipid metabolism to lower plasma cholesterol and TG.

1.2.4.2.2. *Phytosterols*

Phytosterols (or plant sterol/stanols) are structural analogues to cholesterol (*AbuMweis et al. 2008*) found in nuts, seeds, and vegetables that reduce cholesterol absorption. Phytosterols are purported to achieve reduction in cholesterol absorption by a variety of mechanisms: 1) competing with cholesterol for uptake into the enterocyte via NPC1L1; 2) displacing cholesterol (dietary and biliary) from intestinal micelles; 3) preventing esterification within the enterocyte to cholesterol esters thereby preventing incorporation into CM for release into the circulation; or by 4) increasing cholesterol efflux from the enterocyte back into the intestinal lumen (*Plat et al. 2000*; *AbuMweis et al. 2008*; *Calpe-Berdiel et al. 2009*; *Jones et al. 2009*; *van Ee 2009*). Phytosterols are actively re-excreted in bile, leading to low serum levels, except in very rare cases such as sitosterolemia, which is a genetic inability to excrete sterols leading to serum accumulation (*Grundy 2005*; *Jones et al. 2009*). The reduction in cholesterol flow toward the liver from the intestine may increase LDL-r activity or LDL affinity to enhance cholesterol recycling back to the liver to increase hepatic cholesterol stores as well as reduce ApoB production and secretion (*Ruiu et al. 2009*; *Calpe-Berdiel et al. 2009*). Phytosterol supplementation was found to reduce levels of large and medium VLDL particles in both normolipidemic and metabolic syndrome subjects (*Plat et al. 2009*). There does not appear to be a difference in cholesterol-reducing ability between plant sterols and stanols (*Talati et al. 2010*), though certain foods have a greater concentration of plants sterols vs. stanols (e.g. nuts and oils) and there are differences in absorption rates (5% vs. 1%, respectively) (*Derdemezis et al. 2010*). Due to the careful balance of absorption and synthesis to maintain cholesterol homeostasis, phytosterol-induced inhibition of cholesterol absorption may also lead to upregulation of HMGCoA-r to increase cholesterol synthesis (*Jones et al. 2009*) and reduce

lipoprotein production and secretion (*Ruiu et al. 2009*). However, even with the increases in cholesterol synthesis and recycling, the net effect of phytosterol consumption is reduction of plasma cholesterol levels.

Plant sterol supplementation was first successfully used in individuals with familial hypercholesterolemia to effectively lower TC and LDL-c levels (*Becker et al. 1993*; *Moruisi et al. 2006*). In individuals with and without hypercholesterolemia, phytosterols of 1 up to 9 g/day incorporated into foods, such as margarine and yogurt, significantly reduce LDL-c by 10-15% (*Plat et al. 2000*; *Vissers et al. 2000*; *Davidson et al. 2001*; *Mensink et al. 2002*). Addition of phytosterols can help hypercholesterolemic individuals attain recommended targets, even those who are not able to meet current goals using standard pharmacotherapy (*Plana et al. 2008*; *de Jong et al. 2008*). The American Heart Association (AHA) recommends using plant sterols to reduce LDL-c (*McCrindle et al. 2007*). Plant sterols may or may not be retained in the enterocyte, and therefore whether they can affect cholesterol uptake and absorption across a prolonged period of time has yet to be determined (*AbuMweis et al. 2008*). A recent trial concluded that for optimal cholesterol-lowering phytosterols should be given in smaller doses more often over the course of the day, rather than a single larger dose (*AbuMweis et al. 2008*; *AbuMweis et al. 2009*). A dose-response relationship of phytosterol intake and LDL-c reduction up to 3 g/day exists (*Grundy 2005*), and greatest reductions occur with higher baseline LDL-c levels (*Naumann et al. 2008*; *Demonty et al. 2009*). Response to phytosterol supplementation may depend on an individual's natural degree of cholesterol synthesis, as those with higher rates of cholesterol synthesis have been showed to have a lower LDL reduction in response to phytosterol therapy (*Rideout et al. 2010*). Phytosterol intake has not generally been shown to have detrimental effects on plasma levels of fat-soluble vitamins (A, D, E) even at intakes of almost 9 g/day for 10 weeks (*Gylling et al. 2010a*), and levels of serum plant sterols are

comparable with those obtained by lower intakes (*Gylling et al. 2010b*), however this is not universally reported (*Tuomilehto et al. 2009*). Higher intakes do not generally confer greater benefit compared to that obtained with intakes of 2-3 g/day (*Derdemezis et al. 2010*). Typical U.S. and Canada phytosterol consumption is 150-400 mg/day, making supplementation necessary to achieve the recommended 2 g/day for 10% reduction in LDL-c (*Katcher et al. 2009*). If results can be extrapolated from other countries such as Australia, it is estimated that a daily serving of sterol-enriched spread sufficient to lower LDL-c 10% costs $0.17/day; statins to lower LDL-c by 30-40% cost $1.00/day (*Clifton et al. 2009*).

1.2.4.2.3. *Polyunsaturated Fatty Acids*

PUFA like linoleic acid (n-6; LA) and alpha-linolenic acid (n-3; ALA) are essential FA that cannot be made by the body and therefore must be obtained from diet. LA is found in soybean, canola and sunflower oils, whereas ALA acid is found in flax and canola (*Kris-Etherton et al. 2007*). PUFA are ligands for nuclear receptors and transcription factors, such as PPARα which promotes expression of genes involved in FA oxidation (*Russo 2009*; *Minihane 2009*). PUFA may reduce lipoprotein secretion via downregulating MTTP required for lipoprotein assembly (*Minihane 2009*). PUFA suppress the activity of sterol regulatory element binding protein transcription factors SREBP-1c and SREBP-2, which influence a variety of lipid- and cholesterol-related genes, reducing lipogenesis and VLDL assembly (*Le Jossic-Corcos et al. 2005*; *Russo 2009*; *Minihane 2009*). LA-enriched phospholipids enhance secretion of ApoA1 which increases HDL-c (*Pandey et al. 2008*).

American and Canadian dietetic associations recommend 5-10% dietary energy from n-6 PUFA and 0.6-1.2% from n-3 PUFA, whereas actual Canadian intakes are 5% and 0.5%, respectively (*Kris-Etherton et al. 2007*). LA may decrease TC and LDL-c as well as TG in some individuals (*Kris-*

Etherton et al. 2007). In clinical trials, ALA-rich food sources such as flax oil may reduce TC and LDL-c, though this effect is not consistent across all studies and the reduction is minimal (*Goh et al. 1997*; *Egert et al. 2009*; *Pan et al. 2009*). Epidemiological studies suggest ALA has beneficial effects on CVD risk via non-cholesterol related factors (*Kris-Etherton et al. 2007*; *Katcher et al. 2009*).

Long-chain omega-3 fatty acids

Eicosapentanoic acid (EPA) and docosahexanoic acid (DHA) are long-chain n-3 FA that may be formed from ALA to a small degree (*Brenna et al. 2009*), but are typically more efficiently obtained from dietary sources such as marine oils. Fish oil has been shown to be very effective in reducing plasma TG levels, and safe over the long-term (*Miller et al. 1988*; *Saynor et al. 1992*; *Goh et al. 1997*; *Ryan et al. 2009*). Clinical trials have shown that long-term fish oil supplementation can reduce CVD mortality up to 30% (*Lavie et al. 2009*). The TG-lowering effects of EPA and DHA are attributed to effects on reducing: CM and VLDL synthesis thereby reducing entry of these particles into circulation; lipogenesis via SREBP-1c; TG formation by inhibition of DGAT; circulating levels of ApoCIII which may enhance activity of LPL and therefore enhance lipoprotein-bound TG hydrolysis and clearance; and by enhancing the utilization of FA in beta-oxidation via PPARα (*Davidson 2006*; *Jacobson 2008*; *Jung et al. 2008*; *Minihane 2009*; *Maki et al. 2010*). Some studies suggest that fish oil n-3 PUFA may reduce small dense LDL particles (*Kelley et al. 2007*; *Ryan et al. 2009*), which may be due to a shift towards larger and more cholesterol-rich (as well as TG-poor) particles, which are less associated with vascular disease, rather than reduction in particle number (*Maki et al. 2010*). Beyond TG effects, n-3 FA may improve endothelial function and the inflammatory response (*Kris-Etherton et al. 2003*; *Ouguerram et al. 2006*; *Das et al. 2008*; *Valdivielso et al. 2009*).

Arachidonic acid (AA), an n-6 FA, produces the pro-inflammatory 2/4 series of prostanoids and leukotrienes, while EPA produces the less inflammatory 3/5 series (*Flachs et al. 2009*). DHA gives rise to anti-inflammatory lipoxygenases, resolvins, and protectins (*Flachs et al. 2009*).

EPA and DHA at 1-4 g/day are very effective and safe in lowering TG by 20-30% in a variety of individuals, including those with normal or elevated TG levels, metabolic syndrome, and HIV (*Miller et al. 1988*; *Saynor et al. 1992*; *Ebrahimi et al. 2009*; *Woods et al. 2009*; *Ryan et al. 2009*). Greatest reductions in plasma TG levels occur in individuals with higher baseline levels (*Katcher et al. 2009*). Fish oil supplementation of 360 mg/day significantly reduced CRP and tumour necrosis factor (TNF-α), while non-significantly reducing IL-6 and leptin and increasing adiponectin in hyperlipidemic adults (*Micallef et al. 2009*). A cross-sectional study showed that those who consumed >300 g fish per week had 33% lower CRP, 33% lower IL-6, 21% lower TNF-α, and 28% lower serum amyloid A levels (*Zampelas et al. 2005*). Similar reductions in inflammatory markers and TG levels with fish oil therapy may only occur in those individuals with initially elevated levels, and provide little effect in healthy individuals (*Park et al. 2009*; *Pot et al. 2009*). The AHA recommends consumption of 1-4 g/day of EPA/DHA for all adults (*Kris-Etherton et al. 2003*). N-3 FA used in combination with statins to treat mixed dyslipidemia (elevated TC and TG) are considered to act synergistically with statins to lower LDL-c (*Nordøy 2002*). Some individuals experience a slight increase in LDL-c levels while on both treatments, which may depend on genotype (e.g. ApoE) (*Olano-Martin et al. 2010*) or low baseline levels of LDL-c; despite this slight increase in LDL-c the reduction in VLDL-cholesterol concentrations may be large enough to produce a net decrease in total cholesterol levels (*Maki et al. 2010*). Addition of EPA/DHA to statin therapy may enhance reduction of postprandial atherogenic particles such as ApoB100 and ApoB48 (*Valdivielso et al. 2009*).

1.2.4.2.4. *Fibers*

Dietary fiber (particularly soluble fiber) has beneficial effects on blood glucose and lipids (*Cruz et al. 2005*). Soluble fibers are proposed to lower plasma cholesterol by reducing cholesterol absorption and the reabsorption of bile acids, increasing the amount of hepatic cholesterol used to form bile acids and upregulating LDL-r to restore hepatic cholesterol reserves (*Theuwissen et al. 2007*; *Katcher et al. 2009*). Enhancing cholesterol excretion increases hepatic cholesterol used to form bile acids and upregulates LDL-r to restore hepatic cholesterol reserves (*Bazzano 2008*). Colonic fermentation of soluble fiber produces short-chain FA, which may influence hepatic lipid metabolism (*Bazzano 2008*). Fibers slows the rate of glucose absorption leading to lower postprandial insulin concentrations (*Theuwissen et al. 2008*; *Hlebowicz et al. 2008*).

Beta-glucan is a soluble fiber found in grains such as oats, barley, and yeast (*Theuwissen et al. 2007*). Animal studies have reported that beta-glucan may downregulate genes involved in FA synthesis and cholesterol metabolism, as well as proteins involved in intestinal FA uptake (*Drozdowski et al. 2009*). Soluble fibers such as beta-glucan may also positively affect post-meal glucose tolerance, as increased intestinal viscosity may slow the rate of glucose absorption and consequent appearance in blood, leading to reduced postprandial insulin concentrations (*Theuwissen et al. 2008*; *Hlebowicz et al. 2008*). Beta glucan-enriched foods may also modulate feelings of satiety through reducing hormone levels such as ghrelin, peptide Y and glucagon-like peptide 1, which may be related to the degree of viscosity in the supplement used (*Juvonen et al. 2009*; *Vitaglione et al. 2009*).

Beta-glucan added to the diet as supplements or incorporated into food products, such as bread, reduce LDL-c in individuals with elevated blood lipid levels (*Keenan et al. 2007*; *Reyna-Villasmil et al. 2007*; *Liatis et al. 2009*).

Concentrated beta-glucan supplements of 3-5 g/day can reduce TC and LDL-c up to 15% and TG up to 15%, however these effects are not always observed (*Naumann et al. 2006*; *Keenan et al. 2007*; *Queenan et al. 2007*; *Reyna-Villasmil et al. 2007*; *Liatis et al. 2009*). Some studies in individuals that may have altered metabolism such as Type 2 diabetes have not shown a beneficial effect of beta-glucan supplementation (*Cugnet-Anceau et al. 2010*), whereas others have been more effective (*Liatis et al. 2009*); these differences in noted effects could be due to patient population (elevated versus normal LDL-c levels), or modality of supplementation (supplement or type of foodstuff). Independent cholesterol- and TG-lowering effects of beta-glucan may be small but significant and enhance the cholesterol-lowering potential of other interventions (*Brown et al. 1999*; *Reyna-Villasmil et al. 2007*; *Talati et al. 2009*). For example, adding 6g beta-glucan to the AHA Step II diet produced significantly greater reductions in LDL-c (27% vs. 17%) (*Reyna-Villasmil et al. 2007*). The U.S. Food and Drug Administration has approved a health claim for 0.75 g/serving of oat or barley beta-glucan stating that this fiber may reduce risk of heart disease, when consumed as part of a diet low in saturated fat and cholesterol (*Katcher et al. 2009*). However, at least 3 g/day beta-glucan must be consumed to achieve significant cholesterol reduction (*Theuwissen et al. 2008*).

Inulin is an undigestible fructan prebiotic that acts as a prebiotic by altering gut bacteria (*Russo et al. 2008*). Inulin appears to have notable reduction effects on plasma TG levels but has only minimal effects on cholesterol (*Davidson et al. 1999*; *Pereira et al. 2002*). Inulin may reduce plasma TG levels via reducing hepatic lipogenesis, TG synthesis and VLDL secretion (*Pereira et al. 2002*; *Letexier et al. 2003*; *Brighenti 2007*). Results from a recent trial in healthy humans suggest inulin may also reduce postprandial GLP-1 and ghrelin concentrations, as well as increase short-chain FA production which may further modulate gut hormone responses

(*Tarini et al. 2010*). There is also some indication in the literature that inulin may have positive effects on the immune system via effects on intestinal microflora and short-chain FA production (*Seifert et al. 2007*).

A randomized, double-blind, crossover study in healthy males for 22 weeks showed that inulin-enriched pasta reduced plasma TG 23.4%, increased HDL-c 35.9%, and reduced TC:HDL-c 22.2% (*Russo et al. 2008*), as well as delayed gastric emptying (*Russo et al. 2010*). A meta-analysis of existing evidence found that, on average, inulin and related fructans lower TG levels by 7.5% (though from previous studies this can be much higher) (*Pereira et al. 2002*), and even though there was variability in population, duration, and dosage the TG-lowering effect was relatively homogenous (*Brighenti 2007*). Low doses (7-10 g/day) may be more effective to reduce plasma TG than higher doses, and greater effects occur in people with hypertriglyceridemia or enhanced hepatic lipogenesis (*Jackson et al. 1999*; *Beylot 2005*). A 6-month intervention with 10 g/day inulin and oligofructose showed no significant effects on TG or other blood lipids, though the study was performed in normolipidemic humans (*Forcheron et al. 2007*).

1.2.4.2.5. *Soy Protein*

Soy has been shown to increase hepatic LDL-r activity (*Cho et al. 2007*), may protect LDL-c from oxidation, and reduce levels of small dense LDL (*van Ee 2009*). Soy protein may influence expression of SREBP and liver X receptor (LXR), and has been shown to reduce synthesis of FA and cholesterol in hypercholesterolemic subjects (*Wang et al. 2004*). Downregulation of SREBP-1c reduces synthesis of FA and TG, thereby reducing plasma and hepatic lipid levels. Downregulation of SREBP-2 enhances cholesterol clearance leading to reduced plasma levels (*Torres et al. 2006*). Soy's effects may be mediated via effects on insulin levels (*Torres et al. 2006*; *Xiao et al. 2008*).

In clinical trials soy protein significantly reduces TC, LDL-c and TG up to ~10% and may slightly increase HDL-c, though some analyses report more modest effects on TC and LDL-c (~5%) and TG (~7%) (*Zhan et al. 2005*; *Reynolds et al. 2006*; *Hoie et al. 2007*; *Katcher et al. 2009*). Soy protein consumption may also reduce the ApoB:ApoAI ratio (*Pipe et al. 2009*). Effectiveness of soy to reduce cholesterol levels seems to be heavily influenced by baseline lipid level, and to a lesser extent by dosage (at least over 80 mg/day) and length of intervention (particularly for changes in HDL-c) (*Zhan et al. 2005*; *Reynolds et al. 2006*; *Katcher et al. 2009*). The metabolic effects of soy may also depend on whether the soy provided contains protein isolate, isoflavones, or both (*Kerckhoffs et al. 2002*). For lipid-lowering it appears that soy protein may be more important (*Jenkins et al. 2002*; *Wang et al. 2004*), whereas for other effects such as changes in immune markers or hormones may be more dependent on isoflavone concentration (*Jenkins et al. 2002*). The FDA-approved health claim for soy states that diets low in saturated fat and cholesterol and including 25 g/day of soy protein may reduce risk of heart disease; foods must contain at least 6.25 g soy protein/ serving (*Katcher et al. 2009*).

1.2.4.2.6. *Almonds*

Observational studies have frequently reported an inverse relationship between consumption of nuts and risk of CVD (*Kris-Etherton et al. 2008*; *Phung et al. 2009*). Almonds contain many nutrients affecting lipid metabolism and CVD risk, such as PUFA, fiber, phytosterols, vitamin E, and polyphenols (*Chen et al. 2008*). Compared with other nuts, almonds have the lowest peroxide value and highest content of tocopherols (452 μg tocopherols/g oil) (*Maguire et al. 2004*), as well as one of the highest levels of phytosterols (*Chen et al. 2008*). Almonds may reduce oxidation of LDL-c particles (*Chen et al. 2007*; *Jenkins et al. 2008*), and reduce postprandial

glycemia (*Jenkins et al. 2006*).

Meta-analyses suggest almond consumption leads to a modest TC reduction, perhaps in a dose-response manner (*Jenkins et al. 2002*), but may or may not affect levels of LDL-c and TG (*Phung et al. 2009*). A randomized crossover feeding trial for 4 weeks found a 10% reduction in TC and LDL-c in 16 healthy adults consuming 20% of total energy from almonds (corresponding to 56 g or 2 oz of almonds per day) (*Jambazian et al. 2005*). A trial in individuals with Type 2 diabetes found that addition of 100g/day almonds to the diet for 4 weeks reduced TC and LDL-c by 21% and 29%, respectively, and a second study showed that the greatest reduction in TC occurred on the background of a high-fat diet (37% total fat, 10% from almonds) (*Lovejoy et al. 2002*). Jenkins et al (2002) investigated the use of whole almonds in hyperlipidemic subjects following therapeutic diets and found that half-dose almonds (36.5 g/day) and full-dose almonds (73 g/day) were effective in reducing LDL-c by 4.4% and 9.4%, respectively, indicating a dose-response effect (*Jenkins et al. 2002*). Adding 100 g of different forms of almonds (raw, roasted, or as butter) to the free-living diets of 38 hypercholesterolemic patients for 4 weeks significantly reduced LDL-c by 7-12% and TC by 5-7%, and reduced VLDL and TG in some individuals (*Spiller et al. 2003*). The largest reductions in TC and LDL-c occurred in the raw almond group, and there was a nonsignificant increase in HDL-c (+8%) and decrease in VLDL (-15%) and TG (-18%) in the group given almond butter, suggesting that different forms of almonds could be incorporated into the diet for lipid reduction.

1.2.4.2.7. *Red Palm Oil*

Red palm oil is obtained from palm fruit and has approximately equal quantities of saturated FA (SFA) (40-50%) and monounsaturated FA (MUFA) (40%), with the remainder as PUFA (10%) (*Wilson et al. 2005*; *Engelbrecht et*

al. 2009). Red palm oil is unique due to high content of tocopherols, tocotrienols, and carotenoids, which may act as potent antioxidants (*Engelbrecht et al. 2009*). Atherosclerosis is associated with lower plasma levels of tocopherols and tocotrienols (*Riccioni et al. 2008*). Tocopherols may prevent peroxidation of cholesterol and EPA or DHA (*Atkinson et al. 2008*; *Xu et al. 2009*), and preferentially associate with PUFA in cell membranes, stabilizing them (*Atkinson et al. 2008*). Mechanistic benefits of red palm oil on vascular disease are suggested by be inhibition of cholesterol synthesis, platelet aggregation, and reduction in oxidative stress (*Oguntibeju et al. 2009*). Red palm oil feeding can aid in recovery and protection from cardiac ischemia reperfusion injury in animal models, even during hypercholesterolemic feeding, which may be mediated by nitric oxide and PIK3-kinase pathways (*Esterhuyse et al. 2005*; *Esterhuyse et al. 2006*; *Kruger et al. 2007*; *van Rooyen et al. 2008*; *Engelbrecht et al. 2009*). Red palm oil may also reduce tissue plasminogen activator in hyperfibrinogenemic adults (*Scholtz et al. 2004*).

Though it is high in SFA, supplementation of red palm oil has been shown to be neutral or to improve TC (*Scholtz et al. 2004*; *Kruger et al. 2007*; *Salinas et al. 2008*). In free-living adults, red palm oil had neutral effects on plasma lipids compared to palm oil (which increased TC and LDL-c) and sunflower oil (which reduced TC and LDL-c) (*Scholtz et al. 2004*). However, red palm oil given to young healthy men had no significant effects on plasma lipids compared to soybean oil, though plasma carotene, lycopene, and tocopherol levels were enhanced (*Zhang et al. 2003*). Based on this evidence, red palm oil may be an excellent source of antioxidant lipophilic vitamins to provide protection from atherogenesis as well as lipid oxidation, while having neutral effects on plasma lipids.

1.2.4.2.8. *Chocolate*

Chocolate is proposed to have beneficial effects on CVD risk factors through high polyphenol and flavonoid content. Flavonols are associated with increase in vasodilation and antioxidant capacities. In addition, cocoa butter has a large content of stearic acid (18:0) which is a SFA but does not seem to be associated with the plasma cholesterol-raising effects attributed to other SFA such as myristic and palmitic acid (*Kris-Etherton et al. 1994*; *Kris-Etherton et al. 1994*). Proposed beneficial effects on CVD risk factors include lowering plasma cholesterol and blood pressure, raising HDL-c, and improving plasma antioxidant capacity (*Hooper et al. 2008*; *Scheid et al. 2010*).

Recent studies have shown small but significant improvements in HDL-c and reductions in plasma cholesterol and blood pressure. For example, consumption of 45 g/day of high-polyphenol chocolate for 16 weeks raised HDL-c in individuals with Type 2 diabetes in the absence of weight gain or alterations in glycemic control (*Mellor et al. 2010*). Interestingly, in this study addition of chocolate to the daily diet resulted in participants naturally reducing portion size at the following meal, leading to a lack of increase in energy intake. In another group of individuals with impaired glucose tolerance along with elevated plasma cholesterol and blood pressure, 100 g of flavanol-rich dark chocolate consumed for 15 days reduced plasma cholesterol levels 6.5% and systolic blood pressure ~4 mmHg, and also improved insulin sensitivity (*Grassi et al. 2008*). In this study, patients were instructed to reduce energy intake from other dietary sources to compensate for the additional calories supplied by the chocolate. A recent study in HepG2 and Caco2 cells found that cocoa polyphenols increased expression of ApoA1 and decreased expression of ApoB, along with increased expression of SREBP and LDL-r proteins in HepG2 cells (*Yasuda et al. 2011*). If these results persist

in vivo, this may indicate how chocolate increases HDL-c and lowers total and LDL-c. Addition of phytosterols to chocolate-containing cocoa flavanols reduces plasma LDL-c 5-10% and systolic blood pressure in individuals with elevated plasma cholesterol and blood pressure (*De Graaf et al. 2002*; *Allen et al. 2008*). A randomized crossover study in healthy subjects found a small but significant increase (4%) in antioxidant capacity and HDL-c after supplementing an average American diet with cocoa powder and dark chocolate (*Wan et al. 2001*). However, a recent review of clinical trials concluded that there was insufficient evidence for the claims of the effects of cocoa and chocolate on increasing antioxidant capacity (*Scheid et al. 2010*).

It appears that individuals may benefit from controlled daily doses of chocolate, but that these effects may be limited to high polyphenol-containing products, and maintenance of energy balance. Addition of chocolate in combination with other functional nutrients may confer small but additive benefits.

1.2.4.2.9. *Combined interventions*

Dietary components can be effective in reducing blood lipid levels and ameliorating other CVD-related risk factors by acting on a variety of aspects of lipid metabolism, but the magnitude of effect of individual components is generally limited. Therefore, recently there has been a shift towards the investigation of combinations of functional ingredients to achieve more significant effects (*Jenkins et al. 2007*).

A randomized, double-blinded, multiple crossover intervention in mildly hypercholesterolemic adults found that supplementation of 5g beta-glucan lowered LDL-c by 5%, whereas the combination of beta-glucan and 1.5g plant sterols reduced LDL-c by 9.6% (*Theuwissen et al. 2007*). It was determined that beta-glucan increased bile acid synthesis and reduced cholesterol absorption, while plant sterols further reduced cholesterol

absorption. Therefore, a synergistic and additive effect on blood lipid reduction can be achieved when multiple nutrients are used simultaneously. Combination of 2 g/day phytosterols and 1.4 g/day fish oil reduced TC 13% and LDL-c 12.5% compared to either treatment individually, and TG was reduced 22% by fish oil alone and 26% by the combination (*Micallef et al. 2008*). In a study by Castro et al (2007), healthy volunteers were grouped into clusters according to CVD risk level, and then randomized to one of four experimental diet groups for 6 weeks, receiving 460 mg EPA/DHA with or without 580mg soluble fibers, or a placebo (*Castro et al. 2007*). TG and TC were not changed by any diet intervention in any group, however this may be due to small number of subjects in each cluster group, as well as the low doses of fish oil and fiber. The combination of soy protein plus prebiotic resistant starch significantly reduced TC by 5.5% and LDL-c by 7% in mildly hypercholesterolemic individuals over 5 weeks, was sustained during a washout period (*Larkin et al. 2009*). A 6-week intervention of 29 g/day soy combined with oats in postmenopausal women with moderate hypercholesterolemia lowered TC and LDL-c levels further than reductions already achieved by a STEP 1 diet (*Van Horn et al. 2008*). Addition of 1.6g plant sterols to a soy protein beverage reduced TC 4% and LDL-c 7% compared to control soy drink (*Weidner et al. 2008*), whereas another trial showed that a phytosterol-enriched soy beverage providing 1.95 phytosterols/day reduced LDL-c 15% and reduced cholesterol absorption 27% but did not affect cholesterol synthesis in hyperlipidemic subjects (*Rideout et al. 2009*). A 3-week randomized, double-blind, placebo-controlled trial in 60 hyperlipidemic patients found that the combination of 360 mg/day EPA/DHA and 2 g/day plant sterols was more effective than either nutrient individually, reducing CRP 39%, TNF-α 10%, and IL-6 11%, and increasing adiponectin 29.5% (*Micallef et al. 2009*).

The best example of a complete dietary approach to reduce lipids is

the "dietary portfolio" series of investigations by Jenkins and colleagues. A vegetable-based "dietary portfolio" high in soy protein (22.5 g/1000 kcal), soluble fiber (10g/1000 kcal), almonds (23 g/1000 kcal) and plant sterols (1 g/1000 kcal) has been repeatedly shown to effectively reduce TC and LDL-c 25-35% in hypercholesterolemic adults (*Jenkins et al. 2003*; *Kendall et al. 2004*; *Jenkins et al. 2007*; *Esfahani et al. 2010*). The dietary portfolio is comparable to statin therapy (*Jenkins et al. 2005*; *Jenkins et al. 2006*) and may reduce levels of small LDL particles (*Lamarche et al. 2004*). However, compliance to the dietary portfolio and therefore meeting recommended intakes of the key nutrients may be difficult in the long-term, though there will be a portion of the population who are able to adhere to such dietary changes up to 1 year and beyond (*Jenkins et al. 2006*).

Therefore, single nutrients can be effective in reducing plasma lipid levels and other markers of CVD risk. However, the combination of functional nutrients may confer additive benefits, which may be of particular importance in diseases where there are multiple lipid metabolic disturbances or in which the disturbances are not yet characterized.

1.3. Lipid Synthesis

As evidenced by the above discussion, plasma lipid levels and aberrations in lipid metabolism are very important in health and disease development and progression. Elevations in plasma levels of TG or cholesterol can occur due to increased absorption, increased synthesis, reduced clearance, or a combination of these factors. The following discussion will focus on lipid synthesis, mechanisms and influences.

1.3.1. Fatty Acids

FA are grouped by chain length (short, medium, and long) and degree of unsaturation (saturated and unsaturated) (**Table 1-1**). Short-chain FA are

<6 carbons in length, medium-chain are 8-14 carbons, and long-chain are >14 carbons.

Table 1-1: Common fatty acids, notation, and dietary sources

Common Name	ω Notation (Δ notation)	Dietary Sources
Saturated		
Lauric	12:0	Coconut & palm oil
Myristic	14:0	Coconut & palm oil
Palmitic	16:0	Palm oil
Stearic	18:0	Animal fats
Monounsaturated		
Oleic	18:1n9 (18:1Δ9)	Olive & canola oil
Polyunsaturated		
Linoleic*	18:2ω6 (18:2Δ9,12)	Sunflower & safflower oil
Linolenic*	18:3ω3 (18:3Δ9,12,15)	Soybean & canola oil
Arachidonic	20:4ω6 (20:4Δ5,8,11,14)	Eggs & animal fat
Eicosapentaenoic	20:5ω3 (20:5Δ5,8,11,14,17)	Marine sources, supplements
Docosahexaenoic	22:6ω3 (22:6Δ4,7,10,13,16,19)	Marine sources

*denotes essential fatty acids which cannot be synthesized by the human body

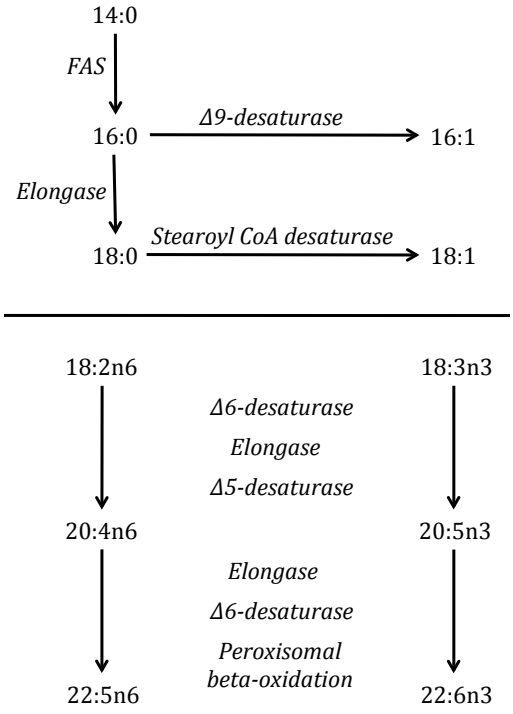
1.3.1.1. De novo lipogenesis

FA oxidation occurs in the mitochondria whereas FA synthesis occurs in the cytosol. Since acetyl CoA is impermeable to the mitochondrial membrane, it is converted to citrate via citrate synthase. Citrate crosses the mitochondrial membrane to enter the cytosol via the tricarboxylate transporter. Citrate acts as a feed-forward activator of FA synthesis. Once in the cytosol, citrate is converted back to acetyl CoA by ATP-citrate lyase. Acetyl CoA is converted to malonyl CoA via acetyl CoA carboxylase (ACC) which is a major rate-limiting enzyme in FA synthesis (*Chavez et al. 2010*). Sequential additions of acetyl CoA elongates the newly-formed FA by two carbon units at a time by fatty acid synthase (FAS), via addition, reduction, dehydration, and reduction (*Chavez et al. 2010*).

SREBPs are proteins that directly activate genes involved in the uptake and synthesis of cholesterol, FA, TG and PL (*Horton et al. 2002*). SREBP-1c increases transcription of genes involved in FA synthesis, whereas SREBP-2 preferentially activates genes involved in cholesterol metabolism such as HMGCoA-r and the LDL-r (*Horton et al. 2002*). SREBP-1c activates FAS, stearoyl-CoA desaturase (SCD-1), and ACC, and activation of SREBP-1c is mediated in part through LXR activation (*Strable et al. 2010*).

Myristic acid (14:0) is a major FA produced through DNL, and can be elongated to palmitic acid (16:0) by FAS. Palmitic acid is the primary FA produced by DNL (*Aarsland et al. 1998*). Palmitic acid can be further elongated or desaturated as shown in **Figure 1-3** (*Strable et al. 2010*; *Green et al. 2010*). Humans are unable to synthesize 18:2n6 (LA) and 18:3n3 (ALA) from precursors due to a lack of the Δ12 and Δ15 desaturases, requiring these FA to be obtained from the diet. As shown in **Figure 1-3**, LA and ALA can undergo further processing to form longer-chain FA.

Figure 1-3: Fatty acid elongation and desaturation.



FA form ester linkages to glycerol to form acylglycerols. Acylglycerols are formed through sequential addition of FA onto the glycerol backbone to form monoacylglycerol, diacylglycerol via monoacylglycerol-acyltransferase, or TG via diacylglycerol-acyltransferase (*Chavez et al. 2010*). TG is the major storage form of lipid in plants and animals, including humans, and is primarily stored in adipose tissue.

1.3.1.1.1. De novo lipogenesis in humans

De novo lipogenesis (DNL) is the synthesis of FA from non-fat sources. DNL occurs primarily in the liver, adipose tissue, and mammary glands (*Pearce 1983*; *Hellerstein 1999*; *Ferré et al. 2007*). Hepatic DNL is considered predominant in humans while adipose tissue DNL is considered minimal, whereas in rodents adipose tissue lipogenesis can be significant (*Pearce 1983*; *Guo et al. 2000*; *Schwarz et al. 2003*; *Diraison et al. 2003*; *Ferré et al. 2007*). DNL is generally considered to contribute negligibly to plasma lipid levels when compared to the influence of dietary TG, particularly for individuals consuming a high-fat or Western diet (*Hellerstein 1999*; *Ferré et al. 2007*). However, these findings may only be true for people with normal body weight and metabolism. Emerging evidence suggests that DNL may be increased in altered metabolic states and therefore contribute meaningfully to plasma lipid levels.

In healthy individuals, DNL has been estimated to contribute ≤10% towards TG levels in fasting conditions, and is typically reported at 2-5% (*Diraison et al. 1998*; *Marques-Lopes et al. 2001*; *Cachefo et al. 2001*; *Timlin et al. 2005*; *Vedala et al. 2006*). However, this rate can be increased upon feeding and there can be large variations in lipogenic response to feeding of up to a 10-fold difference between fasted and fed states and between individuals (*Timlin et al. 2005*; *Barrows et al. 2006*). Aberrations in metabolic state such as insulin resistance and obesity are associated with elevations in DNL

(*Diraison et al. 2002*; *Schwarz et al. 2003*; *Vedala et al. 2006*). DNL therefore can be influenced by hormonal and dietary factors (**Table 1-2**) as discussed below.

Table 1-2: Regulation of de novo lipogenesis by hormones and nutrients

Stimulates DNL	**Suppresses DNL**
Insulin	Glucagon
Obesity*	PUFA
High-carbohydrate diets	High-fat diets
Infection or inflammation	

*may be mediated through insulin resistance and hyperinsulinemia

Insulin promotes hepatic lipogenesis by inducing SREBP-1c gene transcription and accumulation. SREBP-1c, in turn, induces expression of genes involved in FA synthesis such as ACC, FAS, and SCD-1 (*Chahil et al. 2006*; *Chavez et al. 2010*). Insulin also regulates activity of LPL which may increase FA flux to the liver and influence FA synthesis indirectly (*Chahil et al. 2006*). Obesity is related to increased expression of SREBP-1c and DNL enzymes however these effects may be mediated through insulin resistance (*Pettinelli et al. 2009*; *Elam et al. 2010*). States of insulin resistance such as Type 2 diabetes and non-alcoholic fatty liver disease (NAFLD) are associated with elevated DNL (*Diraison et al. 2003*; *Donnelly et al. 2005*; *Wilke et al. 2009*). Glucagon, conversely, suppresses lipogenesis and associated enzymes (*Radenne et al. 2008*).

Dietary carbohydrate in excess of energy needs and hepatic glycogen storage capacity stimulates DNL (*Schwarz et al. 1995*; *McDevitt et al. 2001*; *Parks et al. 2008*; *Strable et al. 2010*). Starch appears to have a neutral effect on plasma TG while monosaccharides can significantly increase plasma TG levels (*Parks et al. 2000*; *Parks 2002*). Fructose in particular has been shown to increase hepatic DNL (*Parks et al. 2008*; *Tran et al. 2010*), whereas

hypercaloric and high-carbohydrate diets may stimulate adipose tissue DNL (*Guo et al. 2000*). Part of this effect of carbohydrate on DNL may be mediated by ChREBP which has been shown to induce expression of ACC, FAS, and SCD-1 (*Ishii et al. 2004*; *Strable et al. 2010*). In contrast, high fat feeding tends to reduce DNL (*Schwarz et al. 2003*). PUFA downregulates SREBP-1c (*Strable et al. 2010*), and SFA and PUFA have been shown to reduce FAS activity (*Radenne et al. 2008*). SFA may not increase DNL but may stimulate TG synthesis instead.

DNL may be increased in states of infection or inflammation, and DNL has been found to be elevated in hyperthyroidism (*Hellerstein 1999*; *Cachefo et al. 2001*). Acute alcohol ingestion also increases DNL, however this is not a major pathway for degradation of ethanol metabolites as acetate released from the liver upon ethanol metabolism served to inhibit FA release from adipose tissue thereby reducing FA availability for TG synthesis (*Siler et al. 1999*).

1.3.2. Cholesterol

Cholesterol plays a variety of important roles in the body. It is a key component in cell membranes and helps to control cellular fluidity, permeability, raft formation, and protein functions (*Levy et al. 2007*). Cholesterol is also a precursor of vitamin D, bile acids, and steroid hormones, such testosterone, estrogen, and cortisol (*Levy et al. 2007*). Dietary intake of cholesterol is approximately 100-300 mg/day from animal products (*Grundy 1983*), although the typical Western diet provides closer to 300-500 mg/day (*Stellaard et al. 2005*; *Wang 2007*), whereas biliary production contributes up to 800-1200 mg/day (*Grundy 1983*; *Ostlund 2002*; *Wang 2007*). Absorption of cholesterol from pooled biliary and dietary sources is approximately 50% (*Bosner et al. 1999*; *Ostlund 2002*). Cholesterol and bile acids that are not absorbed are excreted (*Wang 2007*).

As has been reviewed previously, high plasma cholesterol levels are considered undesirable due to the role of high plasma lipids levels as risk factors in vascular disease. However, there is evidence for reductions in and evidence of low plasma cholesterol levels observed in infection, burn, cancer, and sepsis (*Vyroubal et al. 2008*). In these cases, low plasma cholesterol levels are also unfavourable, and may actually indicate disease progression or be associated with reduced response to treatment. Therefore balance between absorption and synthesis for maintenance of cholesterol homeostasis is essential.

1.3.2.1. Cholesterol synthesis

De novo synthesis of cholesterol is approximately 800-1500 mg/day, with the liver estimated to contribute ~40-50% and the intestine 25-30%, however all cells of the body have cholesterol-synthesizing capabilities (*Dietschy 1984*). The endoplasmic reticulum is the site of cholesterol synthesis and esterification, but the highest concentration of cholesterol is in the plasma membrane which contains 60-80% of total cellular cholesterol (*Maxfield et al. 2002*; *Soccio et al. 2004*). Cholesterol is synthesized from 2 acetyl CoA molecules which combine to form acetoacetyl CoA. Acetoacetyl CoA again binds with acetyl CoA to form HMGCoA, which is further processed by HMGCoA-r, the rate-limiting enzyme for cholesterol synthesis that catalyzes the conversion of HMGCoA to mevalonate (*Stellaard et al. 2005*). Cholesterol is eventually formed through a series of intermediates involving a number of enzymes (*Soccio et al. 2004*).

Cholesterol regulatory mechanisms are highly sensitive to endoplasmic reticulum cholesterol concentration and cellular cholesterol acts as a negative feedback system to influence enzymes involved in cholesterol metabolism as well as LDL-r via SREBP transcription factors (*Horton et al. 2002*; *Maxfield et al. 2002*; *Soccio et al. 2004*). SREBP-2 is located

in the endoplasmic reticulum membrane, and when cellular cholesterol levels are depleted, SREBP-2 translocates to the nucleus to activate gene transcription by binding to sterol response elements (*Horton et al. 2002*). SREBP-2 activates genes involved in cholesterol metabolism such as HMGCoA-r and the LDL-r (*Horton et al. 2002*).

1.3.2.1.1. Cholesterol synthesis in humans

Cholesterol synthesis is reported to contribute ~5% of plasma or erythrocyte cholesterol, but can range from 1-10% (*Diraison et al. 1997*; *Clandinin et al. 1999*; *Diraison et al. 2002*; *French et al. 2002*). There may be diurnal variation in cholesterol synthesis, with highest rates occurring in the overnight and early morning period as opposed to afternoon (*Jones et al. 1990*; *Roe et al. 1991*; *Cella et al. 1995a*), but meal timing may also play a role in diurnal variations in cholesterol synthesis (*Cella et al. 1995b*).

Cholesterol synthesis can be stimulated after feeding (*Mazier et al. 1997*), but increased meal frequency is associated with suppression of cholesterol synthesis (*Jones et al. 1993b*; *Jones 1997*). However, differences in dietary intake may alter cholesterol synthesis, particularly dietary lipid composition. Cholesterol homeostasis is carefully balanced between endogenous and exogenous sources. Increasing dietary cholesterol tends to lower cholesterol synthesis (*Miettinen et al. 1989*; *Jones et al. 1996*), however this may be mediated mainly by changes in hepatic cholesterol synthesis as cholesterol feeding does not affect intestinal cholesterol synthesis (*Dietschy et al. 1971*). When intestinal cholesterol absorption increases there is a rise in cholesterol delivered to the liver from CM, so homeostasis is maintained by reducing hepatic synthesis via HMGCoA-r and by increasing bile acid synthesis (*Stellaard et al. 2005*). LDL-r are also downregulated to reduce uptake of cholesterol from circulating lipoprotein particles (*Stellaard et al. 2005*). Conversely, blocking enterohepatic circulation by biliary obstruction

increases cholesterol synthesis in the intestine as well as the liver (*Dietschy et al. 1971*). Santosa et al (2007) concluded that a change in one "vector" (either synthesis or absorption) causes a compensatory and reciprocal change in the other (*Santosa et al. 2007b*). For example, ezetimibe therapy reduces intestinal cholesterol absorption and leads to an increase in cholesterol synthesis (*Sudhop et al. 2002*), whereas statin therapy reduces cholesterol synthesis accompanied by an increase in absorption (*Gylling et al. 2002*; *Santosa et al. 2007b*).

Dietary FA also influence cholesterol synthesis. Trans FA have been shown to increase cholesterol synthesis (*Matthan et al. 2000*; *French et al. 2002*). Surprisingly, cholesterol synthesis may also be higher with PUFA-feeding and lower during SFA-rich feeding, despite lower and higher plasma cholesterol levels, respectively (*Jones et al. 1994b*; *Jones et al. 1994a*; *Mazier et al. 1997*; *Jones 1997*). The increase in cholesterol synthesis observed with PUFA feeding may be associated more with n-6 FA sources such as corn oil as opposed to sources higher in n-3 FA such as canola oil (*Jones et al. 1994b*; *Cuchel et al. 1996*). This finding also indicates that enhanced cholesterol synthesis does not necessarily contribute to increased plasma levels of cholesterol. There appear to be two categories of individuals, those with higher basal cholesterol synthesis and those with lower, which may affect plasma lipid response to dietary modulation such as cholesterol or phytosterol feeding (*Jones et al. 1993*; *Rideout et al. 2010*) or to drug therapies such as statins and ezetimibe (*Hoenig et al. 2007*; *Hoenig et al. 2010*). Individual differences in cholesterol synthetic rate may be due to genetic polymorphisms; for example, the ApoE2 allele is associated with greater cholesterol synthesis and lower plasma LDL-c, while the ApoE4 allele is associated with lower cholesterol synthesis and higher plasma LDL-c (*Gylling et al. 1992*; *Miettinen et al. 1992*).

CVD is associated with reduced synthesis but greater absorption of

cholesterol (*Rajaratnam et al. 2001*; *Matthan et al. 2009*), whereas individuals with metabolic syndrome and Type 2 diabetes are associated with lower absorption and higher synthesis of cholesterol (*Chan et al. 2003*; *Gylling et al. 2007*) as measured by sterol markers. Obesity may be associated with increased cholesterol synthesis, but this effect has not been consistently shown in all studies (*Griffin et al. 1998*; *Miettinen et al. 2000*; *Diraison et al. 2002*). The relationship of obesity to cholesterol synthesis may be mediated through insulin. Insulin resistance is associated with low absorption and high synthesis of cholesterol, which has been shown to be independent of obesity (*Pihlajamäki et al. 2004*; *Smahelová et al. 2007*; *Hoenig et al. 2010*; *Gylling et al. 2010c*), and both acute and chronic hyperinsulinemia have been shown to stimulate cholesterol synthesis (*Stinson et al. 1993*; *Feillet et al. 1994*; *Griffin et al. 1998*). Weight loss and energy restriction are associated with reduction in cholesterol synthesis, particularly if insulin sensitivity is restored (*Jones 1997*; *Griffin et al. 1998*; *Di Buono et al. 1999*; *Santosa et al. 2007a*). Conversely, glucagon may suppress cholesterol synthesis (*Ingebritsen et al. 1979*).

1.4. Measurement of Lipid Synthesis

Synthesis of FA and cholesterol are mediated by a variety of nutritional, hormonal and disease-related factors, and may contribute to aberrations in lipid metabolism leading to CVD. A variety of methods exist for measurement of lipid metabolic pathways, including flux, absorption, and synthesis. Indirect methods for measuring lipid synthesis utilize measurement of precursors or metabolites, whereas more direct methods available involve use of radio- or stable isotopes combined with different analytical techniques.

1.4.1. Indirect Methods

Indirect calorimetry has been used to estimate whole-body DNL. Respiratory quotients >1.0 reflect conversion of carbohydrate to fat. However, the respiratory quotient over 1 merely indicates that synthesis is greater than oxidation of fat and therefore reflects net synthesis of the whole body as opposed to dynamic actual synthesis (*Hellerstein 1999*).

The sterol balance method is considered the gold standard for measurement of cholesterol flux (*Jones 1997*). The sterol balance method requires precise measurements of food intake and fecal excretion, and attainment of sterol pool equilibrium which can take weeks or months therefore requiring prolonged feeding times (*Jones 1997*). Jones et al. (1998) compared the deuterium method against the sterol balance method for measurement of cholesterol synthesis. These authors found that cholesterol synthesis rates calculated by each method did not differ significantly, though the deuterium method may underestimate absolute cholesterol synthesis in some individuals (*Jones et al. 1998*).

Precursors and metabolites have been used as an indirect measure of cholesterol synthesis and absorption. Levels of sterols and stanols considered to reflect precursors and metabolites have been used to estimate cholesterol synthesis in a variety of populations (*Matthan et al. 2000*; *Gylling et al. 2004*). The assumption is that these compounds transfer into plasma lipoproteins at rates proportional to their formation in the cholesterol synthetic pathway (*Björkhem et al. 1987*). Matthan et al. (2000) compared the precursor/ metabolite method to the deuterium uptake method to determine if these methods were correlated for cholesterol synthesis (*Matthan et al. 2000*). Generally good agreement was found between the concentrations of precursors when they were expressed as absolute (μmol/L) or relative (μmol/mmol of cholesterol) amounts. When compared to the fractional

synthetic rate (FSR), plasma concentrations of desmosterol and lathosterol had better associations than squalene and lanosterol, however there was still ~30% variability between the two methods when the correlation coefficients were considered. Duane (1995) evaluated lathosterol relative to cholesterol synthesis and determined that while lathosterol changed with lovastatin treatment it did not reflect changes in cholesterol synthesis due to dietary cholesterol (*Duane 1995*). Gremaud et al (2002) also found that plasma cholestanol did not correlate with ^{13}C-cholesterol as a marker for cholesterol absorption (*Gremaud et al. 2002*).

Plasma mevalonic acid levels may also be used to assess cholesterol synthesis. While plasma levels of mevalonate may provide additional information about cholesterol synthesis not available via other methods, it may not be able to substitute for sterol balance methods and other more direct and quantitative measurements of daily cholesterol synthesis (*Parker et al. 1984*; *Jones et al. 1992*; *Jones et al. 1996*).

Therefore, using markers of precursors or metabolites is still an indirect measurement of cholesterol synthesis, and is only qualitative (*Jones 1990*). In addition, complete collection of urine or fecal samples are required, which can be labor-intensive and inconvenient.

1.4.2. Directs Methods - Isotopes

The most direct way to measure lipid synthesis would be by direct tissue perfusion or intubation, which is not feasible in most human patient populations for obvious reasons. More direct methods of measurement available involve the use of either radio- or stable isotopes. Depending on the application, an appropriate compound is labeled with an isotope ("tracer") on an atom of interest (carbon, hydrogen, nitrogen, or oxygen). The labelled compound (or a product of its metabolism) can then be traced through to different products in a variety of tissues (e.g. liver or adipose tissue) or pools

(e.g. plasma, urine, feces, or saliva). For example, ^{2}H-cholesterol administered orally dissolved in oil and incorporated into the breakfast meal and ^{13}C-cholesterol administered intravenously solubilized in a lipid emulsion, such as Intralipid, can be used to estimate the origin of excreted cholesterol when feces are collected for several days (*Ferezou et al. 1982*). The manner in which the data is approached and analyzed differs among methods.

1.4.2.1. Background of Use of Stable Isotopes

Regardless of the analytical method employed, either mass isotopomer distribution analysis (MIDA) or isotope ratio mass spectrometry (IRMS), the basic principles behind each are similar. For the purposes here, only stable isotopes will be considered, as radioisotopes are not commonly used for investigations in humans in the present day.

The organic atoms (carbon, hydrogen, nitrogen, and oxygen) exist as a combination of stable and radioactive isotopes. An isotope of an atom has the same chemical properties as the most abundant form of the atom, but a different number of neutrons which affects the mass of the atom. Therefore, isotopes are frequently referred to as "heavy" or "light", with the most abundant form of the atom typically being the lowest in mass (*Wolfe et al. 2005*). For example, carbon has 3 isotopes: ^{12}C the most abundant form, ^{13}C the stable isotope and ^{14}C the radioisotope (*Wolfe et al. 2005*). An isotopomer, by extension, is a molecule that has an isotopic tracer incorporated somewhere in its structure.

Measurement of radioactive isotopes involves determining rate of decay and uses an index of specific activity to measure the absolute amount of tracer present. However the measurement of stable isotopes, since they are "stable" and therefore do not have an activity that decays over time, is based on the ratio of labelled to unlabelled compounds. The "enrichment" of a compound of interest is determined by comparing the amount of tracer to

tracee (tracer to tracee ratio, TTR) (*Wolfe et al. 2005*). The enrichment of a compound is frequently reported as Atom Percent Excess (APE) which essentially indicates what percent of the atoms in a molecule are labelled with the tracer (*Wolfe et al. 2005*).

The quantity of isotopically labeled compounds within a pool of unlabelled compounds (i.e. a mixture) can be determined using mass spectrometry, which measures the molecular weight of a sample. A molecule enriched with one or more heavy isotopes will be heavier in mass compared to an unlabelled molecule, and therefore will be detectable compared to the unlabelled molecule. Mass spectrometry allows for measurement of the mass distribution of molecules (*Brunengraber et al. 1997*).

Caveats to using stable isotope methods in humans include the cost of the isotopes and labelled compounds. In addition, instrumentation required for sample analysis is often costly, complicated, and difficult to operate and maintain (*Rennie 1999*).

1.4.2.2. Mass Isotopomer Distribution Analysis (MIDA)

MIDA is useful when the compound of interest is a polymer formed from monomers, such as a fatty acid formed from repeated units of acetyl CoA. A labeled monomer precursor pool will result in a polymer product that can be labeled multiple times (*Wolfe et al. 2005*). An unlabelled compound will be considered mass 0 (M+0), a compound with one enriched atom will have a slightly higher mass (M+1), and so forth as more labels are incorporated into the polymer (M+2, M+3, etc.).

In MIDA it is not necessary to measure the enrichment in the precursor pool and analysis is performed via gas chromatography mass spectrometry (GC/MS) and mathematical modelling *(Hellerstein et al. 1991; Di Buono et al. 2000)*. This model uses ratios of molar excesses determined by the enrichment of the precursor pool from which they are synthesized, which

negates the need to measure actual precursor enrichment (*Hellerstein et al. 1991*; *Hellerstein et al. 1996*; *Di Buono et al. 2000*). The ratio of each mass isotopomer of theoretically generated molecule of interest is plotted as a function of the precursor pool in order to generate equations for enrichment (*Hellerstein et al. 1992*). FSR can never be 100% due to the contribution of dietary lipids to the body pools, and therefore another variable, the asymptote, is used to define the theoretical maximum isotopic enrichment of newly synthesized molecule expressed as molar excess (*Di Buono et al. 2000*). It is based on the frequency of each isotopomer of de novo molecule for a given precursor. FSR is then calculated as a ratio of the measured enriched product to the theoretical maximum produced over a period of time (*Hellerstein et al. 1992*). Absolute synthetic rate (ASR) can be calculated by multiplying the FSR with the compound pool size, using estimates from previous studies (*Goodman et al. 1980*).

MIDA has the advantages of using GC/MS for analysis, which does not require equipment as specialized as that required for IRMS. However, GC/MS also does not allow for as great precision as IRMS (*Rennie 1999*). The MIDA method has the disadvantages of being invasive as it requires indwelling catheters for isotope delivery, lengthy tracer infusion, and complicated data analysis with mathematical modelling (*Matthan et al. 2000*). Acetate tracer infusion also inflates the acetate pool size (*Jones 1996*), which may influence metabolic pathways. In addition, if using deuterium, higher enrichment is required in order to measure product enrichment by GC/MS, and errors in the correction for natural background enrichments can decrease the accuracy if high enough enrichment is not obtained (*Diraison et al. 1997*; *Diraison et al. 1998*; *Scrimgeour et al. 1999*; *Emken 2001*; *Diraison et al. 2002*). MIDA also requires a homogenous precursor pool with constant enrichment, as well as constant fractional synthetic rate (*Jones 1997*).

1.4.2.2.1. *Application to Fatty Acid and Cholesterol Synthesis*

Measurement of DNL using MIDA usually utilizes ^{13}C-acetate to investigate enrichment in fatty acids, primarily palmitate (*Hellerstein et al. 1993*). One of the issues related to use of ^{13}C-acetate is accurately measuring true precursor labelling, as labeled molecules can be exchanged or diluted with unlabelled molecules (*Dufner et al. 2003*). The acetate pool can vary depending on the nutritional and hormonal state of the individual (*Lowenstein et al. 1975*). Using the MIDA approach, typically only palmitate of either plasma or VLDL TG is measured, as palmitate is considered to be the primary product of DNL (*Diraison et al. 1997*). However, elevations in rates of synthesis for other FA such as stearate and oleate may contribute to DNL as well. Under the MIDA approach, a set of calculations related to probability of enrichment need to be created for each compound of interest, possibly explaining why usually only palmitate is considered.

^{13}C-acetate has also been used to measure cholesterol synthesis, based on the incorporation of repeating units of acetyl-CoA into newly synthesized cholesterol (*Neese et al. 1993*; *Di Buono et al. 2000*). Comparison of using ^{13}C-acetate and MIDA to deuterium incorporation measured by IRMS for estimation of cholesterol FSR has found that the methods correlated for estimations of both FSR and ASR (*Di Buono et al. 2000*).

Deuterium can be used with the MIDA approach, however requires attainment of higher enrichments in body water of >0.3% in order for detection of labelled compounds to be effective using GC/MS (*Diraison et al. 1997*; *Diraison et al. 1998*; *Diraison et al. 2002*). Doses allowing for attainment of greater plasma water enrichments have not been associated with side-effects and are well-tolerated (*Diraison et al. 1997*), therefore choice of dosage and analytical method may come down to financial and technical considerations.

1.4.2.3. Isotope Ratio Mass Spectrometry (IRMS)

In IRMS, there are two or three detectors (instead of one) which will each capture a different ion mass. The IRMS is coupled to a gas chromatograph (GC) and either a pyrolysis (P) or combustion (C) unit. If ^{2}H or ^{18}O are the atoms of interest combustion systems cannot be used as the products produced are water and CO_2, therefore pyrolysis (partial rather than complete combustion) of organic compounds and water containing these isotopes is preferred (*Rennie 1999*). The sample is introduced on the GC, which isolates molecules in a sample one at a time, such as fatty acids. The pyrolysis or combustion unit then converts the incoming molecules into gas, which is introduced to the IRMS for analysis. In this way, each molecule is separated from the others and individual enrichment is determined. The difference in ion current ratios in the sample gas coming from the pyrolysis or combustion unit detected by the IRMS is calculated relative to an isotope ratio standard, which is an internationally agreed-upon standard and differs depending on the isotope used (**Table 1-3**) (*Werner et al. 2001*). Hydrogen isotopes have the largest relative mass difference, and a low natural abundance of deuterium (**Table 1-3**) which makes for the largest variations in natural isotope ratios (*Wolfe et al. 2005*). $^{2}H/H$ ratios are currently expressed as a $\delta‰$ relative to two reference standards, Vienna Standard Mean Ocean Water or Standard Light Antarctic Precipitation, which are used to correct for nonlinear behaviour in instruments and samples preparation or analysis (*Werner et al. 2001*; *Godin et al. 2004*). Investigators typically develop their own secondary standards, such as local tap water, as additional references because the primary international standards are in short supply and have restricted availability (*Werner et al. 2001*).

Table 1-3: International isotope ratio standards

Isotope Ratio	Natural Abundances	International Standard
$^{2}H/^{1}H$	0.015%/99.985%	Vienna Standard Mean Ocean Water Standard Light Antarctic Precipitation Greenland Ice Sheet Precipitation
$^{13}C/^{12}C$	1.11%/98.89%	Vienna Pee Dee Belemnite
$^{18}O/^{16}O$	0.0021%/0.9976%	Vienna Pee Dee Belemnite Vienna Standard Mean Ocean Water

Stable isotopes exist naturally (**Table 1-3**) and therefore have a corresponding natural abundance, though in typically very low proportions. Thus, the measured enrichment determined by IRMS will reflect tracer from both the exogenous tracer given to the patient as well as naturally occurring label (*Wolfe et al. 2005*). A measure of the background or naturally occurring enrichment of label in the sample is therefore required, obtained by taking a sample before the label is given to the patient. A correction for the raw measurement of TTR is made, taking into account the raw measurement of TTR in the sample, the background or naturally occurring enrichment in the sample, and a "skew correction factor" using the natural abundance of the isotope and the number of atoms in the tracer. The "skew correction factor" reflects the fact that some atoms in the tracer and the tracee will already be enriched due to natural abundance, and therefore would not be available for further enrichment (*Wolfe et al. 2005*).

In the past the deuterium/hydrogen ratio ($^{2}H/H$) has been determined by a three-step off-line process, in which the compound is reduced to H_2O and CO_2 by CuO and silver wire, vacuum distillation of H_2O, and finally reduction of H_2O by zinc into hydrogen gas (*Godin et al. 2004*). This process is lengthy and tedious, and therefore an online process using GC coupled with pyrolysis and IRMS and high-temperature conversion elemental analyzer (TC-EA)

coupled to an IRMS has been developed (*Godin et al. 2004*). Hydrogen gas is introduced directly into the ion source of the IRMS via an open split, which has the benefits of being easy, efficient, and avoids the risk of isotopic fractionation (*Godin et al. 2004*). The TC-EA/IRMS represents a simpler system because the compounds do not need to be derivatized, allowing for simpler preparation and avoidance of isotopic dilution, and there are no concerns about GC parameters making the run times shorter (*Godin et al. 2004*). However, not all compounds are appropriate to run on the TC-EA, and sometimes specific injectors (e.g. solid vs. liquid) can be required; typically only plasma water deuterium enrichment is measured by TC-EA while other compounds of interest such as cholesterol and triglyceride are measured by GC/IRMS.

IRMS has the advantage of being highly sensitive and precise, does not require lengthy periods of isotope infusion, and can be used with a short measurement period of up to 24 hours (*Jones et al. 1993a*; *Emken 2001*). However, sample preparation is often lengthy and requires multiple stages, making it labor intensive (*Matthan et al. 2000*).

1.4.2.3.1. *Use of deuterium-labelled water as a tracer*

Deuterium is the most popular stable isotope used in conjunction with IRMS, for use of estimating synthesis of FA and cholesterol. However, the deuterium incorporation method relies on a few assumptions, discussed here.

Assumption #1: Tracer and tracee share the same metabolism.

A basic underlying assumption of stable isotopic methods is that every element in the molecule has an equal chance of being enriched, or replaced, with the isotope. In addition, the tracer is also considered to have the same metabolism as the unlabelled tracee, therefore the labeled molecule will trace

the movement of the unlabelled molecule (*Wolfe et al. 2005*). Deuterated water has become the most commonly used isotope compound used for determination of de novo cholesterol synthesis and lipogenesis. Water is an ideal compound to use as a tracer, as it crosses all membranes and equilibrates in all body water pools and compartments (*Dufner et al. 2003*). Deuterium-labeled water also has the benefits of being relatively inexpensive compared to other isotopically-labeled compounds, and can be administered orally (*Dufner et al. 2003*).

Equilibration across body pools allows sampling of accessible compartments such as plasma and urine, provides an indication of enrichment in otherwise inaccessible pools (*Kelleher 2001*), and avoids issues associated with isotopic tracer dilution across pools, which can happen with carbon-labelled tracers (*Kelleher 2001*). Deuterium-labelled water enters the cell easily and equilibrates rapidly with intracellular water. For example, Bywater et al. (1975) investigated whether deuterium-labelled and regular water are absorbed at equal rates using an isolated rat small intestine model and a practical tracer concentration of 1% D_2O. These investigators found no discrimination at the level of the intestinal mucosa in transport between H_2O and D_2O (*Bywater et al. 1975*). There is relatively little unlabelled water generated in the cell and therefore enrichment of the plasma should reflect enrichment of the precursor pool in the cell (*Wolfe et al. 2005*).

Assumption #2: The tracer should not influence or alter metabolism.

Thompson et al. (1953) found no isotopic effects between deuterium and hydrogen when rats were given deuterium-labelled water over 10 days sufficient to bring the concentration of deuterium in body water to 5% (*Thompson et al. 1953*). Peng et al. (1972) showed that rats fed drinking water containing 5% deuterium oxide showed no behavioural or morphological changes compared to control rats, even when the drinking

water was supplemented for 7 months (*Peng et al. 1972*). When the rats were fed drinking water containing 20% deuterium oxide they experienced morphological changes in kidney, testes, and liver at 6 weeks of feeding; however once feeding of deuterium ceased there was almost complete recovery. Disturbances in synthesis and metabolism of cholesterol and fatty acids has only been found in rats maintained on 20-25% D_2O (achieving approximately 12% enrichment in body water) (*Rabinowitz et al. 1960*; *Peng et al. 1973*). Therefore only at high levels of deuterium administration and incorporation into body water (>5% body water) there appears to be isotopic effects that may negatively affect normal metabolism. Such alterations in metabolism are unlikely to occur with doses of D_2O currently used in human populations, as in humans body water is only enriched by a few tenths of a percent by deuterium (*Brunengraber et al. 1997*). Many human studies using doses of D_2O of 0.5-3g per kg body weight have found plasma water enrichments of <0.5% (*Diraison et al. 1997*; *Gremaud et al. 2001*; *Cachefo et al. 2003*; *Diraison et al. 2003*).

Assumption #3: A consistent fraction of deuterium atoms are incorporated into the compound under different metabolic conditions.

It must be assumed that different metabolic conditions due to diet or disease do not influence the maximum theoretical number of deuterium molecules that can be incorporated into FA or cholesterol (*Leitch et al. 1991*).

Assumption #4: Pool size remains constant over the study period.

In studies where subjects are fed meals, pool size such as plasma TG may not be kept constant due to influx of dietary TG, however according to Leitch et al (1991) it is valid to assume steady-state conditions when comparing time points following an overnight fast (*Leitch et al. 1991*). Plasma

cholesterol levels, on the other hand, do not fluctuate over the day, and inputs of small amounts of dietary cholesterol are unlikely to impact the central pool of cholesterol from which FSR is measured (*Jones et al. 1992*).

1.4.2.3.2. *Application to de novo lipogenesis*

Palmitate, a major FA in the body, has 31 potential hydrogens that can be labelled; one hydrogen on each even-numbered carbon atom comes from water (7 total), all hydrogens on the odd-numbered carbons come from NADPH (14 total), and the rest (even-numbered carbon atom hydrogens and methyl hydrogens) come from acetyl CoA (10 total) (*Murphy 2006*). The amount of deuterium from enriched body water that ends up in the NADPH and acetyl CoA pools will depend on the source of these molecules. Hydrogens from body water can be incorporated into acetyl CoA via glycolysis, cleavage of citrate and acetoacetyl-CoA (*Brunengraber et al. 1997*).

Work by Jungas (1968) in rat adipose tissue investigating tritium and deuterium incorporation into newly synthesized FA estimated 23 hydrogen atoms of a typical FA, here considered to be palmitate, are derived from deuterium in the absence of an isotope effect (*Jungas 1968*). Incorporation was greater in even-numbered positions than odd-numbered positions, however the average ^{3}H incorporated per carbon atom into the FA chain was determined to be approximately 0.87 (*Jungas 1968*). Experiments using fasted tissue incubated with ^{3}H in the absence of substrate and insulin (therefore, when FA synthesis should be minimal) showed very low levels of ^{3}H incorporation (*Jungas 1968*). This indicates that ^{3}H was not incorporated due to reactions associated with beta-oxidation of FA and that synthesis is the major pathway by which ^{3}H would be introduced into the FA molecule. Wadke et al. (1973) found similar results when liver tissue from rats was incubated in deuterium, finding that 22.3 and 24.9 deuterium atoms were incorporated per molecule of palmitate and stearate, respectively (*Wadke et*

al. 1973). Diraison et al (1996) used a MIDA approach to estimate average number of deuterium molecules incorporated into plasma TG palmitate during FA synthesis in rats, and found 22 deuterium molecules incorporated into palmitate (*Diraison et al. 1996*). These investigators proposed that aside from water or NADPH, incorporation of label into palmitate could also occur through labelling of acetyl CoA, potentially through glucose, which is readily deuterated through gluconeogenesis (*Diraison et al. 1996*). Diraison et al (1996) also proposed that free FA synthesized in tissues other than the liver could be recycled back to the liver and contribute to the measured DNL of VLDL-TG (*Diraison et al. 1996*). However, labeled palmitate in the free FA pool occurred after 4 days of deuterium administration, therefore this recycling may not occur with shorter periods of deuterium administration, such as 24 hours. Lee et al (1994) used HepG2 cells to estimate that 17 hydrogen could be replaced with deuterium in palmitate and 20 in stearate (*Lee et al. 1994b*). In a subsequent study a maximum of 22 for palmitate and 24 for stearate in rats was found, however this was after 4 weeks of deuterium labelling, which may be due to incorporation from acetyl CoA (*Lee et al. 1994a*). In general, a value of 0.87 D incorporated per carbon is used for calculations of DNL in most studies (*Jones 1996*).

Isotope incorporation into FA can occur due to natural enrichment, DNL, or chain elongation (*Ajie et al. 1995*). Palmitate is the major product of DNL, however longer-chain FA such as stearate may be enriched to an extent by chain elongation of unlabelled pre-formed palmitate (*Wadke et al. 1973*; *Ajie et al. 1995*).

1.4.2.3.3. *Application to Cholesterol synthesis*

There are three main assumptions that are required when using deuterium to trace cholesterol, in combination with the assumptions of stable isotope methodology previously discussed (*Jones 1990*).

Assumption #1: :A constant fraction of deuterium originates from water during synthesis of cholesterol.

^{2}H equilibrates with total body water and NADPH which are the precursor pools from which 22 of 46 hydrogens for cholesterol synthesis are obtained (*Jones 1990*; *Di Buono et al. 2000*). Of the 22 hydrogens, it is estimated that 7 come from water and 15 from NADPH (*Jones 1990*). These hydrogen are taken from the body water pool and incorporated into the C-H bonds of cholesterol (*Brunengraber et al. 1997*). During the short-term, deuterium incorporation is within 20-22 per molecule cholesterol, whereas over the long-term incorporation is less narrowly defined as the acetyl CoA pool begins to contribute deuterium (*Matthan et al. 2000*).

Using a MIDA approach in rats, Diraison et al (1996) estimated that an average of 20 deuterium molecules are incorporated into cholesterol during synthesis (*Diraison et al. 1996*). Using HepG2 cells, it has been estimated that 20-25 hydrogens in cholesterol had potential to be deuterated (*Javitt et al. 1989*; *Lee et al. 1994b*). However, sometimes quite high levels of deuterium in cell medium were used (*Javitt et al. 1989*). A study in livers from rats fed deuterium-enriched drinking water estimated that a maximum of 30 hydrogens could be deuterated (*Lee et al. 1994a*). However, estimates of higher numbers of deuterium incorporated into cholesterol during synthesis may arise from acetyl CoA due to higher and longer deuterium administration, and are of less concern with short-term testing periods of 24 hours.

Part of the discrepancy in number of deuterium molecules incorporated into cholesterol also arises from how many hydrogens come from NADPH (*Dietschy et al. 1984*). NADPH is metabolized by the pentose phosphate pathway, in which hydrogens from NADPH do not exchange with

water, or the malic enzyme pathway, in which hydrogens from NADPH do exchange with water (*Jones et al. 1993a*). The degree of hydrogen contribution to NADPH from the pentose phosphate pathway to cholesterol synthesis may vary in different tissues or under different metabolic circumstances (*Jones et al. 1993a*). However, the rate of incorporation of deuterium into cholesterol appears to be fairly constant in the liver and intestine, the major organs for cholesterol synthesis, therefore a value of 22 deuterium per cholesterol molecule from water and NADPH is reasonable to assume (*Dietschy et al. 1984*; *Dell et al. 1985*), and this is used by the majority of investigators to calculate cholesterol synthesis (*Jones et al. 1990*; *Jones et al. 1993a*; *Gremaud et al. 2001*).

Assumption #2: C*holesterol rapidly exchanges between sites of synthesis and other pools, such as plasma, liver and intestine*.

Turnover of plasma cholesterol is characterized by three pools consisting of fast, intermediate, and slow-turnover. The M1 pool is considered to be in rapid equilibrium with plasma cholesterol and consists of cholesterol from plasma, blood cells, liver, and intestines (*Goodman et al. 1980*; *Dell et al. 1985*). The liver is believed to contribute significantly to plasma cholesterol levels over other tissues (*Neese et al. 1993*). Entrance and exit of cholesterol from the body only occurs by way of these rapidly exchangeable pools, and therefore production rate in these pools are equivalent to total body turnover rate (*Goodman et al. 1973*; *Mazier et al. 1997*). The M2 intermediate-turnover pool includes cholesterol from viscera and peripheral tissues, whereas the M3 slow-turnover pool includes cholesterol from connective tissue, skeletal muscle, arterial walls, and adipose tissue (*Goodman et al. 1980*; *Dell et al. 1985*). The M1 pool size can be estimated from body weight, plasma cholesterol, and plasma TG (*Goodman et al. 1980*). Free cholesterol is assumed to be in the rapid turnover pool associated with the liver and

intestine, and therefore migrates rapidly between plasma and lipoproteins. It is assumed that flux of cholesterol between other pools is small during time of measurement (*Jones et al. 1990*).

Assumption #3: Enrichment of deuterium in the plasma free cholesterol reflects cholesterol synthesized from the central pool and does not include newly synthesized cholesterol contributed from other pools.

The relatively short-term periods of cholesterol synthesis measured using the deuterium uptake method will not detect cholesterol synthesized in the slower turnover pools (*Jones et al. 1992*). Synthesis in such pools has been estimated to be up to 25% in baboons (*Dell et al. 1985*); however whether this situation is the same in humans is unknown, and therefore the deuterium method may underestimate total net formation rates of cholesterol synthesis yet should estimate the majority of cholesterol synthesized as the method will capture synthesis from the rapid-turnover pool which is the major contributor to total synthesis (*Jones et al. 1992*). In addition, cholesterol does not appear to engage in proton exchange, therefore deuterium enrichment measured in cholesterol reflects new synthesis (*Jones et al. 1993a*).

1.5. Type 1 Diabetes

Type 1 diabetes (T1D) accounts for approximately 10% of all cases of diabetes, the rest comprising mainly Type 2 diabetes (*Daneman 2006*). It occurs mostly in children but can also occur in adulthood (*Haller et al. 2005*). The incidence of T1D is increasing, and is particularly prevalent in certain geographical areas such as Nordic and Western countries, which could reflect genetic or environmental influences (*Rewers et al. 2004*; *Franke et al. 2005*). T1D occurs more commonly in Caucasians, and there are no apparent differences in incidence between males and females (*Pundziute-Lyckå et al.*

2002). There does not appear to be a large heritable genetic link, as only 10-15% of those with T1D has a first-degree relative with the disease (*Redondo et al. 2001*), and risk is higher in those whose father has the disease compared to the mother (*Hämäläinen et al. 2002*; *Rewers et al. 2004*).

T1D is a disorder characterized by the autoimmune destruction of the pancreatic β-cells, leading to an inability to produce insulin. Due to resulting insulitis, patients become dependent on exogenous sources of insulin to regulate plasma glucose levels.

1.5.1. Proposed Causes of Type 1 Diabetes

It has been suggested that stimulation of the immune system to turn against the pancreatic β-cells may be due to genetic susceptibility, environmental factors, or an interaction between these factors. Determining risk factors for development of T1D is complicated by the fact that T1D can occur in childhood but can also occur spontaneously in adults, and appears to have a low association with family history (*Faideau et al. 2005*). Therefore there are suggestions that there are genetic as well as environmental risk factors.

Approximately 20% of Caucasians carry the genotype associated with T1D susceptibility, yet only 5% of those with genetic predisposition appear to progress to overt diabetes (*Knip et al. 2005*). Genetic susceptibility may predispose an individual to be more sensitive to environmental factors or impairments in the innate immune system, such as auto-reactive T cells that are not deleted properly and therefore become reactive to islet autoantigens (*Csorba et al. 2010*). There is evidence to suggest that groups that move from a low-incidence area to a high-incidence area experience an increase in T1D occurrence that mirrors the incidence rate of that region (*Akerblom et al. 1998*), indicating genetic susceptibility cannot be the only contributing factor to development of T1D, and that environmental factors must also play a role.

Suggested environmental causes include viruses and dietary factors. Enteroviruses are suspected, and may act by increasing gut permeability, changing the gut cytokine environment (*Vaarala 2005*), molecular mimicry (*Atkinson 2005*), activation of autoreactive T cells (*Achenbach et al. 2005*), or disruption of the balance between helper and killer T cells (*Faideau et al. 2005*). Cow's milk is usually the first source of foreign dietary protein during infancy in developed countries, and human and bovine insulin differ only by three amino acids leading to the suggestion that there might be molecular mimicry and cross-reactivity with islet antigens (*Vaarala 2002*; *Knip et al. 2005*). Vitamin D is a hormone with immune-modulating capacities, therefore lack of Vitamin D at critical stages in infancy and childhood may affect T1D incidence (*Takiishi et al. 2010*).

1.5.2. Type 1 Diabetes - Pathophysiology

Development of T1D is characterized by four phases: pre-clinical, onset, remission, and establishment (*Rewers et al. 2004*). The pre-clinical phase is generally asymptomatic and can have varying duration, and in fact may not progress to clinical diabetes (*Knip et al. 2005*). During this period autoimmune-driven destruction of the β-cells occurs leading to gradual reduction in insulin secretion (*Rewers et al. 2004*), and appearance of auto-antibodies against islet cell antigens such as glutamic acid decarboxylase and insulin (*Knip et al. 2005*; *Franke et al. 2005*). Islet autoantibodies are present in 70-80% of newly diagnosed T1D patients, compared to 0.5% of the general population (*Haller et al. 2005*). If two or more islet autoantibodies are present, remission is unlikely (*Yu et al. 2000*). As the pre-clinical phase progresses, there is loss of insulin secretion, reduction in insulin response to glucose load, and fasting hyperglycemia (*McCulloch et al. 1991*). The islet of Langerhans are comprised of α, δ, and β endocrine cells (*Faideau et al. 2005*), and yet the immune-mediated attack is targeted specifically against the β-

cells. It has been suggested that incitement of autoimmune attack arises from factors such as molecular mimicry, alteration of self-antigens, or breakdown in central or peripheral tolerance (*Haller et al. 2005*).

At onset of diabetes, patients present with a classic cluster of symptoms including polydipsia, polyuria, polyphagia, and weight loss (*Haller et al. 2005*). Insulin insufficiency leads to plasma hyperglycemia. Once plasma glucose levels exceed the renal reabsorptive threshold, glucose is excreted into the urine, drawing water with it and causing dehydration and thirst (*Haller et al. 2005*). Weight loss occurs due to loss of water, ketones, and glucose, and amino acids are catabolized for gluconeogenesis and FA from adipose tissue for ketogenesis, a process normally inhibited by insulin (*Haller et al. 2005*). There may still be residual β-cell function at this stage (*Knip et al. 2005*), however it is insufficient to handle blood glucose fluctuations.

After insulin therapy has been initiated, there is a transient remission of clinical symptoms which can vary in duration (*Zamaklar et al. 2002*; *Rewers et al. 2004*). Patients may have an improved β-cell function due to exogenous insulin sources and therefore a "rest" for the β-cells (*Eisenbarth 2004*). However, this phase is always temporary and eventually insulin requirements are increased (*Rewers et al. 2004*), at which point T1D is established and irreversible.

1.5.3. Complications of Type 1 Diabetes

1.5.3.1. Acute complications

Acute complications of T1D include ketoacidosis, infections, and hypoglycemia (*Rewers et al. 2004*), whereas chronic complications include microvascular and macrovascular problems. Ketoacidosis is caused by the buildup of ketones used for energy during glucose sparing, leading to osmotic diuresis and acidosis (*Haller et al. 2005*). Hypoglycemia is a major concern in T1D and occurs due to low dietary intake, exercise or insulin overdose,

especially if there is an inappropriate lack of counterregulatory response of glucagon which may be due to the absence of an intraislet insulin signal (*Cryer 2005*). Hypoglycemia is potentially fatal, and is a major limitation to intensive diabetes management. As duration of diabetes progresses, there is loss of the sympathetic neural response by epinephrine to induce physical symptoms of hypoglycemia, which eventually leads to hypoglycemic unawareness (*Cryer 2005*) which is particularly dangerous as the patient is unaware of low glucose levels and therefore does not seek appropriate treatment. However, risk of hypoglycemia must be balanced with long-term risks of micro- and macrovascular complications associated with hyperglycemia.

1.5.3.2. Chronic Complications - Microvascular

Chronic microvascular complications of T1D are retinopathy and nephropathy (while some include neuropathy), whereas macrovascular complications include various forms of CVD. Hyperglycemia may contribute to development or progression of micro- and macrovascular complications by one of four proposed pathways: 1) increased polyol pathway flux, 2) increased advanced glycation end-product formation, 3) activation of protein kinase C isoforms, or 4) increased hexosamine pathway flux (*Brownlee 2001*).

Retinopathy occurs in 80% of patients with long-standing T1D (*Klein et al. 1984*), and increases with duration of diabetes and age at onset (*Klein et al. 1990*; *Orchard et al. 1990*). Retinopathy has been associated with poor survival in diabetes and linked to CVD (*Rajala et al. 2000*). Progression of retinopathy may be slowed by intensive insulin therapy (*DCCT Research Group 1993*), as well as absence of other risk factors such as dyslipidemia (*Chew et al. 1996*).

Nephropathy occurs in 20-30% of diabetic patients (*Rewers et al. 2004*), and is the leading cause of end-stage renal disease in diabetes (*Girach*

et al. 2006). T1D patients with nephropathy are at high risk of death from CVD and renal failure (*Krolewski et al. 1995*). Risk factors for development of nephropathy are hyperglycemia, hypertension, duration of diabetes, age at onset, smoking, and genetic predisposition (*Fogarty et al. 1997*; *Girach et al. 2006*). Glycemic control appears important for prevention of microalbuminuria, whereas risk for progression may be influenced more by presence of hypertension, dyslipidemia, and genetic factors (*DCCT Research Group 1995b*; *Krolewski et al. 1995*; *Bakman et al. 2001*).

Neuropathy is usually asymptomatic and largely untreatable once established, except by palliative measures (*DCCT Research Group 1995a*). In particular, foot problems are the most common reason for hospitalization in patients with T1D (*Dickinson et al. 2002*). Neuropathy is caused by alterations in nerve blood flow leading to neuronal degeneration and impairment of regeneration. Evidence of risk factors for neuropathy may include metabolic control, age, duration of diabetes, presence of other complications, and smoking (*Tesfaye et al. 1996*; *Adler et al. 1997*; *Dyck et al. 1999*). Intensive glycemic control may reduce development of neuropathy (*DCCT Research Group 1995a*).

1.5.3.3. Chronic Complications - Macrovascular

Despite improvements in patient care and pharmacological treatment of risk factors CVD remains a major cause of death in T1D (*Maahs et al. 2010*), and individuals with T1D are at a much higher risk of death from CVD compared to age and sex-matched non-diabetic controls (*Brindisi et al. 2010*). Recent reports also indicate that a significant proportion of T1D patients have asymptomatic but measurable vascular disease (*Weckbach et al. 2009*), even in young individuals (*Krantz et al. 2004*).

Diabetes duration and timing of initiation of intensive glycemic control can determine CVD risk in T1D (*Orchard et al. 2010*). Glycemic control

may have a large impact on development of lesions early in atherosclerosis, but seems to have less of an effect in those with long-term diabetes (*Orchard et al. 2010*). Contribution of hyperglycemia to CVD is still controversial, and Orchard et al (2010) suggest that it may be the improvement in other factors, such as lipoproteins and blood pressure, that accompany lower glycemic levels that contribute to reduced CVD risk (*Orchard et al. 2010*). Hyperglycemia may confer "glucose stabilization" in which glycemia contributes to formation of stable arterial plaques as opposed to vulnerable plaques that would be more likely to lead to serious coronary events (*Orchard et al. 2006*). Still, the DCCT and EDIC trials have reported continued beneficial effects of intensive glycemic therapy even 10 years later, with prior intensively treated individuals showing slower progression of diabetic retinopathy (*White et al. 2010*). In addition, the Pittsburgh EDC study showed that HbA1c was predictive of a fatal CVD event but not a non-fatal event (*Conway et al. 2009*).

Traditional CVD risk factors such as elevated lipid levels are useful to predict CVD risk in T1D, however they will not capture all T1D patients who are at risk (*Orchard et al. 2010*). An atherogenic lipid profile (high TG, LDL-c and TC, and low HDL-c) has been associated with CVD mortality in diabetes (*Grauslund et al. 2010*), however only with modest lipid and lipoprotein abnormalities, whereas nephropathy and retinopathy are associated with more pronounced alterations (*Chaturvedi et al. 2001*). T1D subjects tend to have lower TG and higher HDL-c levels, as compared to people with Type 2 diabetes (*Katulanda et al. 2010*), which would normally confer a protective benefit. The fact that most individuals with T1D have normal lipid levels yet a markedly increased risk of CVD (*Orchard et al. 2010*) has prompted investigations into other irregularities that may contribute to this increased CVD risk, such as qualitative abnormalities in lipoprotein composition or metabolism.

1.5.4. Dysregulations in Lipid Metabolism in Type 1 Diabetes

1.5.4.1. Role of glucose & insulin

As T1D is a state of glucose and insulin irregularities, dysregulation of lipid metabolism could occur due to independent effects of these two factors. For example, glucose concentrations have been shown to upregulate intestinal transporters related to cholesterol and FA absorption (but not efflux) and possibly downregulate genes regulating cholesterol synthesis (*Feingold et al. 1994*; *Tomkin 2008*; *Ravid et al. 2008*). Insulin reduces secretion of ApoB100 from hepatic cells (*Allister et al. 2004*), and inhibits ApoB48 secretion from intestinal cells which may be partially by direct influence as well as partially by suppression of free fatty acids (*Pavlic et al. 2010*). Insulin also inhibits VLDL production and FA release from adipose tissue, and stimulates TG hydrolysis and lipoprotein clearance via activation of LPL (*Vergès 2009*). Insulin resistance is also associated with worse lipoprotein profile in men and women with T1D (*Maahs et al. 2010*). However, as patients with T1D are dependent on exogenous insulin, it is difficult to separate effects that may be due to insulin from those that may be due to some aspect of diabetes per se.

1.5.4.2. Evidence from Animal Studies

Evidence from animal models of T1D suggest altered gene expression of major lipid-modulating factors. SREBP-1c has been suggested to be downregulated after induction of diabetes resulting in reduced FA synthesis, which is only partially restored with insulin therapy (*Bloks et al. 2004*). SREBP-2 and HMGCoA-r may be enhanced in T1D resulting in increased cholesterol synthesis; however, this has not always been observed (*Bloks et al. 2004*) and expression may differ between the intestine and the liver (*Feingold et al. 1994*; *Lally et al. 2007*). For example, Feingold et al (1994)

found an increased expression of HMGCoA-r in the intestine of streptozotocin-injected rats. Also in streptozotocin-injected animals, Lally et al (2007) found no significant difference in intestinal expression of HMGCoA-r but a significantly higher hepatic expression of HMGCoA-r. In comparison, Bloks et al (2004) found a significantly reduced hepatic expression of HMGCoA-r in streptozotocin-treated animals, which did not increase after insulin administration, and no difference in hepatic expression of SREBP2 compared to non-diabetic animals. Hepatic expression of LDL-r may be reduced upon induction of diabetes in animals but is restored with insulin (*Bloks et al. 2004*). Diabetic animals may also have enhanced intestinal and hepatic expression of NPC1L1 and reduced expression of ABCG5/G8, indicating enhanced absorption and less efflux of cholesterol in the intestine and liver (*Bloks et al. 2004*; *Lally et al. 2007*; *Tomkin 2008*). Diabetic animals have also been observed to have greater intestinal cholesterol absorption due to lower resistance of the unstirred water layer and increased passive absorption, which can be corrected with insulin therapy and dietary manipulation (*Thomson 1983*; *Thomson et al. 1983*; *Thomson et al. 1984*). Intestinal and hepatic MTTP have also been found to be upregulated in diabetes, which may contribute to greater CM and VLDL particle synthesis (*Bloks et al. 2004*; *Lally et al. 2007*).

These results indicate that a greater amount of cholesterol may be synthesized and possibly also absorbed in the enterocyte of diabetic animals, which may translate into release of CM particles that are more enriched in cholesterol. Further, there may also be effects of diabetes at the hepatic level causing enhanced hepatocyte cholesterol accumulation and promoting release of cholesterol-rich VLDL particles. In addition, the influence of diabetes is complicated by the effect of insulin therapy as well as potentially different effects on intestinal versus hepatic regulation. While animal studies provide useful information on gene regulation and transporter expression

profiles, often there can be profound hyperlipidemia in diabetic animals with elevations in both plasma TG and TC. This hyperlipidemia is not generally observed in humans, and therefore these animals may not be an ideal model for human T1D lipid metabolism. The studies discussed below provide evidence from human subjects only.

1.5.4.3. Evidence from Humans

Alterations in enzymes involved in modification of the lipid composition of lipoproteins may occur in diabetes. For example, CETP activity has been noted to be higher in T1D patients along with greater transfer of CE between lipoproteins, particularly those with worse glucose control, which may be due to peripheral hyperinsulinemia (*Dullaart et al. 1989*; *Bagdade et al. 1991*; *Bagdade et al. 1994*; *Colhoun et al. 2001*; *Chang et al. 2001*; *de Vries et al. 2005*; *Vergès 2009*). LPL activity may be normal in well-controlled T1D, but reduced in individuals with poor glycemic control and insulin deficiency or resistance (*Nikkilä et al. 1977*; *Groop et al. 1996*; *Caixàs et al. 1998*; *Vergès 2009*). HL activity may be also lower in T1D subjects (*Ruotolo et al. 1994*; *Caixàs et al. 1998*). Irregularities in activities of CETP, LPL, and HL may be improved with insulin therapy (*Ruotolo et al. 1994*; *Bagdade et al. 1994*).

Total ApoB levels have been found to be elevated in T1D (*Albers et al. 2008*; *Bagdade et al. 2009*); however some studies report no such elevation, indicating that increases in ApoB levels may depend on glycemic control (*Duvillard et al. 2005*; *Albers et al. 2008*; *Guy et al. 2009*; *Mazanderani et al. 2009*). However, postprandial ApoB48 levels may be elevated in brittle T1D subjects, indicating reduced clearance efficiency of intestinally-derived lipids (*Su et al. 2009a*). Support for these findings are provided by studies in adults that suggest a delayed CM and CM-R clearance in T1D (*Georgopoulos et al. 1991*). Impaired clearance of CM may be due to reduced lipolysis or tissue

uptake stemming from abnormal particle composition, such as enrichment in cholesterol, or clearance mechanisms (*Georgopoulos et al. 1991*). In addition, it has been suggested that subcutaneous insulin injections produces peripheral hyperinsulinemia while inducing a relative hepatic hypoinsulinemia, which reduces HL-mediated catabolism of VLDL particles (*Annuzzi et al. 2001*).

The type and degree of lipid metabolism abnormality may depend on the level of glucose control in the study population; those with poorer glycemic control may have higher circulating levels of glucose and free FA, which can stimulate VLDL production, as well as low levels of insulin, which may reduce LPL activity resulting in poor clearance of the TG-rich lipoproteins CM and VLDL (*Vergès 2009*; *Sorensen et al. 2010*). There have been few studies on VLDL production rates in T1D, but those available indicate that subcutaneous insulin infusion may reduce VLDL TG production (*Pietri et al. 1983*) but have no effect on VLDL particle production rate (*Duvillard et al. 2005*) in T1D with good glycemic control compared to non-diabetic controls. A study of T1D patients with good control and free from complications found that cholesterol and TG content in VLDL in relation to ApoB was higher compared to matched controls, indicating greater lipid loading of ApoB particles in T1D (*Christ et al. 2001*). Other studies have found that VLDL particles from T1D individuals are enriched in cholesterol or CE and are less enriched in TG compared to non-diabetic individuals (*Rivellese et al. 1988*; *Winocour et al. 1992*; *Dullaart 1995*; *Pérez et al. 1997*; *Duvillard et al. 2005*; *Vergès 2009*)

People with T1D have been found to have a larger proportion of LDL particles present as small and dense as compared to larger, more buoyant particles, which may be atherogenic (*Jenkins et al. 2003*; *Guy et al. 2009*; *Maahs et al. 2010*). LDL particles in T1D have also been found to be more TG-rich (*James et al. 1990*; *Winocour et al. 1992*; *Pérez et al. 1997*; *Duvillard et al.*

2005). Differences in LDL particle characteristics such as proportions of FC, CE, PL, TG, ApoAI and ApoB may be mediated by ApoE genotype, particularly ApoE4 carriers (*Eichner et al. 1992*; *Blaauwwiekel et al. 1998*; *Murdoch et al. 2007*).

From existing evidence it appears that in T1D there may be abnormal metabolism of lipids that begin at the intestinal level, and are further exacerbated by issues related to hepatic metabolism and clearance in the circulation. Clearance issues may be related to alterations in lipoprotein lipid content, enzyme efficiencies, and interactions between lipoproteins and receptors or transfer proteins. In addition, there may be greater cholesterol and TG loading in CM and VLDL particles, which could stem from greater lipid absorption or synthesis. Existing evidence also indicates that normal lipid levels common in T1D may not indicate normal lipid metabolism, therefore investigations are necessary to examine where abnormalities in lipid metabolism lie.

1.5.4.4. De novo lipogenesis and cholesterol synthesis in Type 1 diabetes

Emerging evidence suggests that a significant proportion of T1D patients may have detectable NAFLD. In a recent investigation, NAFLD was present in 44% of the individuals with T1D studied, despite one third of these patients taking lipid-lowering medications (*Targher et al. 2010*). The majority of T1D patients with NAFLD also had serum ALT levels within the normal reference range, indicating that serum liver enzymes are not useful as predictive markers of fatty liver in T1D (*Targher et al. 2010*). Presence of NAFLD in these patients with T1D was associated with a higher prevalence of vascular disease, even after adjustment for risk factors such as age, smoking, diabetes duration, HbA1c and LDL-c (*Targher et al. 2010*). Interestingly, NAFLD was more associated with CVD in individuals with lower BMI and plasma LDL-c and TG. These findings indicate that NAFLD may be present in

T1D individuals even with normal plasma lipid levels, and if so might confer even greater CVD risk. Insulin stimulates DNL, and regulates VLDL synthesis and secretion by targeting ApoB100 for degradation (*Chahil et al. 2006*). Development of NAFLD in T1D may be due to an accumulation of TG-rich particles in the liver combined with insufficient VLDL production due to insulin-mediated ApoB100 degradation. To date there have been no investigations of DNL of individual FA in established T1D individuals.

Cholesterol synthesis has been found to be upregulated in T1D patients beginning insulin therapy (*Feillet et al. 1994*), and in acute hyperinsulinemia (*Abrams et al. 1982*; *Stinson et al. 1993*). However, established T1D has been associated with reduced synthesis and elevated absorption of cholesterol compared to non-diabetics in a series of investigations by Miettinen and Gylling using sterol precursors and metabolites as markers of cholesterol synthesis and absorption (*Miettinen et al. 2004*; *Gylling et al. 2004*; *Gylling et al. 2007*). It has also been suggested that balance between cholesterol absorption and synthesis may not be as well maintained in T1D compared to non-diabetics, as synthesis was reciprocally related to absorption in control subjects across a range of absorption efficiency, but only in the high efficiency absorption in T1D (*Järvisalo et al. 2006*; *Gylling et al. 2007*). Improvement in glycemic control may upregulate cholesterol synthesis but has no apparent effects on cholesterol absorption, indicating that insulin deficiency may downregulate cholesterol synthesis and upregulate cholesterol absorption (*Sittiwet et al. 2007*). In healthy people, those with higher cholesterol absorption efficiency have higher cholesterol in CM and VLDL fractions, implying that enhanced intestinal cholesterol absorption can increase CM cholesterol content (*Agren et al. 2006*). If this finding holds true for T1D as well, it may explain the greater cholesterol contents in CM and VLDL particles that have been noted in these subjects (*Rivellese et al. 1988*; *Winocour et al. 1992*; *Dullaart 1995*;

Pérez et al. 1997; *Duvillard et al. 2005*; *Vergès 2009*). These findings have not been assessed by more direct and quantitative methods, such as stable isotopes.

1.5.4.5. Lipid metabolism in Type 2 diabetes

In contrast to T1D, individuals with Type 2 diabetes typically present with a more atherogenic lipid profile. Type 2 diabetes patients often have elevated levels of TG and low levels of HDL-c, and may have an increase in small dense TG-rich LDL particles (*Chahil et al. 2006*). In insulin resistance typical of Type 2 diabetes there appears to be increased assembly and secretion of TG-rich particles such as CM and VLDL (*Chahil et al. 2006*). In addition, LPL activity may be reduced contributing to impaired clearance, potentially through increased ApoCIII gene expression, whereas HL activity may be increased (*Chahil et al. 2006*). Free FA levels are higher in Type 2 diabetes and may contribute towards increased TG formation and release into VLDL particles, enhancing VLDL production (*Chahil et al. 2006*).

Insulin resistant states are associated with an increase in DNL (*Chahil et al. 2006*; *Wilke et al. 2009*). In addition, patients with Type 2 diabetes may have aberrant responses to dietary therapy (*Schwarz et al. 2003*; *Wilke et al. 2009*). Insulin resistance has also been associated with higher cholesterol synthesis and lower absorption measured by sterol precursors and metabolites (*Smahelová et al. 2007*; *Hoenig et al. 2010*; *Gylling et al. 2010c*). As with T1D, it has been suggested that the reciprocal relationship between cholesterol synthesis and absorption may be impaired in Type 2 diabetes (*Ooi et al. 2009*). Therefore, there may be similarities but also differences in DNL and cholesterol synthesis between T2D and T1D, and generalizations from one disease state to the next cannot be made.

In summary, lipid abnormalities in T1D are still not well-characterized. More evidence is available for abnormalities in Type 2

diabetes, however, generalizations between the two forms of diabetes are inappropriate as there may be significant differences between aetiologies and pathophysiologies between the two diseases. The observation of mostly normal lipid levels in patients with T1D may be masking underling lipid dysregulation. Characterization of aspects of lipid metabolism such as synthesis, absorption, and clearance, may help to decipher protective vs. detrimental metabolic pathways contributing to CVD. Determination of mechanisms contributing to lipid metabolic abnormalities in T1D may also help to guide interventions for treatment and prevention of CVD in a population that has an elevated risk of CVD morbidity and mortality, despite normal lipid levels.

1.6. Chronic Liver Disease and Liver Failure

The liver is a major metabolic organ, involved in the metabolism of nearly all nutrients, drugs, and toxins. This review will focus on chronic liver diseases, which are more common and can be caused by genetic or environmental factors.

1.6.1. Pathophysiology of Liver Failure

Cellular adaptation, degeneration, and finally death contribute to liver injury and disease (*Burt et al. 2007*). An initial inflammatory insult causes hepatocyte injury and hypertrophy due to dysregulation of energy homeostasis and ion pump failure, leading to influx of fluid from the extracellular space and accumulation of macronutrients (*Burt et al. 2007*). Hepatocytes then undergo apoptosis, followed by vascular remodelling and regeneration (*Burt et al. 2007*). Fibrosis in the liver eventually leads to cirrhosis, which can lead to development of neoplasia and carcinoma (*Burt et al. 2007*).

1.6.2. Causes of Liver Disease and Failure

Genetically-caused liver diseases include Wilson's disease, a copper-accumulation disorder, and some forms of cholestasis, which are bile-associated diseases. Cholestasis can be caused by genetic defects in bile formation or bile acid transporters, an autoimmune-mediated response (*Trauner et al. 2002*; *Pratt 2005*), or as a result of a preceding disorder or obstruction in bile secretion or flow (*Trauner et al. 2002*). Cholestasis is characterized by impaired bile secretion leading to inadequate bile in the intestine, and spillover of bile components to the blood, such as bile acids, cholesterol, phospholipids, and bilirubin (*Burt et al. 2007*; *Wagner et al. 2009*).

Liver disease is a frequent complication associated with chronic alcohol abuse (*Hoek et al. 2004*). Alcoholism is present in ~10% of the population, and is the third preventable cause of death in the United States (*Levitsky et al. 2004*; *Mandayam et al. 2004*; *Lucey et al. 2009*). Metabolism of alcohol produces acetaldehyde, which is toxic, and is further metabolized to acetate, which can be used for FA and cholesterol synthesis or metabolized for energy via the citric acid cycle (*Burt et al. 2007*; *McKillop et al. 2009*). FA oxidation is reduced, coupled with enhanced lipolysis, leading to increased TG synthesis and contributing to formation of fatty liver, which is present in up to 90% of individuals that abuse alcohol (*Mandayam et al. 2004*; *Burt et al. 2007*). Enhanced TG and cholesterol deposition in hepatocytes reduces cellular membrane fluidity and permeability, and also displaces the nucleus, impairing cellular function (*Burt et al. 2007*). Prolonged alcohol abuse can cause progression to hepatitis in up to 35% of individuals, and finally to fibrosis and cirrhosis in ~10% of individuals who stop consuming alcohol but up to 70% of individuals who continue to abuse alcohol (*Hoek et al. 2004*; *Levitsky et al. 2004*; *Mandayam et al. 2004*). Alcohol-related cirrhosis leading

to death accounts for up to half cirrhosis-related deaths worldwide (*Mandayam et al. 2004*).

Hepatitis strains B (HBV) and C (HCV) are the most common forms of hepatitis in individuals with liver disease in developed countries. HBV is prominent in China, Asia, and Africa and is passed by body fluids, but can be prevented by vaccination (*Burt et al. 2007*). HBV is mostly asymptomatic and may exist in an inactive or carrier state, however 15-40% of individuals with chronic HBV infection may develop liver failure leading to death (*Fattovich 2003*; *Burt et al. 2007*). Along with alcohol, HCV is a leading cause of liver disease and it is estimated that 3% of the world's population are infected (*Burra 2009*; *Douglas et al. 2009*). HVC-related cirrhosis is a primary reason for liver transplantation, and reoccurrence of infection after transplantation is high (*Burra 2009*; *Douglas et al. 2009*). HCV is not preventable, as the virus can mutate which helps it elude the body's humoral immune response, and causes damage due to the immune response directed at the virus. Steatosis is common in HCV (*Safdar et al. 2004*), and progresses to hepatitis in 60-80% of individuals (*Syed et al. 2010*). HCV is also highly associated with development of hepatocellular carcinoma (HCC), accounting for up to 75% of hepatic cancer cases worldwide (*Douglas et al. 2009*). HCV is associated with insulin resistance which may be predominantly peripheral over hepatic (*Douglas et al. 2009*; *Milner et al. 2010*), and Type 2 diabetes which is three times more common in individuals with HCV compared to those without HVC and those with HBV (*Douglas et al. 2009*).

More recent phenomenons causing liver problems are NAFLD and non-alcoholic steatohepatitis (NASH). NAFLD is defined as hepatic fat accumulation of 5-10% by weight (*Lazo et al. 2008*). NAFLD is estimated to be present in ~30% of the population, and in 75% of obese individuals, whereas up to 20% of obese individuals may have NASH (*Cheung et al. 2008*; *Lazo et al. 2008*). The pathophysiology of NAFLD and NASH are still under

investigation, however it is proposed that NAFLD and progression to NASH occurs via a "two-hit" process. The first hit is imbalance in FA metabolism leading to development of steatosis (NAFLD), and the second is induction of an inflammatory state (NASH) that can eventually lead to fibrosis and cirrhosis (*Burt et al. 2007*; *Jou et al. 2008*). It is hypothesized that fatty liver occurs due to the combination of enhanced lipolysis leading to increased FA delivery to the liver, increased DNL, reduced hepatic lipid clearance, and reduced FA oxidation leading to accumulation of TG in lipid droplets causing cellular dysfunction (*Burt et al. 2007*; *Fabbrini et al. 2008*; *Cheung et al. 2008*; *Jou et al. 2008*). Development of fatty liver is associated with obesity, particularly visceral obesity, and insulin resistance (*Cheung et al. 2008*; *Jou et al. 2008*). The combination of steatosis and inflammation in NASH leads to fibrosis and cirrhosis in up to 50% of patients (*Burt et al. 2007*; *Jou et al. 2008*).

Advanced liver disease often leads to development of HCC, which is the third leading cause of cancer death worldwide (*Roberts et al. 2005*; *Sherman 2010*). While HCC is often a primary reason for transplantation and death, it is not a primary cause of liver failure (*Sherman 2010*). There is usually a preceding disease that induces liver damage leading to cirrhosis, which makes the liver susceptible to cell regeneration and repair errors (*Burt et al. 2007*). Alcohol, HBV, HCV, and NAFLD or NASH are particularly associated with development of HCC (*Mandayam et al. 2004*; *Burt et al. 2007*; *Sherman 2010*). HCC is generally asymptomatic and is extremely malignant, therefore upon presentation the patient is usually at an advanced stage and HCC is only discovered upon presentation of other symptoms due to liver failure, such as jaundice (*Burt et al. 2007*; *Sherman 2010*). HCC is proposed to develop by an initial insult that causes a genetic defect which cannot be repaired and is irreversible, damage which is then promoted via replication and progresses (*Burt et al. 2007*).

1.6.3. Complications of Liver Failure

Patients with advanced liver disease have a myriad of metabolic derangements. Protein-energy malnutrition is common, particularly in alcohol abuse, as well as presence of micronutrient deficiencies (*Halsted 2004*; *O'Brien et al. 2008*). Metabolic derangements stem from both nutritionally-related factors, such as inadequate dietary intake, and metabolic factors such as abnormal digestion and absorption, enhanced protein catabolism, and altered glucose and lipid metabolism (*Halsted 2004*).

Cirrhosis is usually irreversible liver damage and the final stage of chronic liver disease (*Burt et al. 2007*), and is a major cause of death (*Mandayam et al. 2004*). It develops by necrosis that is not repaired, fibrosis, and loss or transformation of liver architecture (*Anand 2001*; *Burt et al. 2007*). The most common causes of hepatic cirrhosis are alcohol and hepatitis due to HCV, HBV, and NASH. Cirrhosis can lead to impaired metabolism of macronutrients, vitamins and minerals, and cause portal hypertension, ascites, and encephalopathy (*Burt et al. 2007*). Hyperinsulinemia coupled with reduced insulin sensitivity and hyperglycemia are prevalent in cirrhosis, and insulin clearance rate may be impaired (*Barzilai et al. 1991*; *Bayraktar et al. 1995*). Eventually advanced cirrhosis will lead to metabolic decompensation (*Anand 2001*; *Burt et al. 2007*; *Durand et al. 2008*).

Cirrhosis is associated with development of portal hypertension (*Cárdenas et al. 2003*). Portal hypertension causes splanchic blood vessel formation and vasodilation which limits blood flow through the systemic circulation and causes arterial underfilling (*Cárdenas et al. 2003*; *Mandell et al. 2008*). Development of ascites causes disturbed electrolyte and protein metabolism, and can lead to encephalopathy due to accumulation of toxic substances in the blood (*Burt et al. 2007*). Hepatic encephalopathy is neurologic impairment caused by incomplete toxin removal, which

accumulate in the blood and can impair the brain (*Burt et al. 2007*).

1.6.4. Dysregulation of Lipid Metabolism in Liver Failure

Liver damage that has progressed to cirrhosis and liver failure affects metabolism of carbohydrates, protein, and lipids. Lipid-related changes include alterations in lipoproteins as well as enzyme functions. The following review will focus on changes in lipid metabolism associated with liver failure and cirrhosis due to alcohol, HCV, and HCC, excluding genetic cholestatic disorders and NAFLD/NASH.

Generally in advanced liver disease and cirrhosis, plasma levels of TG and TC are reduced (*Miller 1990*; *Iglesias et al. 1996*; *Jiang et al. 2006*; *Varghese et al. 2007*). In alcoholic cirrhosis, plasma TC and TG can be elevated (*Jiang et al. 2006*; *Varghese et al. 2007*). Elevations in TG may stem from high plasma free FA due to lipolysis of adipose tissue for energy. However, in advanced disease plasma TC and TG levels tend to be lower (*Rössner et al. 1979*; *Zambon et al. 1995*). In fact, low plasma cholesterol levels may be indicative of worsening HCV, presence of steatosis, and predict response to treatment (*Serfaty et al. 2001*; *Siagris et al. 2006*; *Corey et al. 2009*). Bile acid synthesis may be reduced in cirrhosis, and bile acid uptake may be impaired (*Miettinen 1972*; *Yoshida et al. 1999*; *Burt et al. 2007*).

Alterations in enzyme activities may affect lipoprotein composition and metabolism. For example, during cirrhosis there may be reduction in lecithin:cholesterol acyltransferase activity, which may impair formation of CE and HDL metabolism as well as altered lipid composition of LDL (*Avgerinos et al. 1983*; *De Martiis et al. 1983*; *Nayak et al. 1988*; *Miller 1990*; *Tahara et al. 1993*; *Zambon et al. 1995*; *Cabré et al. 2005*). CETP may not be altered in cirrhotic patients (*Tahara et al. 1993*; *Iglesias et al. 1996*), whereas LPL and HL activity may be normal or reduced (*Avgerinos et al. 1983*; *Iglesias et al. 1996*; *Cabré et al. 2005*). It has been indicated that levels of all

apolipoproteins are reduced in cirrhotic patients, though to varying degrees (*Zambon et al. 1995*). ApoB levels may or may not be reduced in cirrhosis (*Nayak et al. 1988*), but may be lower in advanced liver disease such as HCC, hepatic coma, and decompensated cirrhosis (*Cordova et al. 1984*; *Nayak et al. 1988*; *Iglesias et al. 1996*; *Jiang et al. 2006*).

A study in rats using artificial labelled CM found that in cirrhotic animals lipolysis of circulating CM was faster compared to normal rats, whereas remnant particle removal was slower (*Damião et al. 1993*). Patients with cirrhosis due to alcohol were found to have higher fasting CM levels (*Avgerinos et al. 1983*), however other studies have found a lower CM response following an oral fat load in cirrhotic patients (*Cabré et al. 2005*). As yet it is unclear how the intestinal lipid response is affected during advanced liver disease, though it could by proposed that there could either by a greater CM secretion to compensate for reduced hepatic lipoprotein synthesis, or elevated plasma CM levels due to reduced clearance by hepatic receptors, or both.

In HCV there is impaired synthesis and secretion of VLDL, potentially due to inhibition of MTTP (*Negro et al. 2009*; *Syed et al. 2010*). HCV particles circulate as lipoviral particles rich in TG, ApoB100, and ApoE that can resemble VLDL particles (*Syed et al. 2010*). It is proposed that HCV virus replication utilizes the VLDL secretion pathway, and causes a reduction in rate of secretion of VLDL to increase the ability of the virus to co-assemble with VLDL for transport (*Syed et al. 2010*). Impaired VLDL synthesis and secretion contributes to the low plasma levels of LDL frequently seen in HCV and cirrhosis (*Varghese et al. 2007*). In alcoholic cirrhosis and HCC a reduction in levels of lipoproteins including HDL, VLDL, and consequently LDL, is noted , which may stem from impaired synthesis and secretion (*Rössner et al. 1979*; *Jiang et al. 2006*; *Burt et al. 2007*). VLDL particles may contain less cholesterol and TG in cirrhotic patients which may stem from

reduced synthesis or reduced lipid incorporation into VLDL particles (*Rössner et al. 1979*; *Iglesias et al. 1996*; *Ferré et al. 2002*).

HCV infection may downregulate PPAR-α, leading to reduction in FA oxidation, which may contribute to FA available for TG formation and development of steatosis (*Syed et al. 2010*). Alcoholism is also associated with downregulation of PPAR-α, contributing to steatosis (*Reddy et al. 2006*). Plasma free FA may be increased in alcoholic cirrhosis and HCC (*Wilcox et al. 1978*; *Jiang et al. 2006*), however in advanced cirrhosis plasma FA may be reduced (*Cabré et al. 1996*). Incorporation of dietary FA into VLDL-TG may be lower in cirrhotic patients, which may be due to reduced hepatic TG and VLDL synthesis (*Cabré et al. 2005*). Reduced incorporation of dietary FA into CM has also been observed, which could indicate an alternate absorption pathway for fat during cirrhosis, suggested to be via the portal systemic shunts that form due to portal hypertension (*Cabré et al. 2005*). It has been reported that there are low levels of PUFA in plasma lipid fractions of patients with advanced cirrhosis, including LA, ALA, AA, EPA, and DHA (*Wilcox et al. 1978*; *González et al. 1992*; *Cabré et al. 1996*; *Burke et al. 2001*). PUFA deficiency may arise from malnutrition or impaired activity of the Δ5 and Δ6 desaturases (*González et al. 1992*; *Cabré et al. 1996*).

1.6.4.1. De novo lipogenesis and cholesterol synthesis in liver failure

Increased hepatic lipid synthesis may contribute to development of steatosis. Steatosis is a pre-disposing factor for development of fibrosis and hepatocyte damage leading to cirrhosis (*Reddy et al. 2006*).

1.6.4.1.1. De novo lipogenesis

It has been proposed that HCV infection upregulates hepatic DNL of FA by activation of SREBP-1c and FAS in cellular models of HCV (*Waris et al. 2007*; *Yang et al. 2008*; *Negro et al. 2009*; *Syed et al. 2010*). However, a study in HCV patients examining liver biopsy samples found no significant differences

in SREBP-1c expression between HCV patients and subjects with normal liver function (*McPherson et al. 2008*). There was a negative correlation between SREBP-1c expression and degree of steatosis and fibrosis, indicating those with worse liver function tended to have lower SREBP-1c expression (*McPherson et al. 2008*). Expression of FAS showed no relationship with steatosis, but higher FAS expression was associated with greater insulin resistance, whereas SREBP-1c expression was not associated with degree of insulin resistance (*McPherson et al. 2008*). The differences noted between cellular models and the reported human study may be due to acute vs. chronic effects of HCV infection. An ex vivo analysis of adipocytes from cirrhotic and healthy patients found that adipocyte lipogenesis was higher in cirrhotic patients than controls (*Harewood et al. 1982*). DNL may also be upregulated in alcoholic steatosis and cirrhosis (*Tietge et al. 1998*; *Syn et al. 2009*), however, this effect has largely been inferred. Studies in which hepatic DNL has been directly measured and found to be enhanced have predominantly only been carried out in patients with NAFLD, using deuterated water analyzed by MIDA (*Diraison et al. 2003*), or labelled acetate to estimate DNL (*Donnelly et al. 2005*). Therefore, studies using more direct methods are warranted in non-NAFLD liver failure patients.

1.6.4.1.2. Cholesterol Synthesis

It has been proposed that hepatic cholesterol synthesis is reduced in HCV infection through viral-replication mechanisms (*Corey et al. 2009*). This downregulation in cholesterol synthesis may stimulate upregulation of LDL-r thereby lowering plasma cholesterol levels, which may account for the lower plasma cholesterol levels noted in HCV infected individuals (*Corey et al. 2009*). Hepatic cholesterol synthesis has also been suggested to be impaired in HCC, chronic liver disease, and cirrhosis, as evidenced from radioactive studies in animals and methods using sterol markers and ex vivo techniques in humans (*Bissell et al. 1972*; *Miettinen 1972*; *Miller 1990*; *Zimmermann et al.*

1992; *Jiang et al. 2006*). The reduction of cholesterol synthesis has largely been inferred and has not been confirmed by direct and quantitative methods, such as stable isotopes.

In summary, individuals with severe hepatic damage experience alterations in lipid metabolism. Liver failure patients often have low plasma lipid levels, considered desirable in the general population. However, low plasma lipid levels may not indicate normal lipid metabolism, and in the case of liver failure may actually be indicative of established or accelerating hepatic damage. In addition, while plasma lipid levels may be low, this does not mean that lipid synthesis is reduced in liver failure. On the contrary, alcohol, HCV, and cirrhosis have been inferred to upregulate lipid synthesis. Upregulation of lipid synthesis in cirrhotic patients would be of particular importance since development and progression of steatosis is involved in development and worsening of cirrhosis. Determination of lipid synthesis in patients with cirrhosis may help to guide treatment modalities as well as lead to development of new markers for characterization of disease progression.

1.7. Transplantation

1.7.1. Islet Transplant

Islet transplant (ITx) is a relatively novel treatment for T1D. The concept of transplanting isolated islets instead of whole pancreas for amelioration of diabetes began with significant work in animal models, following by trial cases in human subjects (*Warnock et al. 1983*; *Warnock et al. 1987*; *Warnock et al. 1992*). The Edmonton Protocol developed in 1999 was a key turning point for ITx (*Shapiro et al. 2000*). Despite moderate success with ITx, the procedure is not without side-effects, and consideration and management of such side-effects are necessary.

1.7.1.1. Islet Physiology and Role of Insulin

The pancreas is composed of 95-98% exocrine cells and <5% endocrine cells (*Kobayashi et al. 2004*). The endocrine cells, the islets of Langerhans, are made up of alpha, beta, delta, and PP cells. The most important cells for glucose regulation are the α- and β-cells. β-cells comprise 80-85% of all cells in the islets, and are insulin-secreting cells, while α-cells secrete glucagon (*Kobayashi et al. 2004*). β-cells can detect blood glucose levels and therefore play a major role in maintaining glucose homeostasis. When insulin is impaired or insufficient, the peripheral organs are unable to take up glucose and blood glucose levels consequently remain high resulting in hyperglycemia.

Plasma glucose concentrations are maintained between 4 and 7 mmol/L via tightly controlled interactions between intestinal absorption, production by the liver, and uptake by peripheral tissues, influenced by the actions of insulin and glucagon (*Saltiel et al. 2001*). Insulin is the primary regulator of blood glucose concentration; it inhibits hepatic production while stimulating glucose uptake by tissues such as muscle and adipose. In addition to its glycemic effects, insulin also influences protein and fat metabolism through its anabolic actions, promoting the storage of FA and amino acids in adipose, liver, and muscle tissues, by stimulating lipogenesis, glycogenesis, and protein synthesis, and inhibiting lipolysis, glycogenolysis and protein catabolism (*Saltiel et al. 2001*; *Meshkani et al. 2009*). Any dysregulation of insulin, whether reduced secretion or tissue sensitivity, results in alterations in macronutrient metabolism and can lead to increased fasting and postprandial glucose and lipid levels (*Saltiel et al. 2001*).

Insulin stimulates cellular glucose uptake via a complex set of signalling mechanisms. Upon reaching the target tissue cell, insulin binds to the insulin receptor (*Meshkani et al. 2009*). Activation of the insulin receptor

pathway stimulates translocation of glucose transporters. The majority of insulin-stimulated glucose uptake (~75%) is by skeletal muscle, and a small fraction by adipose tissue, both of which utilize the GLUT4 transporter (*Saltiel et al. 2001*; *Huang et al. 2007*). GLUT4 resides in cellular vesicles which cycle from intracellular stores to the plasma membrane (*Huang et al. 2007*). Hepatic glucose uptake is independent of insulin; however insulin still exerts other effects such as inhibiting glycogenolysis and gluconeogenesis, and stimulating glyconeogenesis (*Saltiel et al. 2001*). Insulin accomplishes this via direct effects on the liver as well as by influencing substrate availability. Insulin can modify free FA supply from visceral fat, which is less insulin-sensitive than subcutaneous fat (*Saltiel et al. 2001*). In addition, insulin increases transcription of FAS and ACC, key enzymes in lipogenesis, possibly through transcription of SREBP-1c (*Saltiel et al. 2001*; *Meshkani et al. 2009*). Insulin also inhibits adipose tissue lipolysis and therefore free FA flux to the liver by inhibiting HSL (*Saltiel et al. 2001*; *Meshkani et al. 2009*).

1.7.1.2. Exogenous Insulin Therapy and Pancreatic Transplant

Endogenous insulin is released from the pancreas into the portal circulation where it is estimated that 50-80% is removed by the liver (*Kautzky-Willer et al. 1993*; *Meier et al. 2005*; *Toffolo et al. 2006*), resulting in a lower proportion released into the systemic circulation for tissue exposure. Exogenous insulin, on the other hand, is injected and therefore released directly into the systemic circulation. Insulin injections translate into tissues exposed to more insulin while the liver is exposed to less, leading to an altered insulin gradient. In addition, exogenous insulin cannot respond to moment-to-moment transient in vivo changes in blood glucose. This problem of trying to match exogenous insulin dose with in vivo insulin requirements is one of the greatest challenges faced by the diabetic individual, as too much insulin can lead to hypoglycemia while too little can lead to prolonged states of hyperglycemia, which may have implications for development of long-term

complications (*DCCT Research Group 1993*). Current methods of insulin delivery include multiple daily injections and continuous subcutaneous insulin infusion via a pump. Continuous insulin infusions via a pump have been shown to lead to greater improvements in HbA1C than traditional multiple daily injections, and may also reduce glycemic fluctuations (*DCCT Research Group 1993*; *Bruttomesso et al. 2009*; *Derosa et al. 2009*). The insulin pump allows for delivery of insulin similar to normal pancreatic secretion by mimicking basal and bolus secretion of insulin in response to food intake (*Valla 2010*). Insulin pumps allow for greater flexibility and quality of life (*Bruttomesso et al. 2009*). Recent development of continuous insulin infusion pumps coupled to a sensor allows for monitoring of blood glucose in real-time, and may lead to fewer incidences of hypoglycemia (*Bergenstal et al. 2010*).

Given limitations of insulin therapy, the only other option to treat T1D is to replace the β-cells, either by pancreas or islet transplantation. Pancreatic transplantation has been shown to be an effective therapy for T1D patients who are also undergoing simultaneous renal transplantation (*Frank et al. 2004*). Pancreas and islet transplants are both found to be highly effective in obtaining insulin independence. Pancreas transplants may provide a more reliable and longer restoration of normoglycemia but are also associated with longer hospital stays, more readmissions to hospital, and greater number of complications, and may not improve morbidity or mortality in T1D individuals (*Hirshberg et al. 2003b*; *Frank et al. 2004*). ITx may be more expensive than whole-pancreas even with the shorter hospital stays and lower rate of readmission, due to the need for multiple islet donors in order to establish insulin independence. However, it must be kept in mind that typically pancreas used for ITx is initially rejected for use in whole organ transplant, leading to the possibility of less viable tissue (*Frank et al. 2004*).

Given the risks inherent in whole pancreas transplantation, ITx

presents an attractive option. ITx is associated with considerably less morbidity due to a less complex and invasive surgery, as well as fewer complications than with whole organ transplants. Additionally, there is the possibility of re-transplantation should the graft fail.

1.7.1.2.1. Population

The supply of islets is severely limited; therefore it is restricted to those for whom it confers the greatest benefit. As such, only those with "brittle" diabetes are considered, defined as those with undetectable C-peptide levels and widely varying inadequate glucose control despite best efforts at insulin therapy, as well as severe recurrent and reduced awareness of hypoglycemia (*Shapiro et al. 2006*). Exclusion criteria may include advanced coronary artery disease, HbA1c <12%, and inadequate renal reserve (*Shapiro et al. 2006*). As with any transplant, the benefits of ITx must outweigh the risks associated with complications related to surgery and immunosuppression.

1.7.1.2.2. Procedure

Isolation of islets is based on the Ricordi method. The procedure involves obtaining pancreas tissue from a cadaveric donor followed by: 1) pancreatic volume expansion using collagenase, 2) pancreatic digestion and collection of tissues, and 3) islet purification (*Ricordi et al. 1986*; *Ricordi et al. 1989*). Liberase solution is infused through the main pancreatic duct to cause pancreatic expansion, after which the enlarged pancreas is put in a Ricordi chamber filled with the same Liberase enzyme solution which induces tissue digestion (*Kobayashi et al. 2004*). Digestion is stopped when the exocrine tissue dissociates from the islets, because dissociated islets are difficult to purify and collect, and cellular activities are damaged (*Kobayashi et al. 2004*). Islets are then isolated by gravity centrifugation and the tissue solution collected (*Kobayashi et al. 2004*). Islets are infused into the portal vein of the

recipient, where they adhere to liver tissue and secrete insulin.

1.7.1.3. Islet Transplant - Outcomes and Success

Initial results of ITx clinical trials were disappointing. Fewer than 12% of patients remained insulin-independent for longer than one week, and approximately 8-10% remained independent at one year post-transplant (*Warnock et al. 1992*; *Hirshberg et al. 2003b*). The drug protocol in these transplants typically included antibody induction, Cyclosporine, Azathioprine, and glucocorticoids (*Shapiro et al. 2000*). The turning point in ITx occurred in 2000 in which a glucocorticoid-free immunosuppressive regimen was utilized (*Shapiro et al. 2000*). This regimen, now termed the "Edmonton Protocol", was groundbreaking in their initial results. Glucocorticoids are potent immunosuppressives; however they are associated with significant side-effects, including weight gain, hypertension, and most critically in this setting cause insulin resistance and hyperglycemia. In place of these drugs, the Edmonton Protocol utilized a combination of Sirolimus, Tacrolimus, and Daclizumab, which allowed for a best attempt at minimizing damage to the β-cells (*Shapiro et al. 2000*; *Kobayashi et al. 2004*). In addition, patients were selected for transplantation if they had not yet had onset of any long-term complications. The Edmonton protocol also used multiple transplants from a variety of donors to achieve insulin independence, avoided xenoprotein-enriched media to minimize islet immunogenicity, and avoided *in vitro* culture of islets to minimize cold ischemia time; these factors may have contributed to the success of this protocol (*Hirshberg et al. 2003b*; *Frank et al. 2004*; *Digon 2009*).

Success by Edmonton Protocol

In the initial report, all 7 patients initially transplanted with the Edmonton Protocol did not require exogenous insulin once a sufficient

number of islets had been transplanted, which was typically two transplants (*Shapiro et al. 2000*). In addition, initial transplantation with suboptimal islet mass reduced severe hypoglycaemic episodes in all patients (*Shapiro et al. 2000*). In all patients there was a reduction in serum glucose concentrations and glycemic fluctuations, normalization of HbA1c, and detectable C-peptide levels at 3 and 6 months post-transplant. In addition, results from oral glucose-tolerance tests conveyed that none of the patients met current American Diabetes Association criteria for diabetes, although the response at 120 min was impaired in some subjects and two had fasting glucose concentrations above the normal range (*Shapiro et al. 2000*). Reports on additional patients from the Edmonton group provide similar results in improvement in HbA1c and glycemic control, as well as insulin independence in ~80% at 1 year post-transplant (*Ryan et al. 2001*; *Ryan et al. 2002*). While these initial results were impressive, the long-term results are less favourable. A five-year follow-up of Edmonton Protocol islet patients showed that while majority (~80%) of patients remained C-peptide positive, the proportion remaining insulin independent was <10% (*Ryan et al. 2005*). However, those that resumed insulin therapy typically required lower insulin doses than pre-transplant, indicating some treatment benefit.

Success by Other Groups

The International Trial of the Edmonton Protocol evaluated the reproducibility of the Edmonton Protocol's success at 9 international sites (*Shapiro et al. 2006*). At one year, 44% of patients had insulin independence, 28% had partial function, and 28% had lost graft function (*Shapiro et al. 2006*). Of the patients who were insulin independent at one year, only a third were still insulin independent at two years follow-up, and this proportion decreased further at 3 years (*Shapiro et al. 2006*). The Collaborative Islet Transplant Registry collates information on ITx from 25 American and

Canadian institutions involved in ITx. In a 2008 summary, the Registry reported that of 325 patients 50% maintained insulin independence at 1 year post-transplant, which was reduced to 35% at 2 years, and 23% at 3 years post-ITx (*Alejandro et al. 2008*). At 3-years post-transplant, 23% of patients were insulin independent, 29% insulin dependent with detectable C-peptide, 26% had lost function, and 22% were unknown due to missing data (*Alejandro et al. 2008*).

The majority of transplant groups report 50-80% of patients are insulin independent at one year post-transplant, however the proportion of patients retaining graft function appears to decline to 25-50% by 3 years post-transplant (*Hirshberg et al. 2003a*; *Froud et al. 2005*; *Warnock et al. 2008*; *Vantyghem et al. 2009a*). While infrequent, long-term insulin independence is possible in some individuals, as evidence is now available on select exceptional cases maintaining insulin independence >10 years (*Berney et al. 2009*). Compared to patients on the insulin pump, ITx recipients had better glycemic control and reduced insulin needs out to 3 years post-transplant (*Vantyghem et al. 2009b*). When compared to intensive medical therapy involving management of glucose, lipids and blood pressure, after 3 years ITx recipients had better glycemic control as well as slower progression of retinopathy, while renal function was not different between the two groups (*Warnock et al. 2008*).

Therefore, despite somewhat disappointing outcomes for long-term insulin independence, ITx still offers other benefits associated with metabolic control and quality of life. Reduction in insulin requirements and incidence of hypoglycemia, as well as improvements in glycemic control and hypoglycemia unawareness may be particularly important to the diabetic patient, and should not be undervalued. Such improvements may contribute to the overall improvement in quality of life of diabetic patients after ITx (*Tharavanij et al. 2008*).

Graft Failure and Obstacles to Success

The reasons for failure of islet function following transplant are not yet clear as there are many possible factors, such as β-cell exhaustion, development of insulin resistance, suboptimal transplant site, immunosuppressive toxicity, rejection, and recurrent autoimmune damage (*Frank et al. 2004*). The most prominent obstacles to islet transplantation progression are procedure-related complications, shortage of islets available for transplantation, and use of immunosuppressants (*Hirshberg et al. 2003b*).

Procedural-related complications include internal bleeding, gall bladder puncture and portal vein thrombosis (*Hirshberg et al. 2003b*). While the liver has the advantage of being an easy site for transplantation and allows for portal insulin delivery thereby avoiding the systemic hyperinsulinemia that occurs with exogenous insulin therapy, it may not be an ideal site for islet engraftment (*Rajab 2010*). Approximately half of islets from donor tissue are lost during isolation and injection, due to thrombotic and immune-mediated mechanisms (*Vardanyan et al. 2010*; *Rajab 2010*). In addition, inadequate revascularization of intraportally transplanted islets contributes to low islet oxygenation and consequent cell death (*Lau et al. 2009a*; *Lau et al. 2009b*). Graft rejection may occur due to autoimmune damage (*Frank et al. 2004*) or other causes (*Toso et al. 2009*). The hepatic environment may also induce islet dysfunction, providing glucose-rich portal blood and proximity to hepatocytes which contain glycogen and have high gluconeogenic capacity (*Mattsson et al. 2004*; *Vardanyan et al. 2010*). It is not yet clear if islet function is completely restored in ITx, which may be due to either inadequate islet mass transplanted or deleterious effects of immunosuppressive agents. For example, intrahepatic islets have been shown to have lower insulin content and stimulated insulin release (*Mattsson et al. 2004*; *Rickels et al. 2005*; *Rickels et al. 2006*; *Lau et al. 2007*). In addition, the

neuroendocine hypoglycemic response is improved in some but not all ITx reports, however the glucagon response may not be fully restored as compared to non-diabetic individuals, which could be due to either low α-cell number or impaired glucagon response (*Meyer et al. 1998*; *Paty et al. 2002*; *Rickels et al. 2005*; *Lau et al. 2006*; *Rickels et al. 2007*).

Prioritization of cadaveric pancreata are currently given to whole pancreas transplants, and only allocated to ITx if deemed unsuitable or declined, leading to a relative shortage in islets available for transplantation (*Hirshberg et al. 2003b*; *Frank et al. 2004*). In addition, isolation and purification of adequate islets from donor pancreata are inefficient (*Hirshberg et al. 2003b*). Immunosuppressive agents have adverse side-effects, and are often toxic to β-cells (*Frank et al. 2004*; *Digon 2009*). Concentrations of these drugs are highest in the portal circulation surrounding the intrahepatic islets, which could be protective against recurrent autoimmunity or detrimental due to toxic effects on the β-cell (*Desai et al. 2003*).

1.7.1.4. Islet Transplant - Complications

Side-effects of islet transplantation, like those of many types of transplantation, are associated with the use of immunosuppressive drugs. Commonly reported serious adverse events associated with ITx are elevated liver function enzymes, hemorrhage associated with surgical procedure, pneumonia, portal vein thrombosis, and ovarian cysts in pre-menopausal women, whereas non-serious adverse events include mouth ulcers, anemia, diarrhea, edema, hypoglycemia, vomiting, and lymphopenia (*Hirshberg et al. 2003a*; *Ryan et al. 2004*; *Froud et al. 2005*; *Hafiz et al. 2005*; *Shapiro et al. 2006*; *Alejandro et al. 2008*). About half of patients may develop a serious adverse event, and 15% may experience multiple adverse events (*Hafiz et al. 2005*). Most complications are associated with the immediate post-transplant

period and resolve over time (*Rafael et al. 2003*; *Hafiz et al. 2005*). However, some side-effects, such as hyperlipidemia, persist over the course of immunosuppression.

1.7.1.4.1. Hyperlipidemia and Cardiovascular Disease after Islet Transplant

Incidence of hyperlipidemia

In the Collaborative Islet Transplant Registry's database of ITx patients, 32% of patients were on a lipid-lowering medication pre-transplant, which increased to 61% of patients post-ITx at up to 3 years post-transplant (*Alejandro et al. 2008*). Other reports have indicated that increases in LDL-c post-transplant may require pharmacologic therapy in 50-90% of patients, due to either new onset or exacerbation of hyperlipidemia (*Ryan et al. 2001*; *Ryan et al. 2002*; *Hirshberg et al. 2003a*; *Ryan et al. 2005*; *Froud et al. 2005*; *Hafiz et al. 2005*). Plasma TG levels may increase marginally during the post-ITx period, but most reports indicate that plasma TG levels are not affected (*Ryan et al. 2001*; *Froud et al. 2005*; *Hafiz et al. 2005*).

Animal models have indicated lipid accumulation in hepatocytes surrounding islets as well as upregulation of lipogenic regulatory factors such as SREBP-1c in hepatocytes around islets, and suggest that localized steatosis may contribute to β-cell failure (*De Paepe et al. 1995*; *Lee et al. 2007*). Imaging and biopsy techniques in human ITx patients have observed mild and localized steatosis in 20-50% of patients at ≥1 years post-transplant (*Markmann et al. 2003*; *Bhargava et al. 2004*; *Shapiro et al. 2006*; *Toso et al. 2009*), and one report found that presence of steatosis was associated with declines in graft function (*Venturini et al. 2010*). Presence of steatosis surrounding intraportal islets suggests lipogenesis may be upregulated, either due to local hyperinsulinemia or effects mediated by immunosuppressive medications.

Incidence and Mortality of CVD in ITx patients

As reviewed previously, hyperlipidemia is associated with CVD (*Castelli et al. 1992*; *Wilson et al. 1998*; *Alagona 2009*; *Chapman et al. 2010b*). In addition, individuals with T1D are at an increased risk of morbidity and mortality from CVD. Interestingly many individuals with T1D have normal plasma lipid levels, and yet still experience CVD at rates similar to individuals with Type 2 diabetes, who have elevated lipid levels and other metabolic abnormalities (*Orchard et al. 2010*; *Maahs et al. 2010*; *Brindisi et al. 2010*). ITx may induce hyperlipidemia to a extent that pharmacological treatment is required, associated with use of immunosuppressive medications (*Ryan et al. 2001*; *Ryan et al. 2005*; *Hafiz et al. 2005*). However, ITx is also associated with greater glycemic control, which clinical trials in individuals with T1D have shown to be of crucial importance in prevention of micro- and macrovascular complications (*White et al. 2010*). It is currently unknown the long-term effects of increasing hyperlipidemia, a known risk factor for CVD, in a population already sensitive to CVD morbidity and mortality.

Little evidence exists to date on the effects of ITx alone on CVD mortality in T1D individuals. The majority of evidence for effects of insulin secretion restoration on CVD morbidity and mortality comes from islet or pancreas transplants in tandem with kidney transplantation. A study of ITx patients who also received a kidney transplant showed improvement in cardiovascular function at 3 years post-transplant, measured by cardiac ventricular function and intimal medial thickening (*Fiorina et al. 2005*). Another study in islet and kidney-transplanted patients found that patients who maintained graft function had greater survival and fewer cardiovascular deaths compared to a group that experienced graft failure, who also had a worse atherosclerotic profile and endothelial function (*Fiorina et al. 2003*). Patients receiving pancreas after kidney transplants may show improvement in atherosclerotic lesions and regression of atherosclerosis even out to 4

years post-transplant, however it is unclear whether these beneficial effects are due to restoration of insulin metabolism or renal function (*Jukema et al. 2002*).

1.7.2. Liver Transplant

The first successful liver transplants (LTx) with graft survivals of ≥6 months in humans was in 1967 (*Starzl et al. 1968*). Since that time, modifications of procedural and immunosuppressive factors have contributed to improvements in survival. Presently, survival in LTx recipients is 85-90% at 1-year, 75-90% at 5-years, 55-80% at 10-years, ~70% at 15-years, and ~50% at 20-years post-transplant (*Vogt et al. 2002*; *Borg et al. 2008*; *Pfitzmann et al. 2008*; *Adam et al. 2009*; *Watt et al. 2010b*).

1.7.2.1. Liver Transplant - Population

Contraindications for LTx include extrahepatic malignancy, active alcohol or drug use, and advanced cardiopulmonary disease; however other criteria are also considered, such as age >65 years, diabetes, renal dysfunction, portal vein thrombosis, HIV, and active psychiatric illness (*Adam et al. 2009*; *Dawwas et al. 2009*). Primary indicators for LTx are cirrhosis (50-60%), cancer (10-15%), and acute liver failure (<10%) (*Adam et al. 2009*). Alcohol and hepatitis C are the primary causes of cirrhosis (~20% and 15%, respectively) (*Adam et al. 2009*). LTx is currently indicated in HCC in individuals according to the Milan criteria, with small single lesions ≤5 cm or up to three lesions ≤3 cm (*Mazzaferro et al. 1996*; *Adam et al. 2009*).

In the U.S. and Canada, patients on the LTx waiting list are prioritized using model for end-stage liver disease (MELD) scoring, which predicts patient's risk of mortality. Therefore the MELD system works on a "sickest first" basis as opposed to "first come, first served" (*Adam et al. 2009*; *Dawwas et al. 2009*). MELD score is based on bilirubin, creatinine, and prothrombin time. Highest priority is given to patients experiencing fulminant hepatic

failure or those requiring retransplantation for hepatic artery thrombosis or primary nonfunction (*Adam et al. 2009*). Patients with HCC are automatically assigned extra MELD points, indicating the expected expedited mortality associated with this disease (*Cabrera et al. 2010*).

Livers for transplant come from cadaveric or live donors. Criteria for assessing usability of a donor graft include age of the donor, cause of death, presence and degree of steatosis, cold ischemia time, and presence of HBV or HCV (*Adam et al. 2009*; *Dawwas et al. 2009*). Corticosteroids are used for immunosuppression in the immediate post-transplant period, and are typically withdrawn over a few months (*Adam et al. 2009*). Maintenance immunosuppression includes calcineurin inhibitors, either Cyclosporin or Tacrolimus, and antimetabolites such as mycophenolate mofetil (MMF) and Azathioprine, while the mammalian target of rapamycin (mTOR) inhibitor Sirolimus is also used in some cases (*Adam et al. 2009*). Calcineurin and mTOR inhibitors are potent immunosuppressives but can have severe adverse effects, therefore other medications such as MMF and Azathioprine are added in order to reduce dosage of these drugs (*Benten et al. 2009*).

1.7.2.2. Liver Transplant - Outcomes

Chronic rejection in LTx patients is small in patients with long-standing survival (*Knechtle et al. 2009*). Diseases at high risk of recurrence after LTx include HBV, HCV, autoimmune conditions, and HCC (*Adam et al. 2009*). HBV can be treated post-transplant with antiviral medications, reducing the recurrence rate to 10% of patients (*Adam et al. 2009*). HCV recurrence post-transplant is more common as antivirals only have a modest effect, and recurrence of HCV negatively affects overall graft and patient survival (*Adam et al. 2009*). HCV recurrence occurs in 60-90% of patients, and within 10 years up to 40% of patients progress to cirrhosis (*Gane et al. 1996*; *Hübscher 2009*). For patients transplanted for HCC, the risk of HCC

recurrence is ≤10% within 2 years, however those with previously larger tumours the risk is higher (*Mazzaferro et al. 1996*; *Yao et al. 2002*). Recurrence of HCC is highly associated with <1 year survival after recurrence (*Yao et al. 2002*).

1.7.2.3. Liver Transplant - Complications

Short-term mortality after LTx is caused by primary graft dysfunction, acute rejection, severe infection, or technical complications associated with the transplant procedure (*Benten et al. 2009*). Long-term morbidity and mortality associated with LTx result from effects induced by immunosuppressive medications. The calcineurin inhibitors Cyclosporine and Tacrolimus are associated with hypertension, hyperglycemia, hyperlipidemia, lymphoma, and nephrotoxicity (*Benten et al. 2009*). Sirolimus is less associated with hypertension, hyperglycemia, and nephrotoxicity, but is considered more hyperlipidemic than Cyclosporine and Tacrolimus (*Benten et al. 2009*). MMF and Azathioprine are not associated with hyperlipidemia, hyperglycemia, nephrotoxicity, but are associated with bone marrow suppression and gastrointestinal toxicity (*Benten et al. 2009*). There have been reports of some patients able to be weaned off immunosuppression without graft rejection. While this induction of tolerance is unusual, it presents an opportunity to strive in the future towards maximizing the tolerogenic properties of the liver (*Benseler et al. 2007*).

Renal dysfunction occurs in as many as 50% of LTx patients at 5-years post-transplant, and may be influenced by hypertension, calcineurin inhibitors, diabetes, and coronary artery disease (*Farkas et al. 2009*; *Adam et al. 2009*; *Karie-Guigues et al. 2009*; *Choquette et al. 2010*). Declines in renal failure are associated with reduced graft and patient survival (*Farkas et al. 2009*).

Hypertension is also common after LTx, occurring in 50-70% of

patients, and is believed to be caused by vasoconstriction of systemic and renal vessels induced by calcineurin inhibitors (*Bianchi et al. 2008*; *Benten et al. 2009*; *Watt et al. 2010a*). Reduction in use of calcineurin inhibitors is associated with improvement in hypertension (*Benten et al. 2009*), though hypertension in more common with Cyclosporine use than Tacrolimus (*Bianchi et al. 2008*).

Weight gain is common after LTx, and obesity affects <50% of patients (*Sheiner et al. 2000*; *Adam et al. 2009*; *Pagadala et al. 2009*; *Watt et al. 2010a*). Weight gain may be due to corticosteroids, or improvement in quality of life affecting appetite.

LTx patients post-transplant will either remain diabetic (if diabetic before transplant) or develop new-onset diabetes, leading to presence of diabetes in up to 60% of LTx patients >6 months post-transplant (*Heisel et al. 2004*; *Tietge et al. 2004*; *Laryea et al. 2007*; *Bianchi et al. 2008*; *Kuo et al. 2010*; *Watt et al. 2010a*). An additional 30% of LTx patients may have impaired glucose tolerance (*Tietge et al. 2004*). Development of diabetes post-transplant is influenced by HCV, immunosuppressive agents, and other patient factors such as obesity (*Delgado-Borrego et al. 2004*; *Heisel et al. 2004*; *Kuo et al. 2010*). Diabetes may be caused by insulin resistance or defects in insulin secretion (*Fernandez et al. 1999*; *Henchoz et al. 2003*; *Tietge et al. 2004*). Tacrolimus is associated with greater incidence of new-onset diabetes than Cyclosporine therapy (*Heisel et al. 2004*; *Kuo et al. 2010*). Diabetes post-transplant is associated with increased rate of fibrosis, hepatic artery thrombosis, and mortality (*Watt et al. 2010a*).

Features of the metabolic syndrome include hypertension, obesity, and hyperlipidemia. As immunosuppressive agents have exacerbating effects on blood pressure, weight gain, and plasma lipid levels, it is not surprising that metabolic syndrome is being increasingly identified in LTx patients (*Pagadala et al. 2009*; *Watt et al. 2010a*). Metabolic syndrome has been

reported in up to 60% of patients >6 months post-transplant (*Laryea et al. 2007*; *Bianchi et al. 2008*). Major vascular events are much more common in individuals with metabolic syndrome post-transplant (*Laryea et al. 2007*; *Pagadala et al. 2009*).

Development of fatty liver may occur in up to 30% of LTx patients with no prior evidence of fatty liver, which may progress from NAFLD to NASH (*Seo et al. 2007*; *Dumortier et al. 2010*). A study by Dumortier et al (2010) found that 31.3% of LTx patients undergoing liver biopsy had some degree of steatosis, with half showing low-grade steatosis, a third showing moderate steatosis, and the rest showing severe steatosis defined as steatosis in more than two-thirds of hepatocytes (*Dumortier et al. 2010*).

Risk of recurrence of previous or development of new extrahepatic cancers, as well as mortality from cancer, are elevated in LTx patients compared to the general population (*Benten et al. 2009*). Risk of HCC recurrence has been discussed previously. Extrahepatic cancers are reported to occur in up to 15% of LTx patients (*Herrero et al. 2005*; *Oo et al. 2005*). In one report, 1 in 4 patients who survived >3 months post-transplant developed neoplasia; however the majority of these were cutaneous carcinomas which are not associated with increased mortality (*Herrero et al. 2005*).

1.7.2.3.1. Hyperlipidemia and Mortality after Liver Transplant

Hyperlipidemia occurs in ~50% of LTx patients >6 months post-transplant, with hypercholesterolemia occurring in ~15-65% and hypertriglyceridemia occurring in ~30-70% of LTx patients (*Gisbert et al. 1997*; *Sheiner et al. 2000*; *Guckelberger et al. 2005*; *Laryea et al. 2007*; *Bianchi et al. 2008*; *Benten et al. 2009*; *Adam et al. 2009*). In addition, low HDL-c levels are prevalent in up to 50% of LTx patients (*Laryea et al. 2007*; *Bianchi et al. 2008*). Increases in plasma lipid levels after transplant may be due to

immunosuppressive medications, or may also be influenced by development of diabetes or obesity which are also common in the post-transplant period (*Adam et al. 2009*). Glucocorticoid reduction is associated with reduction in TC and TG (*Guckelberger et al. 2005*). Elevated plasma lipids are more common with Cyclosporine use than Tacrolimus, and conversion from Cyclosporine to Tacrolimus is generally associated with a reduction in levels of cholesterol and TG (*Canzanello et al. 1997*; *Rabkin et al. 2002*; *Bianchi et al. 2008*; *Beckebaum et al. 2009*). However, some studies suggest long-term use of Cyclosporine and Tacrolimus >5 years show no difference in plasma lipids, suggesting that over time the differences noted between the two medications in prevalence of hyperlipidemia equilibrate (*Atillasoy et al. 1998*; *Guckelberger et al. 2005*).

Major causes of death post-transplant in LTx patients include hepatic-related causes such as chronic rejection and disease recurrence, and non-graft-related causes such as malignancy and CVD (*Pruthi et al. 2001*; *Vogt et al. 2002*; *Watt et al. 2010b*). Death post-transplant in LTx patients due to graft failure are 28-32%, malignancy 14-22%, CVD 11-20%, infection 9-22% and renal failure 6% (*Borg et al. 2008*; *Pfitzmann et al. 2008*; *Mells et al. 2009*; *Watt et al. 2010b*). Graft failure and death due to disease recurrence, infection and malignancy are reported to be major causes of death within the first 5 years post-transplant, while the majority of CVD-related events may occur later after LTx (*Borg et al. 2008*; *Guckelberger 2009*).

Predicted 10-year risk of CVD is higher in LTx patients compared to the general population (*Neal et al. 2004*). Relative risk of CVD in LTx patients may be as high as 3, and risk of death is 2.5 compared to matched subjects (*Johnston et al. 2002*). Major vascular events such as stroke, transient ischemic attack, myocardial infarction and sudden cardiac death occurs in ~20% of LTx patients, though some patients may have preexisting heart disease or risk factors (*Pruthi et al. 2001*; *Laryea et al. 2007*; *Borg et al. 2008*).

Prevalence of severe CVD defined as >70% arterial stenosis, as opposed to incidence of mortality, is estimated to be ~15% in patients >50 years of age (*Carey et al. 1995*). Hypertension, hypercholesterolemia, and hypertriglyceridemia are associated with vascular events (*Laryea et al. 2007*; *Borg et al. 2008*; *Pfitzmann et al. 2008*). Type of immunosuppression may also be important as Cyclosporine is more highly associated with CVD and CVD-related mortality than Tacrolimus (*Rabkin et al. 2002*), however this is not reported in all studies possibly due to differences in follow-up time (*Guckelberger et al. 2005*).

The above evidence suggests that restoration of organ function after either islet or liver transplant may improve mortality. However, immunosuppressive medications are associated with significant side-effects. A primary side-effect of some immunosuppressive agents is hyperlipidemia. The mechanisms associated with immunosuppressive medication-induced hyperlipidemia have not been elucidated, however could be related to synthesis, absorption, or clearance. Measurement of lipid synthesis in post-transplant patients may help to clarify potential mechanisms leading to hyperlipidemia after transplant, and further help to guide appropriate treatment plans.

1.7.3. Graft Rejection and Immunosuppressive Medications

Graft rejection is a major issue in organ transplantation such as LTx and ITx. Recognition of the transplanted graft as foreign can be through direct or indirect mechanisms (*Aw 2003*; *Martinez et al. 2005*). Direct allorecognition involves migration of antigen-presenting cells from the graft to other lymphoid organs, where T-cells are stimulated directly. Indirect allorecognition involves antigen-presenting cells from the host picking up donor antigens sluffed off by the graft tissue circulating in the periphery, causing T-cell stimulation. The immune response consists of antigen

presentation, stimulation, T-cell activation, and lymphocyte proliferation (*Aw 2003*; *Taylor et al. 2005*).

The immune system can cause graft rejection through T-cell-mediated cytotoxicity, delayed type hypersensitivity, and antibody-mediated damage (*Martinez et al. 2005*). Allograft tolerance is the primary goal of transplantation, defined as a state of long-term acceptance of foreign tissue without requirement for generalized immunosuppressive agents, along with an intact immune system capable of mounting an adequate immune response upon presentation of other antigens (*Schroeder et al. 2003*; *Aw 2003*). The success of organ transplantation relies on the prevention of graft rejection. As mechanisms for inducing tolerance are not yet available for clinical practice, prevention of graft rejection is accomplished through pharmatherapy.

Immunosuppressive medications act to suppress various aspects of the immune system in order to prevent detection of the foreign tissue graft and the ensuing immune-mounted attack. As each type of medication acts on a different part of the pathway, a combination of drugs is given so as to maximize immunosuppression. Immunosuppressive medication is given in two phases, induction and maintenance; induction is usually started before transplant and can be a combination of medications or simply higher doses, while maintenance involves a combination of drugs but at lower doses than utilized in the induction phase (*Aw 2003*). The following section will discuss major immunosuppressive medications currently used in clinical practice and describe the mechanisms through which they accomplish immunosuppression.

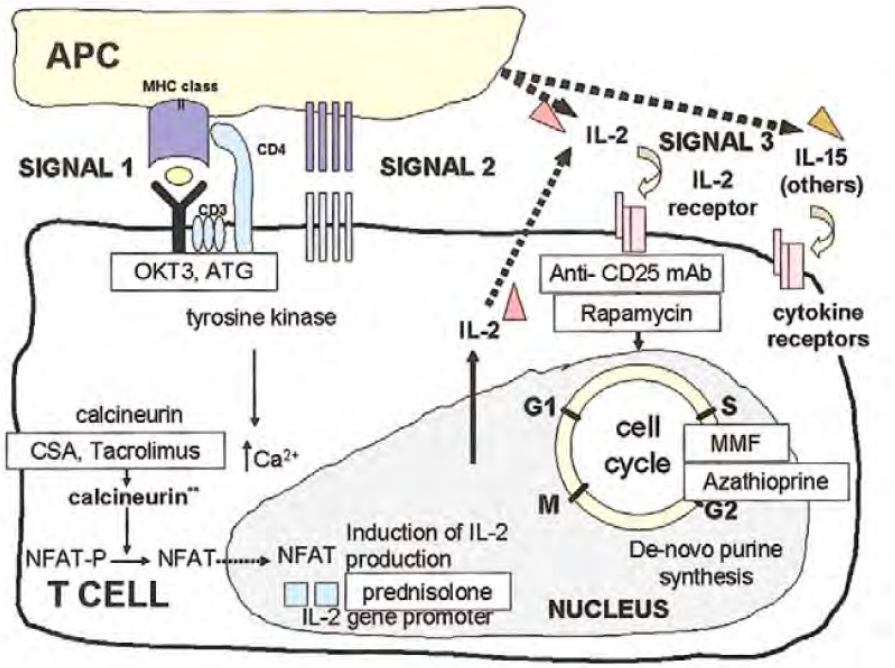
1.7.3.1. Mechanisms of Immunosuppression and Side-Effects

As stated, immunosuppressives act on some aspect of the immune system to interrupt lymphocyte proliferation and signalling mechanisms (**Figure 1-4**). However, these medications are not specific to immune cells in

their mechanisms of action. Therefore, inevitably there are side-effects of these drugs stemming from action on other cell types. The most notable side-effects include hypertension, diabetes, and dyslipidemia.

Corticosteroids, such as prednisone, block the nuclear activating factors, such as the IL-2 gene promotor, which regulates cytokine gene transcription and secretion, thereby impairing activation of T and B cells (*Allison 2000*; *Aw 2003*; *Taylor et al. 2005*; *McPartland et al. 2007*). Corticosteroids are potent immunosuppressives but have broad effects leading to numerous side-effects, such as hyperlipidemia, diabetes, hypertension, and weight gain (*McPartland et al. 2007*; *Linden 2009*; *Bai et al. 2010*). For these reasons, corticosteroids are typically limited to the early post-transplant period (*Taylor et al. 2005*) and eventually tapered off, sometimes as early at 6 weeks post-transplant in some transplant centers (*McPartland et al. 2007*).

Figure 1-4: Immunosuppressive agents and their specific sites of action



From: Aw MM (2003) J Pediatr Surg 38:1275-1280.

Sirolimus binds to the intracellular receptor FKBP12 of calcineurin

which then binds to mTOR in the AKT/protein kinase B signalling pathway, interrupting cell signalling and replication mechanisms (*Taylor et al. 2005*; *McPartland et al. 2007*; *Dowling et al. 2010*). Sirolimus blocks intracellular signalling mechanisms and inhibits T-cell activation and response to cytokines, including signals from IL-2 important for T-cell proliferation (*Aw 2003*; *McPartland et al. 2007*). Sirolimus is less associated with hypertension, hyperglycemia, and nephrotoxicity, but is more hyperlipidemic than Cyclosporine and Tacrolimus (*Benten et al. 2009*).

Calcineurin inhibitors disrupt signalling mechanisms by impairing calcineurin and interfering with IL-2 production which is important in mounting an immune response (*Aw 2003*; *McPartland et al. 2007*). Cyclosporine binds to cyclophilins while Tacrolimus (also called FK506) binds to FKBP-12, forming complexes and inhibit calcineurin's phosphatase activity (*Allison 2000*; *Aw 2003*). Inhibition of calcineurin activation prevents movement of NFAT transcription factors to the nucleus of activated T-cells, which are involved in gene transcription of signalling factors (*Allison 2000*). Cyclosporine impairs T-helper responses and antibody synthesis (*Linden 2009*). Cyclosporine and Tacrolimus are associated with hypertension, hyperglycemia, hyperlipidemia, diabetes, lymphoma, and nephrotoxicity, though each to varying degrees (*McPartland et al. 2007*; *Benten et al. 2009*; *Bai et al. 2010*).

Antiproliferative agents such as Azathioprine and MMF inhibit purine biosynthesis thereby preventing differentiation and proliferation of lymphocytes (*McPartland et al. 2007*). Azathioprine inhibits adenosine synthesis, making it relatively non-specific in its effects on all proliferative cells (*Allison 2000*). Azathioprine is metabolized to 6-thioguanine nucleotides that act as purine antagonists which inhibit RNA, DNA, and protein synthesis (*Allison 2000*). MMF inhibits the rate-limiting enzyme in guanosine synthesis, which provides a more targeted approach as lymphocytes are unable to use

the “salvage” pathway for guanosine synthesis (*Aw 2003*; *Taylor et al. 2005*). MMF and Azathioprine are not associated with hyperlipidemia, hyperglycemia, nephrotoxicity, but are associated with bone marrow suppression and gastrointestinal toxicity causing nausea and diarrhea (*Taylor et al. 2005*; *McPartland et al. 2007*; *Benten et al. 2009*).

Sirolimus, Cyclosporine, and Tacrolimus are metabolized through cytochrome P450 in the liver, as are many drugs and foods, therefore particular attention needs to be taken to avoid drug interactions with other medications such as some calcium channel blockers and statins, and foods such as grapefruit juice (*Zimmerman 2004*; *McPartland et al. 2007*; *Benten et al. 2009*).

1.7.3.2. Immunosuppressive-induced Post-Transplant Hyperlipidemia

Post-transplant hyperlipidemia is a common occurrence across a variety of organ transplant groups (*Moore et al. 2001*). Prevalence of hyperlipidemia tends to be higher in other transplant groups such as heart and renal as compared to ITx and LTx (*Deleuze et al. 2006*; *Sánchez-Lázaro et al. 2010*). Hypercholesterolemia has been reported in ~80% of heart transplant patients at 1-year post-transplant, though this may be due greater and longer use of steroids post-transplant (*Sánchez-Lázaro et al. 2010*). Up to 70% of renal transplant patients may have dyslipidemia characterized by elevated levels of TG, LDL-c, and ApoB as well as lower levels of HDL-c (*Kimak et al. 2007*).

1.7.3.2.1. Potential mechanisms for immunosuppressive-induced hyperlipidemia

Elevation in plasma lipids could result from increased synthesis or absorption, reduced clearance, or a combination of these factors. The following sections will discuss possible mechanisms of immunosuppressive-induced hyperlipidemia. Only major immunosuppressive medications known

to induce or exacerbate hyperlipidemia will be discussed, including corticosteroids, Sirolimus, and the calcineurin inhibitors Cyclosporine and Tacrolimus.

Corticosteroids are suggested to increase ACC and FAS, VLDL synthesis and activity of HMGCoA-r, and reduce LDL-r and LPL activity (*Chan et al. 1981*; *Moore et al. 2001*; *Charco 2002*). Corticosteroids may also inhibit CETP activity, which may affect lipid transfer between HDL and ApoB-containing lipoproteins (*Lemieux et al. 2002*; *Kimak et al. 2007*).

Sirolimus may inhibit LDL-r activity which could contribute to impaired clearance of lipoprotein particles leading to elevated plasma lipid levels (*Ma et al. 2007b*; *Ma et al. 2007a*). This finding is further supported by the observation that cholesterol accumulation in HepG2 cells as well as hepatic cells from guinea pigs was reduced by Sirolimus (*Aggarwal et al. 2006*; *Ma et al. 2007b*; *Ma et al. 2007a*). Studies in renal transplant patients suggest Sirolimus may increase CETP activity and reduce LPL activity, compounding reduction in clearance of lipoprotein particles (*Hoogeveen et al. 2001*; *Tory et al. 2008*). This is supported by the finding that renal transplant patients treated with Sirolimus have shown elevated ApoB levels indicating greater VLDL and LDL particle concentration (*Tur et al. 2000*; *Hoogeveen et al. 2001*; *Morrisett et al. 2002*). This increase in lipoprotein numbers may be mediated by the increase in plasma free FA concentrations also observed in these patients (*Morrisett et al. 2002*). In addition, kinetic analysis found that the elevated ApoB-containing lipoprotein levels were due to reduced catabolism of these particles (*Hoogeveen et al. 2001*). In these patients TG synthesis, assessed by infusion of isotopically-labeled palmitate, was also reduced by Sirolimus (*Morrisett et al. 2002*). However, in guinea pigs a physiologically-relevant dose of Sirolimus was associated with LDL and particularly VLDL particles that were larger and more TG-rich (*Aggarwal et al. 2006*).

Cyclosporine has been used in clinical practice since 1978, while Tacrolimus has been used since early 1990 (*Todo et al. 1990*; *Linden 2009*). In a rat model, Cyclosporine treatment did not affect hepatic expression of HMG-CoA-r activity (*Vaziri et al. 2000*), whereas a study in HepG2 cells suggested an upregulation of HMGCoA-r expression (*Gueguen et al. 2007*). Greater cholesterol content in VLDL and LDL particles has been observed in renal transplant patients receiving Cyclosporine, potentially indicating cholesterol synthesis may be upregulated (*Ichimaru et al. 2001*). Cyclosporine is transported via LDL particles and can bind to the LDL-r, which may reduce clearance of plasma lipoprotein particles, and may also interfere with LDL-r synthesis (*Rayyes et al. 1996*; *Ruiu et al. 2005*; *Subramanian et al. 2007*). Renal transplant patients treated with Cyclosporine have elevated levels of ApoB, potentially indicating reduced clearance of ApoB-containing particles (*Tur et al. 2000*; *Ichimaru et al. 2001*). Further, Cyclosporine may increase CETP and reduce LPL activity, leading to altered composition and reduction in clearance of lipoprotein particles (*Superko et al. 1990*; *Derfler et al. 1991*; *Vaziri et al. 2000*; *Tory et al. 2008*). Cyclosporine administration into rat hepatocytes in vitro increased FA synthesis and palmitate esterification in TG suggesting Cyclosporine may increase TG formation, whereas Tacrolimus did not show the same effects (*Brown et al. 2007*).

Considerably less evidence is available for the role of Tacrolimus in lipid metabolism as compared to Cyclosporine. Potentially this is because the shift from Cyclosporine to Tacrolimus therapy is considered to be a treatment for post-transplant hyperlipidemia (*Neal et al. 2001*; *Lucey et al. 2005*; *Roy et al. 2006*). However, Tacrolimus is still associated with considerable hyperlipidemia and may be comparable in lipid-raising ability to Cyclosporine in the long term (*Charco et al. 1999*; *Chin et al. 2000*; *Woodside et al. 2005*). Part of the controversy in prevalence of hyperlipidemia between Cyclosporine and Tacrolimus therapy may be influenced by dose and

duration of concomitant corticosteroid therapy, which tends to be lower with Tacrolimus administration (*Moore et al. 2001*). While it could be inferred due to the observation that most of the aberrations on lipid metabolism due to Cyclosporine appear to be through modulation of calcineurin activity, it cannot be said for certain whether Tacrolimus may exert influences on lipid metabolism similar to Cyclosporine.

In contrast to Cyclosporine, Tacrolimus has not been shown to impair CETP activity but has been shown to reduce LPL activity, which may impair clearance of lipoprotein particles and contribute to increased plasma lipid levels (*Tory et al. 2008*; *Tory et al. 2009*). Renal transplant patients treated with Tacrolimus have been shown to have elevated levels of ApoB, as well as ApoCIII (*Tur et al. 2000*; *Ichimaru et al. 2001*). It is unknown whether the elevated ApoB-containing lipoprotein particles observed in Tacrolimus therapy is due to impaired clearance by LDL-r as has been shown in patients receiving Cyclosporine as previously discussed. It is difficult to say, as Cyclosporine is carried by LDL particles which may be why Cyclosporine appears to have direct effects on particle and receptor characteristics, whereas Tacrolimus is primarily transported in the blood by red blood cells bound to proteins such as α-acid glycoprotein and albumin (*Vicari-Christensen et al. 2009*). Greater cholesterol content in VLDL particles has also been observed in renal transplant patients receiving Tacrolimus, which could potentially be due to increased cholesterol synthesis or to reduced cellular cholesterol efflux (*Ichimaru et al. 2001*; *Karwatsky et al. 2010*).

As can be appreciated, the majority of direct evidence for aberrations in lipid metabolism induced by immunosuppressive medications has come from in vitro cellular and animal models, or from indirect measurements of enzyme activities. Despite the prevalence of hyperlipidemia across transplant populations, there have been very few assessments of lipid synthesis as a possible mediator of hyperlipidemia in post-transplant human subjects. The

following section will describe data available on human studies of post-transplant hyperlipidemia.

1.7.3.2.2. De novo lipogenesis and Cholesterol Synthesis in Post-Transplant patients

DNL using labelled acetate was estimated in 5 stable (>1 year post-transplant) LTx recipients compared to 8 control subjects by Minehira et al (2001). It was found that hepatic DNL was not different between LTx and control subjects, reported at ~3% (*Minehira et al. 2001*). However, these results are difficult to interpret as 3 of the LTx patients were on prednisone and Cyclosporine therapy, 1 was on prednisone and Tacrolimus, and 1 was on Cyclosporine alone; individual DNL data was also not provided. In addition, liquid nutrition was provided hourly, which is not representative of normal free-living meal consumption and timing. FA profiles of Tacrolimus-treated renal transplant patients showed lower PUFA content as well as lower levels of AA but higher levels of 16:0 (palmitic acid), and 18:1 (oleic acid) in LDL particles compared to control subjects (*Cofan et al. 1999*). This finding may indicate upregulation of DNL as 16:0 and 18:1 are major products of FA synthesis.

A study in patients undergoing LTx found that markers of cholesterol absorption and synthesis began to stabilize after transplantation to approach levels observed in healthy individuals (*Nikkilä et al. 1992*). However, this study is difficult to interpret due to the short period of follow-up after transplantation of 1 month, when prednisone therapy was still prevalent in addition to Cyclosporine, as well as a mixed group of patients transplanted for either acute liver necrosis or primary biliary cirrhosis. Despite this, at 1-month post-transplant markers of cholesterol absorption were still higher than control subjects, and markers of cholesterol synthesis were normal in patients transplanted for acute liver failure while patients transplanted for primary biliary cirrhosis had lower markers of cholesterol synthesis. In

contrast, in pediatric renal, liver, and cardiac transplant patients treated with Cyclosporine and prednisone it was found that LTx patients had higher markers of cholesterol synthesis and lower markers of cholesterol absorption compared to renal and cardiac patients as well as control subjects (*Siirtola et al. 2006*). In the LTx patients, plasma TC levels were positively associated with markers of cholesterol synthesis and inversely associated with markers of cholesterol absorption. Alternatively, renal transplant patients treated with Cyclosporine and prednisone were found to have lower markers of cholesterol synthesis compared to control subjects (*Sutherland et al. 1995*). In these patients, markers of cholesterol synthesis were positively correlated with plasma ApoB and VLDL levels. Elevated plasma ApoB-containing lipoprotein levels could be due to impaired clearance, which may then cause intracellular cholesterol synthesis to be upregulated.

As can be appreciated from the above discussion, the impact of immunosuppressive therapy on cholesterol and FA synthesis in human transplant patients is far from clear, and is hampered by mixed immunosuppressive regimens and patient groups. In addition, the above investigations have included patients on steroid therapy; this is confounding because steroids may exert independent and possibly opposite effects on cholesterol and FA synthesis compared to Cyclosporine, Tacrolimus, and Sirolimus therapy. In addition, many transplant centers currently try to wean patients off steroid therapy early in the post-transplant period to minimize steroid-related side-effects. Therefore, effect of steroid therapy on lipid synthesis and consequent risk for CVD-related morbidity and mortality is not relevant to most stable long-term transplant recipients. Studies investigating effects of Cyclosporine, Tacrolimus, and Sirolimus in the absence of steroid therapy on lipid metabolism in human patients in free-living conditions are therefore warranted.

1.7.3.3. Treatment of Post-Transplant Hyperlipidemia

Post-transplant hyperlipidemia, like hyperlipidemia occurring in the general population, can be treated using diet or drugs. The following section will describe efficacies of pharma- and dietary therapy for hyperlipidemia in post-transplant patients.

1.7.3.3.1. Treatment of Post-Transplant Hyperlipidemia - Drugs

Statins are the most widely used lipid-lowering agent in the general population, and this is no different in the transplant population. Statins can effectively lower TC and LDL-c in renal and liver transplant populations (*Massy et al. 1995*; *Zachoval et al. 2001*; *Ichimaru et al. 2001*; *Imamura et al. 2005*; *Wissing et al. 2006*). Caution must be taken, however, as certain statins, such as atorvastatin, lovastatin and simvastatin, are metabolized by the same enzyme systems as Sirolimus, Cyclosporine, and Tacrolimus and therefore drug monitoring and selection is important (*Moore et al. 2001*; *Zimmerman 2004*). In a review of statin use in LTx patients, adverse events (comprising muscle pain and weakness) leading to discontinuation of statin therapy occurred in 6 of 69 (8%) of patients; all were on Cyclosporine and either pravastatin or atorvastatin (*Martin et al. 2008*). Co-administration of Sirolimus and Cyclosporine can lead to elevated plasma levels of statins (*Amundsen et al. 2010*), however this effect does not appear to be reported with Tacrolimus co-administration (*Lemahieu et al. 2005*). Statin therapy has been associated with reduced CVD morbidity and mortality in the general population, however effect of statin therapy on survival in the transplant population is not clear. Some reports have indicated a beneficial effect of statin therapy on cardiac events as well as CVD-related- or all-cause mortality (*Wiesbauer et al. 2008*; *Soveri et al. 2009*), while others have reported no association with improved patient survival or CVD events and mortality (*Navaneethan et al. 2009*; *Younas et al. 2010*). Discrepancies between studies

may be related to type of immunosuppressive medication as well as transplant population.

Ezetimibe is a relatively newer cholesterol-lowering agent that may be useful in hyperlipidemia organ transplant recipients. Ezetimibe effectively lowers plasma cholesterol levels to a further extent than that achieved by high-dose statin and can be effective even as monotherapy, which may be beneficial for patients that cannot achieve target LDL-c levels with statin monotherapy (*Kohnle et al. 2006*; *Buchanan et al. 2006*; *Chuang et al. 2007*; *Almutairi et al. 2009*). Co-administration of ezetimibe with statins may lower levels of TC and LDL-c as well as TG, and may allow for reduction of statin dosages (*Rodríguez-Ferrero et al. 2008*; *Yoon et al. 2009*). Co-administration of Sirolimus and ezetimibe in healthy subjects was shown to have no influences on blood levels of either drug, and led to reductions in sterol markers of cholesterol absorption indicating efficacy of ezetimibe action (*Oswald et al. 2010*). Ezetimibe may interfere with Cyclosporine metabolism, leading to increased plasma levels of either drug (*Koshman et al. 2005*; *Bergman et al. 2006*), however this effect is not always observed by increased plasma levels (*Kohnle et al. 2006*; *Yoon et al. 2009*).

Pharmacological therapy can be effective for post-transplant hyperlipidemia, but concern exists for drug interactions. In addition, the mechanisms contributing to the increase in plasma lipids with immunosuppressive therapy are not yet elucidated, therefore there is the potential that other lipid-related mechanisms could be targeted for more effective treatment.

1.7.3.3.2. Treatment of Post-Transplant Hyperlipidemia - Dietary Therapy

Dietary modification is consistently cited as the safest treatment for hyperlipidemia in the general population as well as transplant patients, and should be the first route of treatment pursued (*Kobashigawa et al. 1997*;

Kasiske et al. 2004; *Wenke 2004*). However, effectiveness of lifestyle interventions in the general population are mixed due to compliance issues. The challenge of preventing or treating hyperlipidemia in the transplant population is compounded by the presence of immunosuppressive medications, which may alter normal nutrient metabolism.

Phytosterol-containing foods provided to renal transplant patients on Cyclosporine therapy in amounts sufficient to provide 2g/day of phytosterols was effective in reducing plasma TC levels by 10%, especially in patients using statins (*Sutton et al. 2009*). Additional lowering of TC and LDL-c up to 20% was also observed in cardiac transplant patients on steroid and Cyclosporine therapy consuming phytosterol-enriched margarine providing 2.5g/day phytosterols in addition to statin therapy (*Vorlat et al. 2003*). Substitution of 25g/day animal protein for soy protein for 5 weeks reduced plasma TC and LDL-c levels up to 10% in renal transplant patients on steroid and Cyclosporine therapy (*Cupisti et al. 2007*).

Meta-analyses have found a consistent TG-lowering effect of fish oil in renal transplant patients (*Tatsioni et al. 2005*). Fish oil supplements of 4g/ day significantly reduced plasma TG levels ≥20% in cardiac transplant patients on Sirolimus or everolimus therapy (*Celik et al. 2008*). Fish oil supplementation of 6g/day significantly reduced plasma TG 7% and had no effects on acute rejection rate or renal function compared to a soy oil control group in renal transplant patients maintained on steroid and Cyclosporine immunosuppression (*Hernández et al. 2002*). However another trial using 6g/day fish oil for 12 months in renal transplant patients on steroid and Cyclosporine therapy showed no reduction in plasma TG levels (*Santos et al. 2000*). Fish oil in combination with statin therapy in renal transplant patients significantly lowered plasma TC, LDL-c, and TG levels as well as ApoB levels (*Grekas et al. 2001*). Addition of 6g/day fish oil to an AHA diet and statin for 3 months effectively lowered plasma TC and TG levels by 10% and 25%,

respectively, in renal transplant patients resistant to diet-alone (*Castro et al. 1997*). Fish oil is considered safe in transplant patients and does not appear to affect graft rejection (*van der Heide et al. 1993*; *Kooijmans-Coutinho et al. 1996*; *Santos et al. 2000*).

From the above studies, it is evident that dietary nutrients can be effective in reducing plasma lipid levels in post-transplant hyperlipidemic patients. However, when given as single interventions, the magnitude of lipid-lowering may be small (≤10%), whereas when given with a statin generally there is a much greater effect. This indicates that clinically effective reduction of plasma lipid levels may not be possible with single nutrient interventions in post-transplant patients. Interventions utilizing whole-diet or dietary pattern approaches have met with some success in post-transplant patients.

Compliance to an intervention comprising a dietary plan following the AHA Step 1 guidelines and an increase in physical activity in cardiac transplant patients on Cyclosporine or Tacrolimus therapy was associated with lower body weight and fat mass, as well as lower TC by 10-20% and TG by 30% (*Guida et al. 2007*; *Guida et al. 2009*). An exercise and diet intervention based on the National Cholesterol Education Program also improved body composition and overall health in LTx patients on steroid and Tacrolimus therapy (*Krasnoff et al. 2006*). The AHA diet has also been shown to moderately reduce plasma TC and LDL-c by 10% and TG by 7%, as well as body fat, in renal patients on Cyclosporine and steroid immunosuppression after 4-6 months of therapy (*Lopes et al. 1999*; *Barbagallo et al. 1999*). Reductions in TC and LDL-c may be greater for those who have higher baseline LDL-c levels (*Barbagallo et al. 1999*). However other studies have not shown significant effects of the AHA diet in renal transplant populations in reduction of LDL-c, though there may be changes in other parameters (*Moore et al. 1990*). The AHA diet was not effective in hyperlipidemic renal transplant patients on steroids and Cyclosporine in reducing plasma levels of

cholesterol, and only marginally reduced plasma TG levels in those with BMI <26 kg/m^2 despite evidence of dietary changes among participants (*Tonstad et al. 1995*).

A saturated fat and cholesterol-restricted diet also emphasizing PUFA effectively normalized plasma lipid levels in 65% of renal transplant patients on steroid and Cyclosporine therapy, with the remaining patients requiring medication (*Sapan et al. 2009*). A PUFA-rich, low-cholesterol diet also reduced plasma TC by 15% and TG by 30% within 2 weeks in nine renal transplant patients taking prednisone and Cyclosporine (*Nelson et al. 1988*). A low-glycemic diet in renal transplant patients emphasizing whole grains, vegetables, low animal protein, MUFA, and PUFA effectively lowered TC and LDL-c by 10% and TG by 20% (*Stachowska et al. 2005*), and renal transplant patients predominantly consuming a Mediterranean-style diet have been shown to have a lower incidence of metabolic syndrome (*Nafar et al. 2009*). Implementation of a Weight Watchers-style points system and structured support program to monitor food intake in long-term cardiac transplant patients on steroid and Cyclosporine did not have significant effects on plasma lipid levels, however did result in lower body weight and blood pressure (*Salyer et al. 2007*).

Renal transplant patients on steroids and Cyclosporine following a diet restricted in total and saturated fat and cholesterol showed small but significant reductions in TC, LDL-c and body weight after 8 weeks, however only 50% of patients were considered compliant and target TC levels were only achieved by 20% of patients (*Zaffari et al. 2004*). Counselling renal transplant patients on a fat- and cholesterol-restricted diet similar to the one previously described can lower plasma TC and LDL-c, however also may not be effective enough for most patients to reach target levels of TC and LDL-c (*Hines 2000*).

The above evidence suggests that dietary treatment programs can

have some success in reducing post-transplant hyperlipidemia, however, as with any lifestyle intervention, there can be issues of compliance. Dietary interventions may only lower TC levels by 10-15%, which may not be enough to achieve target lipid levels in some patients. Interventions emphasizing PUFA appear to have a significant impact on reduction of plasma TG levels. Evaluation of the efficacy of dietary programs in post-transplant patients is also complicated by the prevalence of corticosteroid usage in almost all trials discussed above. Corticosteroids are particularly hyperlipidemic and may reduce effectiveness of dietary interventions. In addition, the majority of trials have been in patients receiving Cyclosporine therapy, whereas currently Tacrolimus therapy is gaining popularity and is more frequently used in some transplant centers. Despite modest efficacy, dietary therapy should be a cornerstone of post-transplant patient management, and further research into combinations of dietary patterns as well as individual nutrients or components should be explored.

1.8. References for Literature Review

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2. Rationale, Hypotheses, and Objectives

2.1. Lipid dysregulation in Type 1 diabetes

Individuals with T1D are at a much higher risk of death from CVD compared to age and sex-matched non-diabetic controls (*Brindisi et al. 2010*), and CVD remains a major cause of death in T1D (*Maahs et al. 2010*). This risk is despite the finding that most individuals with T1D have normal lipid levels (*Orchard et al. 2010*). Therefore, there may be other irregularities in T1D that may contribute to increased CVD risk, such as qualitative abnormalities in lipoprotein composition or metabolism. Greater synthesis of cholesterol or FA may alter lipoprotein composition which could affect interaction of lipoproteins with modifying enzymes, receptors, and tissues. Alternatively, lower lipid synthesis may indicate that absorptive pathways are more important in lipid regulation in diabetes. Identifying dominant regulatory aspects may help to guide therapies for prevention of hyperlipidemia and vascular disease. T1D has been associated with reduced synthesis and elevated absorption of cholesterol in a series of investigations by Miettinen and Gylling using sterol precursors and metabolites as markers of cholesterol synthesis and absorption (*Miettinen et al. 2004*; *Gylling et al. 2004*; *Gylling et al. 2007*). However, these findings have not been assessed by more direct methods, such as stable isotopes, which is a more sensitive method of measurement. The majority of patients with T1D are treated with statins, whether for direct need or prophylaxis; however, if cholesterol synthesis is not elevated perhaps other therapies could be investigated that may be more effective and appropriate for this population.

T1D patients who receive an ITx are estimated to benefit from freedom or reduction of insulin injections, as well as greater glycemic control, which have shown to be of crucial importance in prevention of micro- and

macrovascular complications in individuals with T1D (*White et al. 2010*). However, transplantation may induce hyperlipidemia associated with use of immunosuppressive medications (*Ryan et al. 2001*; *Ryan et al. 2005*; *Hafiz et al. 2005*). Further, hyperlipidemia has been shown to increase risk of CVD death in people with T1D. It is currently unknown the long-term effects of increasing hyperlipidemia, a known risk factor for CVD, in individuals with T1D, who are a population already sensitive to CVD morbidity and mortality.

2.1.1. Hypothesis and Objective 1: Lipid synthesis in Type 1 diabetes

The hypothesis is that de novo lipogenesis is elevated and cholesterol synthesis is reduced in brittle Type 1 diabetes compared to non-diabetic control subjects, which may contribute to increased risk for CVD morbidity and mortality. To investigate this, de novo lipogenesis and cholesterol synthesis will be determined using the deuterium incorporation method in subjects with brittle Type 1 diabetes and compared to age-, sex-, and BMI-matched control subjects.

2.2. Lipid dysregulation in liver failure

The liver is a major metabolic organ, involved in metabolism of nearly all nutrients, including lipids. Disturbances in lipid metabolism may contribute to or exacerbate underlying liver disease as well as maintenance of homeostasis and function. Generally in advanced liver disease and cirrhosis, plasma levels of TG and TC are low (*Miller 1990*; *Iglesias et al. 1996*; *Jiang et al. 2006*; *Varghese et al. 2007*). However, development and progression of steatosis is a prominant feature of cirrhosis and decline in hepatic function, which may arise due to increased lipid synthesis (*Burt et al. 2007*; *Fabbrini et al. 2008*; *Cheung et al. 2008*; *Jou et al. 2008*). Altering fat and cholesterol synthetic pathways may be a feature of hepatitis C virus that contributes to viral reproduction as well as decline in hepatic function (*Waris et al. 2007*; *Yang et al. 2008*; *Negro et al. 2009*; *Corey et al. 2009*; *Syed et al.*

2010). To date measurements of synthesis of cholesterol and fatty acids in liver failure patients have not been carried out using direct and quantitative methods, such as stable isotopes.

2.2.1. Hypothesis and Objective 2: Lipid synthesis in Liver Failure

The hypothesis is that cholesterol synthesis is impaired in patients with liver failure, while de novo lipogenesis may be increased. To investigate this, de novo lipogenesis and cholesterol synthesis will be determined using the deuterium incorporation method in subjects with liver failure and compared to healthy control subjects.

2.3. Lipid dysregulation in post-transplant patients

Post-transplant hyperlipidemia is a common occurrence across a variety of organ transplant groups (*Moore et al. 2001*), and many transplant groups use similar medications such as Sirolimus, Cyclosporine, and Tacrolimus. Therefore, findings from one transplant group, such as liver transplant, may translate to other transplant groups. Elevation in plasma lipids could result from increased synthesis or absorption, reduced clearance, or a combination of these factors. Cyclosporine may increase cholesterol and FA synthesis (*Gueguen et al. 2007*; *Brown et al. 2007*), however it is unknown whether Tacrolimus has similar effects. Given that Tacrolimus therapy is gaining popularity and becoming more frequently used in transplant centers, it is important to elucidate the independent and potentially divergent effects of Cyclosporine and Tacrolimus on lipid synthesis and mechanisms related to hyperlipidemia, to properly direct appropriate treatment modalities.

The majority of direct evidence for aberrations in lipid metabolism induced by immunosuppressive medications has come from cellular and animal models, or from indirect measurements of enzyme activities. Despite the prevalence of hyperlipidemia across transplant populations, there have

been very few assessments of lipid synthesis as a possible mediator of hyperlipidemia in post-transplant human subjects. Previous estimations have been hampered by short follow-up time after transplantation (*Nikkilä et al. 1992*) or concomitant use of corticosteroids (*Minehira et al. 2001*; *Siirtola et al. 2006*). Current immunosuppressive protocols favour cessation of steroid use within the first few months post-transplant, therefore it is important to ascertain effects of immunosuppressive agents on lipid metabolism in the absence of steroid usage as it is more relevant to present-day transplant populations. In addition, trials to date have utilized cholesterol markers to estimate synthesis, therefore an examination using a more sensitive and quantitative method such as stable isotopes is warranted.

2.3.1. Hypothesis and Objective 3: Lipid synthesis in post-transplant patients

The hypothesis is that de novo lipogenesis and cholesterol synthesis is higher in post-transplant patients, resulting from immunosuppressive medications and contributing to post-transplant hyperlipidemia. To determine this, de novo lipogenesis and cholesterol synthesis will be determined using the deuterium incorporation method in stable islet or liver transplant patients, compared to healthy control subjects.

2.4. Role of diet in post-transplant hyperlipidemia

Statins can effectively lower TC and LDL-c in renal and liver transplant populations (*Massy et al. 1995*; *Zachoval et al. 2001*; *Ichimaru et al. 2001*; *Imamura et al. 2005*; *Wissing et al. 2006*); however, certain statins are metabolized by the same enzyme systems as Sirolimus, Cyclosporine, and Tacrolimus therefore drug monitoring and selection is important (*Moore et al. 2001*; *Zimmerman 2004*). Dietary modification is consistently cited as the safest treatment for hyperlipidemia in the general population as well as transplant patients, and should be the first route of treatment pursued

(*Kobashigawa et al. 1997*; *Kasiske et al. 2004*; *Wenke 2004*). However, the challenge of preventing or treating hyperlipidemia in the transplant population is compounded by the presence of immunosuppressive medications, which may alter normal nutrient metabolism. Dietary nutrients can be effective in reducing plasma lipid levels in post-transplant hyperlipidemic patients. For example, a consistent TG-lowering effect of fish oil has been found in renal transplant patients (*Tatsioni et al. 2005*), and phytosterol-containing foods can be effective in reducing plasma TC levels by 10% (*Sutton et al. 2009*). Dietary interventions may only lower TC levels by 10-15%, which may not be enough to achieve target lipid levels in some patients. However, evaluation of the efficacy of dietary programs in post-transplant patients is also complicated by the prevalence of corticosteroid usage in many of the trials available in the literature. Corticosteroids are particularly hyperlipidemic and may reduce effectiveness of dietary interventions. Further, as stated previously, present immunosuppressive protocols favour cessation of steroid use shortly after transplant, meaning that results from previous trials may not necessarily translate to present-day populations. In addition, the majority of trials have been in patients receiving Cyclosporine therapy, whereas currently Tacrolimus therapy is more frequently used in some transplant centers and may be less hyperlipidemic.

Single nutrient interventions can be effective in lowering plasma levels of TG or TC. For example, phytosterols can significantly reduce LDL-c by 10-15% (*Plat et al. 2000*; *Vissers et al. 2000*; *Davidson et al. 2001*; *Mensink et al. 2002*). However it is likely that the combination of nutrients will be more effective as a greater number of lipid-related mechanisms will be targeted. For example, phytosterols lower TC levels as previously stated, while fish oil can reduce plasma TG levels by 20-30% (*Miller et al. 1988*; *Saynor et al. 1992*; *Ebrahimi et al. 2009*; *Woods et al. 2009*; *Ryan et al. 2009*). A "dietary portfolio" has also repeatedly been shown to effectively reduce

plasma lipid levels in adults (*Jenkins et al. 2003*; *Kendall et al. 2004*; *Jenkins et al. 2007*; *Esfahani et al. 2010*). The efficacy of dietary interventions incorporating whole foods, however, are frequently impeded by issues related to compliance (*Jenkins et al. 2006*). Therefore, development of a novel food supplement that is portable and easy to incorporate into daily meals may confer greater benefit.

2.4.1. Hypothesis and Objective 4: Efficacy of dietary intervention in post-transplant hyperlipidemia

The hypothesis is that a dietary novel food supplement intervention incorporating a variety of nutrients will reduce plasma lipid levels, de novo lipogenesis, and cholesterol synthesis in post-transplant patients. To determine this, a multi-nutrient dietary intervention will be administered to stable islet and liver transplant patients as well as control subjects for 4 weeks to determine efficacy of the diet in reduction of plasma lipids and lipid synthesis. De novo lipogenesis and cholesterol synthesis will be determined using the deuterium incorporation method before and after the intervention.

2.5. References for Rationale, Hypothesis, and Objectives

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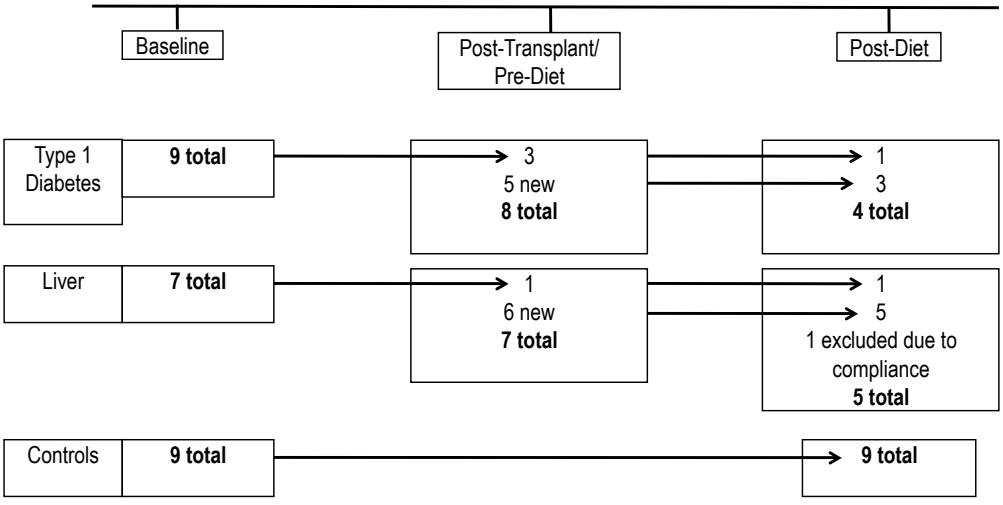
3. Methods

3.1. Participants

3.1.1. Recruitment Criteria

Participants aged 18-64 were included. Subject recruitment was initiated November 2005 and ended June 2009. This study was approved by the Health Research Ethics Board of the University of Alberta. For descriptions of subjects recruited for each study, please consult the individual sections (Chapters 4-7). **Figure 3-1** describes final sample sizes obtained for the study.

Figure 3-1: Final sample sizes, indicating number of subjects who were followed over time and newly recruited.



3.2. Study Protocol

Prior to each testing day, participants completed a questionnaire

listing medication use, a 3-day food record to estimate usual dietary intake, a 3-day physical activity record to estimate habitual activity patterns, and a fecal frequency record to assess presence of diarrhea. Participants were provided with food scales to assist in completion of food records, and pedometers for the physical activity records. The food record was analyzed for total caloric and macronutrient intake prior to testing day using Food Processor (ESHA Research, Versions 10.0-10.4). The 3-day average caloric intake was used as the basis for determination of quantity of food items on the testing day.

3.2.1. Testing Day

Transplant subjects were tested pre-transplant and/or post-transplant and after dietary intervention. Control subjects were tested before and after dietary intervention. Testing days were the same for each period.

On testing days, participants arrived at the University of Alberta Human Nutrition Research Unit after an overnight fast. T1D participants were instructed to fast unless they experienced hypoglycemia, which they were told to treat as usual. Fasting blood samples were drawn and an IV catheter inserted for subsequent blood draws. Participants ingested a loading dose of deuterium-labelled water (D_2O; D-175; 99.9 atom% D; CDN Isotopes) of 1.0 g/kg body water estimated at 60% body weight. Participants were given a maintenance dose of D_2O (1 g/kg body water) diluted in 1.5 L of water to be consumed throughout the day to maintain plasma deuterium levels at a plateau. D_2O was prepared by slow filtration through a 0.22 micron, 25 mm Cameo 25ES Syringe Cellulosic sterile and pyrogenic filter (GE Water & Process Technologies, Fisher Scientific) to remove any contaminants that may be present. After D_2O administration, participants consumed a breakfast meal and post-prandial timing was initiated upon completion. Height and weight were measured using electronic scales, and resting metabolic rate by

metabolic cart (SensorMedics Vmax Spectra 29n Nutritional Assessment Instrument; Vmax Program Software IVS-0101-12-3A; Viasys Healthcare). Participants also underwent body composition analysis, either by DEXA (General Electric Lunar Prodigy High Speed Digital Fan Beam X-Ray-Based Densitometer; enCORE 9.20 software; GE Healthcare) or BodPod (software version 3.2.5, Life Measurement Inc.), as well as measurement of blood pressure and waist circumference. Trunk, android, and gynoid fat were assessed by DEXA. Blood samples were drawn at 2, 4, 6, and 8 hours following breakfast. Lunch was provided after the 4-hour blood sample. An afternoon snack was provided after the 8-hour blood sample, and a supper meal and evening snack was provided for participants to consume at home. Participants were instructed to consume only those foods provided, to not eat after 8 PM, and to arrive the next morning fasted at the University of Alberta Hospital outpatient lab for a 24-hour blood sample.

The total gram quantity of each food for testing day meals was scaled for each participant to mimic normal total caloric intake, estimated from the 3-day food records. An individual's normal caloric intake was calculated as a proportion of the generic testing day caloric quantities which was set to make up 1000 kcal, and the amounts of foods for each individual subject adjusted to make up their usual caloric amount. The breakfast meal was comprised of whole wheat toast with margarine (Becel Original, non-hydrogenated; mainly canola and sunflower oils; Unilever Canada), eggs scrambled with margarine (Becel) and 1% milk using a ratio of 2 eggs to 1 tsp margarine to 1/4 cup milk, and apple slices. The lunch meal was a commercially prepared Lean Cuisine dish consisting of pasta, chicken, and vegetables in a marinara sauce, and 1% milk. The afternoon snack was sliced vegetables including broccoli, green pepper, mushrooms and carrots, with a low-fat ranch salad dressing dip. The evening meal was a turkey sandwich consisting of whole wheat bread, margarine, mustard, tomato, deli smoked turkey, and low-fat cheddar

cheese, and an apple, and the evening snack was a commercially prepared hummus dip (Tribe Classic; Tribe Mediterranean Foods Inc.; Taunton, MA) and whole wheat pita bread slices. The meals provided 20% of calories from protein, 45% from carbohydrates, and 35% from fat, with 240 mg dietary cholesterol per 1000 kcal. Additional non-caffeinated beverages such as black tea or coffee with no added sugar or dairy was permitted. Meals were provided throughout the day in a sequential fashion to represent typical food consumption in a free-living situation and to elicit a typical post-prandial response. In addition, it would have been dangerous to request individuals with brittle diabetes to fast more than 4 hours during the day. Participants with diabetes self-administered long- and rapid-acting insulin doses and injections, which was typically just before meals were consumed. The time, amount, and type of insulin was recorded in the chart.

Use of Deuterium Oxide in Hydropic Subjects

Individuals in liver failure often have ascites and peripheral edema related to portal hypertension as a complication of advanced cirrhosis. This increase in body fluid volume could potentially affect deuterium equilibration. Through investigation of D_2O as a method for estimating total body water, Faller et al. (1954) found that while normal participants have equilibration of D_2O with all body fluids, including blood, liver, and urine, within two hours, hydropic participants with ascites or edema due to cardiac decompensation or nutritional reasons had slower attainment of D_2O equilibration between serum and ascitic/edematous fluids and urine (*Faller et al. 1955*). Participants attained equilibration between these body fluid depots prior to 8 hours after administration, and all fluids were equilibrated by 24 hours. A loading dose of 0.9g D_2O per kg body weight was given, without a maintenance dose. It was anticipated for the present study that the loading dose plus maintenance dose of 1g D_2O per kg body water, estimated

at 60% body weight, would be sufficient to increase serum deuterium levels to a plateau in a short period of time, and would be sufficient by 24 hours for reliable calculation of fractional synthesis rate of FA and cholesterol. In addition, since volume of ascitic fluid could not be measured directly, it was assumed that the extra fluid volume would be at least partially accounted for in the total body weight. Analysis of plasma water (PW) enrichment showed no differences between liver failure and control groups at 24h (9446 ± 466 vs. 10163 ± 606 delta; p=0.35) and any postprandial timepoint (**Appendix C**; p>0.05), or in change in PW enrichment from baseline at 24h (9571 ± 465 vs. 10285 ± 605 delta; p=0.35) or any timepoint (**Appendix C**; p>0.05). Further analysis of liver failure patients with (n=4) and without (n=3) mild to moderate ascites showed no differences in total PW enrichment at 24h (Ascites 9575 ± 514 vs. No Ascites 9274 ± 984 delta; p=1.00) and postprandial timepoints (**Appendix C**) or change in PW enrichment from baseline at 24h (Ascites 9696 ± 512 vs. No Ascites 9405 ± 983 delta; p=1.00) or postprandial timepoints (**Appendix C**).

3.3. Procedures

3.3.1. Assessment of Plasma Lipids and Inflammatory Markers

Blood was drawn into either SST or K_2EDTA Vacutainer (BD Canada; Mississauga, ON) tubes. To obtain plasma for analyses, tubes were centrifuged at 4°C for 10 min at 3000 RPM on a Jouan CR4.22 centrifuge using a Jouan M4.4 rotor. After centrifugation, plasma was stored at -80°C for further analyses. Serum samples were also sent to the University of Alberta Hospital laboratory for analysis of plasma glucose, insulin, and lipid panel. Insulin was analyzed by immunoassay using the sandwich principle on the Elecsys 2010. Total and HDL cholesterol (TC and HDL-c), TG, and glucose were determined by the SYNCHRON LX System using standard kits. LDL-c was calculated using the Friedewald equation. Other data not obtained

during this study protocol were collected from the Clinical Islet and Liver Transplant programs, including HbA1c, electrolytes, and liver enzymes.

Cytokine analysis was performed on fasting (0h) serum samples only. Analysis was performed on a Luminex 100 IS Total System and StarStation analysis software (Luminex Corporation, Austin, TX). Custom multiplex kits were purchased from Invitrogen Canada Inc. (Burlington, ON) for analysis of TNF-a, IL-6, IL-10, CRP, and fibrinogen.

3.3.2. Lipoprotein separation

Lipoproteins were separated from 2 mL of plasma within 24 hours of collection by sequential non-equilibrium density-gradient ultracentrifugation (*Wilke et al. 2009*). Briefly, plasma was ultracentrifugated using a 0.196 molal NaCl solution on a Beckman Optima Centrifuge using a MLS 50 rotor for 25 min at 25,000 RPM at 20°C, and the chylomicron fraction removed. A 0.196 molal NaCl solution was applied and the solution centrifuged for 30 min at 25000 RPM and 20°C using a MLS 50 rotor to remove the chylomicron fraction. VLDL was extracted by using a 0.196 molal NaCl solution and centrifugation for 3 hours at 100,000 RPM at 20°C using a TL-100 centrifuge and Beckman TLA 100.2 100K rotor. The VLDL fraction was removed and stored at -80°C.

3.3.3. Lipid extraction and isolation

After separation, plasma and VLDL lipids were extracted and isolated using a modified Folch procedure (*Folch et al. 1957*) and thin layer chromatography (*Wilke et al. 2009*). 100-150 µL of plasma was used for lipid extraction and isolation, whereas all of the VLDL sample obtained from centrifugation was used for lipid extraction and isolation. Plasma and VLDL lipids were extracted using 2:1 chloroform:methanol and 0.25% calcium chloride, washed with 3-4 mL chloroform/methanol/H_2O (86:14:1), and dried under nitrogen gas. Sample and standard (33.3-50 uL) were plated in

triplicate on a G plate and placed in a glass tank using a solvent system of 80:20:1 petroleum ether:ethyl ether:acetic acid. The developed plate was sprayed with 0.1% ANSA and held under a UV lamp to identify fluorescent lipid bands. Lipid fractions, including TG, total phospholipids (TPL), free cholesterol (FC), and cholesteryl ester (CE) were obtained.

A FA standard was added to TG (C15:0) and TPL (C17:0) fractions before saponification and methylation to produce FA methyl esters. CE fractions under three cycles of saponification and thin layer chromatography to yield FC fractions. Briefly, 1.0 mL 0.5N KOH in methanol was added to each tube and the tubes heated at 110°C for 1 hour. After cooling, 1 mL 14% BF_3 in methanol and 2 mL hexane were added to each tube, and tubes again heated for 1 hour at 110°C. Tubes were cooled and 1 mL double distilled water was added, vortexed, and allowed to separate. The top layer was removed and placed in a microvial and dried under nitrogen. 200 µL hexane was added, the tube vortexed, and transferred to a micro-insert glass tube and dried to ~50 µL under nitrogen. Plasma and VLDL TG samples for analysis of fatty acid composition and synthesis were analyzed in duplicate at all timepoints (0, 2, 4, 6, 8, and 24 h).

3.3.4. Gas chromatography

FA methyl ester samples from plasma- and VLDL-TG samples and TPL were analyzed using either a Varian Star 3600 gas chromatograph using a 25 m 0.22 mm BP 20 column or Varian 3900 gas chromatograph (Varian Inc.) using the Galaxie Chromatography Data System (V.1.9.301.220) with a 30m 0.25 mm BP20 column. A TG-FA standard (GLC 461; Nu-chek Prep Inc.) was used to identify peaks, and FA concentrations were determined by comparison to the internal standard used (C15:0 for TG or C17:0 for TPL). Samples were analyzed for total major FA concentration and contribution of individual major FA to total concentration. Major FA were considered to be

those FA 14-18 carbons in length as well as 20:4n6, 20:5n3, and 22:6n3.

3.3.5. Isotope ratio mass spectrometry

3.3.5.1. Deuterium enrichment in plasma and VLDL TG

Plasma- and VLDL-TG samples were analyzed using either a Delta PlusXL IRMS (Finnigan Mat) with HP 6890 (Agilent; Mississauga, ON) and PAL GC1 autosampler (analyses 2007-2008) or Delta V Plus Isotope Ratio Mass Spectrometer with Trace GC Ultra and Triplus Autosampler (Thermo Scientific; West Palm Beach, FL) (analyses 2009-2010). A 30 m 0.22 mm BP-20 column was used for FA analysis. For each run oven temperature was set at 90°C and was increased to 230°C over the run. Sample injection size was 1.3 µL, helium flow 1.0 mL/min, and injector temperature set at 240°C. The D/H reactor temperature was 1420°C. The FA areas and deltas were averaged for calculations of lipogenesis. Fasting de novo FA synthesis (DNFA; %) was calculated from 24h fasting plasma- and VLDL-TG samples, and postprandial samples were calculated from post-breakfast meal plasma- and VLDL-TG samples.

3.3.5.2. Deuterium enrichment in plasma and VLDL cholesterol

Calculation of cholesterol fractional synthesis rate (FSR; % pool/day) is typically made from deuterium enrichment in FC (*Jones et al. 1993*; *Diraison et al. 1997*). Red blood cells contain only FC obtained from plasma, but may underestimate cholesterol synthesis in the short-term (<12 hours) period (*Jones et al. 1993*). Therefore, plasma cholesterol was chosen for estimation of whole-body cholesterol synthesis, and VLDL-cholesterol was chosen for estimation of hepatic cholesterol synthesis. Incorporation of deuterium into CE is usually low and delayed compared to FC, but can represent a loss of label from the FC pool and underestimate total cholesterol synthesis if it is not measured (*Neese et al. 1993*; *Jones et al. 1993*; *Diraison et*

al. 1997). Therefore analysis of CE was included in this investigation, as newly synthesized FC moves into the CE pool, and omitting analysis of CE may underestimate total cholesterol synthesis (*Jones et al. 1993*). Jones et al (1990) found that deuterium incorporation into FC was highest during early morning, lowest in the afternoon (1400 to 1800) and highest at 0600 (*Jones et al. 1990*). CE-FSR was found to be less variable, and peaked during early morning. Other investigators have found that cholesterol synthesis may be maximal around 2200 (± 2 hours), and lowest around 1130 (± 0.5 h), however variations can occur due to changes in meal timing (*Cella et al. 1995*). Therefore, 0, 4, 8, and 24-hour timepoints were chosen to capture synthesis during the morning (0-4 hrs), afternoon (4-8 hrs), and full 24 hours. FC and CE samples were run separately to determine FSR for each fraction. VLDL-FC (V-FC) was measured in the fasting state only. Following the methods of Gremaud et al (2001) and Pouteau et al (2003), FC and CE samples were derivatized to cholesteryl acetate by addition of 150 µL acetic anhydride and 40 µL pyridine and heated for 10 min at 90°C (*Gremaud et al. 2001*; *Pouteau et al. 2003*). To remove reagents, samples were heated at 80°C under nitrogen until dry. Samples were rehydrated with 400 µL hexane, dried overnight, and rehydrated with 80 µL hexane. Plasma cholesterol samples were analyzed using a Delta V Plus Isotope Ratio Mass Spectrometer with Trace GC Ultra and Triplus Autosampler (Thermo Scientific; West Palm Beach, FL) using a 30 m, 0.25 mm DB-5 column (J&W Scientific) with 0.25 um film thickness. Oven temperature was set at 140°C and was increased to 310°C for the run, and to 320°C for post-run. Sample injection size was 1.0 µL, helium flow 1.3 mL/min, and injector temperature 280°C. Samples were analyzed in duplicate, and the deltas averaged for calculations. Fasting FC-FSR (% pool/day) and CE-FSR (% pool/day) were calculated from the 24h fasting plasma samples, and postprandial samples were calculated from the post-breakfast meal plasma samples.

3.3.5.3. Plasma water deuterium analysis

Undiluted plasma for deuterium analysis was filtered by centrifugation in 10 K polyethersulfone membrane VWR Centrifugal filters (VWR International) for 1 hour at 14,000 RPM on a Jouan A-14 centrifuge. Samples for plasma water analysis were injected on a High Temperature Conversion-Elemental Analyzer (Finnigan TC/EA) with an AI/AS 3000 autosampler (Thermo Scientific; West Palm Beach, FL) coupled to the Delta V Plus Isotope Ratio Mass Spectrometer. Sample injection size was 0.3 µL, GC temperature set at 90°C, pyrolysis reactor temperature set at 1400°C, and helium flow at 2.0 mL/min. After each injection the syringe was rinsed twice with double distilled water. Samples were introduced in sequential order of expected enrichment (i.e. baseline, 2 h, 4 h, etc.) to reduce memory effect and number of injections required. Each sample was injected four to six times and the last 3 values were averaged for use in calculations. Plasma water samples were analyzed at all timepoints.

3.4. Calculations

Fatty acid concentration (for plasma or VLDL) was calculated by:

FA (µg/mL) = µg Std (AreaFA/AreaStd)*(1000/Sample µL)

The Std is the FA standard used in sample preparation/analysis (C15:0 or C17:0), AreaFA is the area of the FA peak determined by GC, AreaStd is the area of Std peak determined by GC, Sample is the volume of sample used in preparation (amount plated during TLC), and the constant 1000 is used to correct the volume from µL to mL.

De novo fatty acid synthesis (DNFA %) was calculated by (*Konrad et al. 1998*; *Wilke et al. 2009*):

DNFA (%) = [Δδ FA‰/Δδ PW‰ x 0.477] x 100%

Δδ FA‰ and Δδ PW‰ represent the change in FA or plasma water (PW) enrichment (measured-background). The constant 0.477 is derived based on estimations from Jungas (1968) that 0.87g-atoms ^{3}H per g-atom carbon are incorporated into a given FA (*Jungas 1968*), and represents an average FA present in a TG molecule. This constant can be replaced with constants for individual FA calculated from the number of hydrogens available for potential replacement with deuterium (enrichment). The correction factor for each individual FA is based on calculations by Leitch and Jones (1991) (*Leitch et al. 1991*). The correction factors are 0.487 for myristic acid (14:0), 0.449 for palmitic acid (16:0), 0.480 for palmitoleic acid (16:1), 0.477 for stearic acid (18:0), and 0.447 for oleic acid (18:1) as per Wilke et al (2009) (*Wilke et al. 2009*).

The fractional synthesis rate (FSR) of cholesterol was calculated by (*Di Buono et al. 2000*; *MacKay et al. 2010*):

FC-FSR (% pool/day) = (Δδ FC)/(Δδ PW x 0.478) x 100%

FC-FSR represents fractional synthetic rate (% pool/day) of FC. The Δδ FC and Δδ PW represent change in deuterium enrichment in FC or plasma water compared to baseline enrichment. The constant 0.478 represents the maximal ratio of hydrogen in newly synthesized cholesterol that may come from deuterium, from either body water or NADPH (*MacKay et al. 2010*). This equation is the same for calculation of FSR for FC and CE.

3.5. Statistics

Statistical analysis was performed using GraphPad Prism (V. 5.0c; GraphPad Software Inc.). Two groups were compared by Mann-Whitney t-tests for non-parametric data. Comparisons between matched participants were made using paired t-tests. Three groups were compared by Kruskal-Wallis one-way ANOVA with Dunn's post-tests for non-parametric data.

Relationships between variables were calculated using Spearman's rank correlation test for non-parametric data. Changes before and after dietary intervention within groups were compared using paired t-tests. Significance was set at $p<0.05$. Data are presented as mean ± SEM.

3.6. References for Methods

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4. Lipid synthesis in Type 1 diabetes

Despite improvements in patient care and pharmacological treatment of risk factors CVD remains a major cause of death in T1D (*Maahs et al. 2010*). Individuals with T1D are at a much higher risk of death from CVD compared to age and sex-matched non-diabetic controls (*Brindisi et al. 2010*), despite the fact that most individuals with T1D have normal lipid levels (*Orchard et al. 2010*). Traditional CVD risk factors such as elevated lipid levels are useful to predict CVD risk in T1D, but will not capture all at-risk T1D individuals (*Orchard et al. 2010*).

SREBP-2 and HMGCoA-r may be enhanced in rats with T1D resulting in increased cholesterol synthesis, but this has not always been observed (*Bloks et al. 2004*) and expression may differ between the intestine and the liver (*Feingold et al. 1994*; *Lally et al. 2007*). For example, Feingold et al (1994) found an increased expression of HMGCoA-r in the intestine of streptozotocin-injected rats, while Lally et al (2007) found no difference in intestinal expression of HMGCoA-r but significantly higher hepatic expression of HMGCoA-r. In comparison, Bloks et al (2004) found reduced hepatic expression of HMGCoA-r in streptozotocin-treated animals, which did not increase after insulin administration, and no difference in hepatic expression of SREBP2 compared to non-diabetic animals. Hepatic expression of LDL-r may be reduced upon induction of diabetes in animals but is restored with insulin (*Bloks et al. 2004*). Diabetic animals may also have enhanced intestinal and hepatic expression of NPC1L1 and reduced expression of ABCG5/G8, indicating enhanced absorption and less efflux of cholesterol in the intestine and liver (*Bloks et al. 2004*; *Lally et al. 2007*; *Tomkin 2008*). Intestinal and hepatic MTTP have also been found to be upregulated in diabetes, which may contribute to greater CM and VLDL particle synthesis (*Bloks et al. 2004*; *Lally et al. 2007*).

Changes in lipid metabolism in T1D may be mediated through impaired insulin regulation. Insulin normally promotes hepatic lipogenesis by inducing SREBP-1c gene transcription and accumulation. SREBP-1c induces expression of genes involved in FA synthesis such as ACC, FAS, and stearoyl-CoA desaturase (*Chahil et al. 2006*; *Chavez et al. 2010*). This may be of interest given that a recent investigation found NAFLD present in 44% of individuals with T1D, despite one third of these individuals taking lipid-lowering medications (*Targher et al. 2010*). However, hepatic SREBP-1c has been suggested to be downregulated after induction of diabetes in rats resulting in reduced FA synthesis, which can be partially or fully restored with insulin therapy (*Shimomura et al. 1999*; *Bloks et al. 2004*). Insulin resistance is associated with low absorption and high synthesis of cholesterol (*Pihlajamäki et al. 2004*; *Hoenig et al. 2010*), and both acute and chronic hyperinsulinemia stimulate cholesterol synthesis (*Feillet et al. 1994*; *Griffin et al. 1998*). However, T1D in humans has been associated with reduced synthesis and elevated absorption of cholesterol compared to individuals without diabetes in a series of investigations by Miettinen and Gylling using sterol precursors and metabolites as markers of cholesterol synthesis and absorption (*Miettinen et al. 2004*; *Gylling et al. 2004*; *Gylling et al. 2007*). These findings have not been assessed by more direct and sensitive methods, such as stable isotopes. To date there have been no investigations of DNL of total or individual FA in individuals with T1D.

The deuterium incorporation method is a useful tool for studying in vivo synthetic pathways and has been used in a variety of populations (*Guo et al. 2000*; *Gremaud et al. 2001*; *Diraison et al. 2003*). Deuterium incorporation is particularly useful for clinical populations as it can be used to estimate synthesis of FA and cholesterol simultaneously via sampling of different lipid compartments. In addition, deuterium is safe, can be used with a short measurement period of up to 24 hours, rapidly equilibrates across body

pools, and analysis by isotope ratio mass spectrometry provides a highly sensitive and precise measurement of in vivo synthesis (*Jones et al. 1993*; *Kelleher 2001*; *Emken 2001*).

The objective of this study was to confirm whether synthesis of cholesterol is lower and if FA synthesis is elevated in individuals with brittle T1D compared to healthy non-diabetic subjects.

4.1. Methods - Subject recruitment

Individuals with T1D were recruited from waiting lists for the University of Alberta Clinical Islet Transplant program. Inclusion criteria for participants wishing to undergo islet transplant includes brittle diabetes characterized by severe recurrent hypoglycemia and hypoglycemic unawareness (*Shapiro et al. 2000*). Exclusion criteria includes unstable coronary artery disease, active proliferative retinopathy or severe macroproteinuria (*Ryan et al. 2005*). Subjects were maintained on short- and long-acting insulin regimens, primarily insulin lispro (Humalog®) and insulin glargine (Lantus®), respectively. Subjects with type 1 diabetes who were taking lipid-lowering medications were instructed to stop medications 1 week before the testing day to allow a wash-out period before testing. A one-week period was determined as adequate due to the relatively short half-life of most statin medications, with the longest half-life being estimated at up to 30 hours for Atorvastatin (*Bellosta et al. 2004*). Of the eligible T1D subjects, 50% declined to participate, for reasons including out of town and difficulty scheduling with existing appointments for islet transplant clinic, or disinterest. Nine subjects with T1D completed testing. The serum and VLDL from one T1D subject was not obtained, therefore data for FA composition, concentration, and synthesis and cytokines was omitted for this individual.

Healthy non-diabetic subjects (Control) were recruited by advertisements at the University of Alberta and diabetes clinics in Edmonton

as a control group. Control subjects were not taking lipid-lowering or other medications, were non-smokers and normotensive, had fasting plasma LDL-cholesterol (LDL-c) <3.4 mmol/L, and HDL-cholesterol (HDL-c) >0.90 mmol/L, and did not have diagnosis or family history or premature CVD, diabetes, or other metabolic disorders. Control subjects were sex, and age- and BMI-matched (within 10%) to the subjects with diabetes; one T1D subject was not able to be matched. Of approximately 20 people that expressed interest in the study, only 8 were able to be matched with a person with T1D. An additional Control subject was recruited as her spouse was matched, and she was within the age- and BMI-range of the T1D subjects.

For details regarding methods and procedures please refer to Chapter 3: Methods.

4.2. Results

4.2.1. Subject characteristics

The majority of T1D subjects were taking statin medication (7 of 9), and medications for hypertension (all were taking at least one type of anti-hypertensive drug including ACE inhibitor, beta blocker, and calcium channel blocker). Approximately half (4 of 9) T1D subjects were taking thyroid medication, and two of these subjects were also taking a diuretic.

There were no significant differences between Control and T1D groups in major anthropometric markers (**Table 4-1**). There were no significant differences in fasting levels of plasma lipids, insulin, or inflammatory markers between Control and T1D groups (**Table 4-2**). Although fasting blood glucose levels were not significantly different between groups, they were slightly higher in the T1D subjects. Inflammatory markers tended to be higher in T1D compared to Control, though the difference was not statistically significant (**Table 4-2**). Postprandial plasma levels of TC and TG were not significantly different between T1D and Control, nor was the

incremental area under the curve for either TC or TG (**Appendix A**), though plasma TG levels tended to be higher following the lunch meal at 6 h and 8 h in the T1D group compared to Control group (**Figure 4-1**).

There were no significant differences in total caloric intake and most dietary markers between groups (**Table 4-3**). Percent of energy from carbohydrate was significantly lower and from fat significantly higher in the T1D group compared to Control.

Table 4-1: Anthropometric characteristics of control and type 1 diabetes subjects.

Measurement	**Control (n=9)**	**Type 1 Diabetes (n=9)**	**p-value**
Males:Females	3:6	4:5	n/a
Age (years)	54 ± 4	53 ± 4	0.93
BMI (kg/m^2)	25 ± 0.8	26 ± 1	0.67
WC (cm)	87 ± 4	93 ± 4	0.45
WC (cm) - Males	94 ± 1	98 ± 3	>0.05
WC (cm) - Female	83 ± 6	89 ± 6	>0.05
Body fat (%)	33 ± 3	31 ± 5	0.81
Body fat (%) - Males	26 ± 4	21 ± 4	>0.05
Body fat (%) - Females	36 ± 3	40 ± 3	>0.05
RMR (kcal/day)	1366 ± 58	1442 ± 53	0.23
RMR (kcal/day) - Males	1383 ± 109	1549 ± 32	>0.05
RMR (kcal/day) - Females	1358 ± 76	1334 ± 66	>0.05
Systolic blood pressure (mmHg)	119 ± 7	138 ± 8	0.13
Diastolic blood pressure (mmHg)	74 ± 6	88 ± 5	0.22

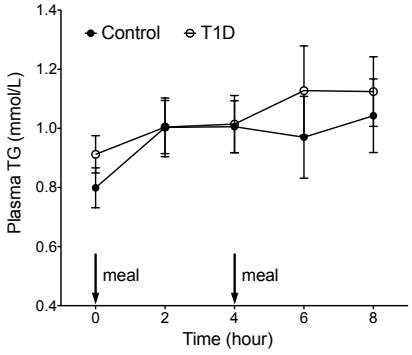
Data expressed as mean ± SEM; two-tailed

Table 4-2: Fasting lipid, glucose, insulin, and cytokine levels of control and type 1 diabetes subjects.

Fasting Concentration	**Control (n=9)**	**Type 1 Diabetes (n=9)**	**p-value**
TC (mmol/L)	5.1 ± 0.1	4.8 ± 0.2	0.73
LDL-c (mmol/L)	3.1 ± 0.2	2.9 ± 0.3	0.63
HDL-c (mmol/L)	1.5 ± 0.1	1.5 ± 0.1	0.48
TG (mmol/L)	0.80 ± 0.1	0.91 ± 0.1	0.20
Glucose (mmol/L)	5.0 ± 0.1	7.1 ± 1.1	0.057
Insulin (mU/L)	5.7 ± 1	3.4 ± 1	0.20
C-peptide	*not measured*	0.01± 0.01	n/a
HbA1c (%)	*not measured*	8.9 ± 0.5	n/a
TNF-α (pg/mL)	0.70 ± 0.2	0.78 ± 0.2	0.28
CRP (mg/L)	1.9 ± 0.7	2.4 ± 0.8	0.55
Fibrinogen (ng/mL)	1264 ± 166	1650 ± 240	0.32
IL-6 (pg/mL)	3.5 ± 2	2.2 ± 0.4	0.73
IL-10 (pg/mL)	0.31 ± 0.1	0.32 ± 0.1	0.92

Data expressed as mean ± SEM; # n=8 for TNF-α, CRP, fibrinogen, IL-6, and IL-10; two-tailed

Figure 4-1: Postprandial plasma TG levels of control and type 1 diabetes subjects.



Data expressed as mean ± SEM

Table 4-3: Dietary intake of control and type 1 diabetes subjects.

Background Dietary Intake	**Control (n=9)**	**Type 1 Diabetes (n=9)**	**p-value**
Total kcal	1607 ± 126	1689 ± 191	1.00
Total kcal - Males	1911 ± 330	2104 ± 305	>0.05
Total kcal - Females	1455 ± 52	1357 ± 114	>0.05
CHO (%)	55 ± 3	44 ± 3 *	0.02
Protein (%)	19 ± 1	21 ± 2	0.49
Fat (%)	24 ± 2	36 ± 3 *	0.02
SFA (%)	7.6 ± 0.8	11 ± 2	0.077
MUFA (%)	7.6 ± 0.9	11 ± 2	0.26
PUFA (%)	4.4 ± 0.6	5.7 ± 1	0.44
Cholesterol (mg)	201 ± 44	181 ± 31	0.93
Fiber (g)	22 ± 4	19 ± 3	0.67
Sugars (g)	90 ± 7	47 ± 8 *	<0.001

Data expressed as mean ± SEM; *p<0.05; two-tailed

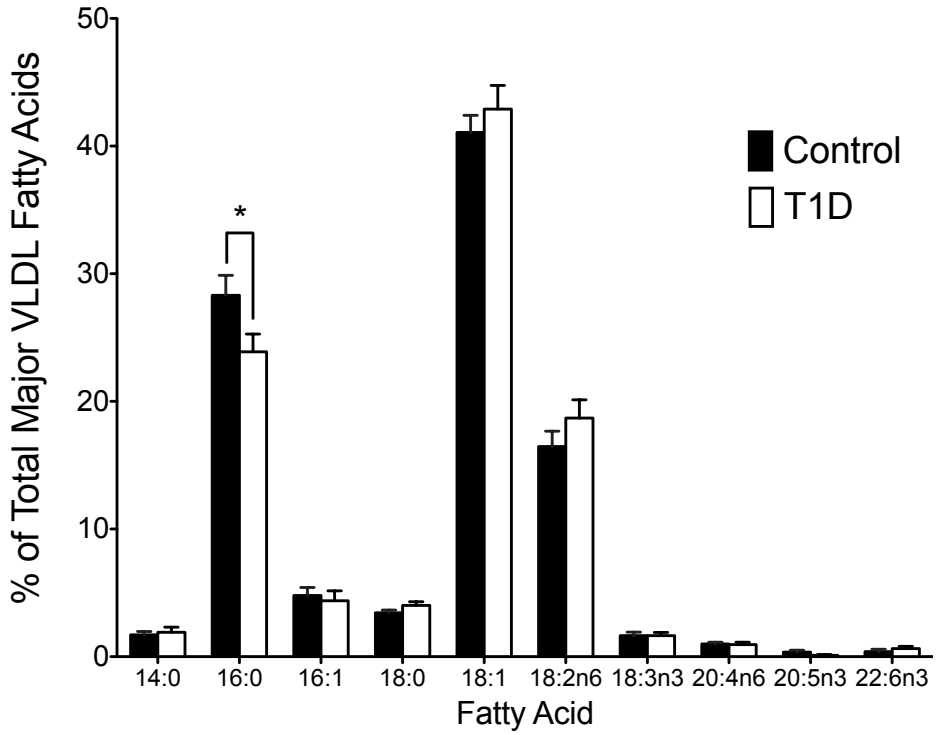
4.2.2. Fatty acid composition

Concentration of fasting VLDL-TG FA did not differ between T1D and Control groups (314 ± 87 μg/mL and 151 ± 34 μg/mL respectively). Contribution of individual FA to overall fasting VLDL-TG was not different between groups except for 16:0 which was lower in the T1D group compared to Control (**Figure 4-2**).

Total major FA composition of fasting plasma-TG showed a similar distribution pattern to VLDL-TG FA composition. Contribution of individual FA to overall fasting plasma-TG were not significantly different between groups except for 16:0 which was lower in T1D compared to Control (22 ± 0.8 % vs. 27 ± 1.3 %, respectively; p<0.05). Concentration of fasting TPL-FA

was not significantly different between T1D and Control (503 ± 192 μg/mL and 732 ± 101 μg/mL, respectively). Contributions of individual FA to fasting TPL-FA did not differ between groups.

Figure 4-2: Fasting VLDL TG fatty acid composition of control and type 1 diabetes subjects.



Data expressed as mean ± SEM; *p<0.05

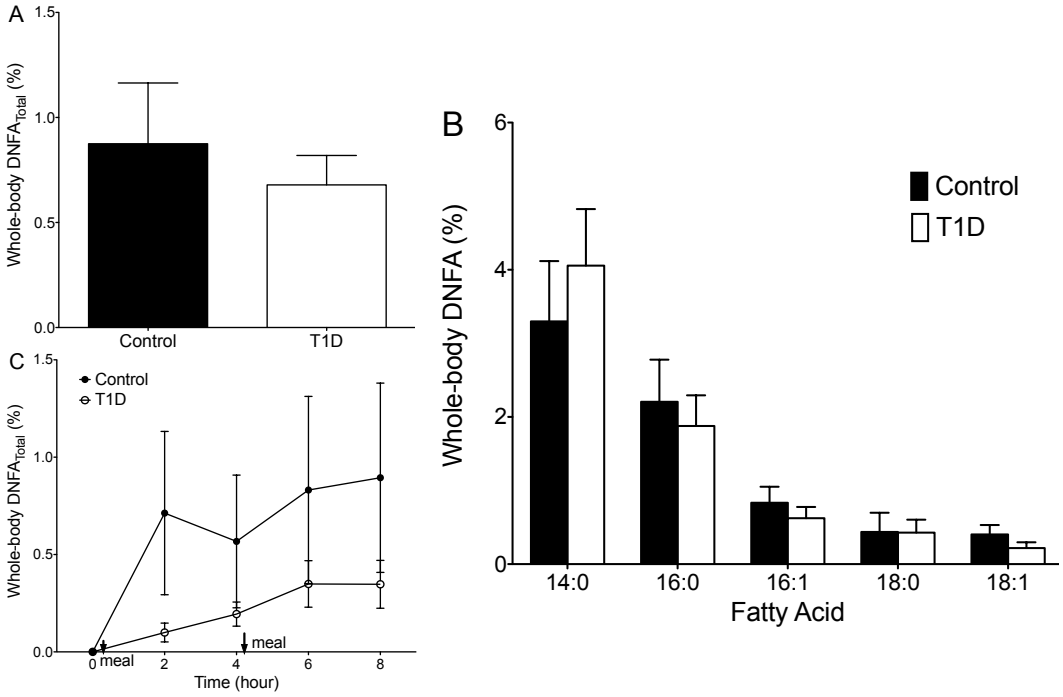
4.2.3. Fatty acid synthesis

Total PW enrichment was not significantly different between groups at 24 h or at any postprandial timepoint measured (**Appendix C**). Change in PW deuterium enrichment from background levels was not significant at any timepoint except at 6 h where change from baseline was higher in Controls than T1D (**Appendix C**).

Fasting whole-body DNFA estimated from plasma-TG FA was not significantly different between T1D and Control groups (**Figure 4-3A**). Fasting whole-body DNFA ranged from 0-2.7% in Control and 0-1.37% in

T1D. Similarly, fasting synthesis of individual FA from plasma-TG including 14:0, 16:0, 16:1, 18:0 and 18:1 were not significantly different between groups (**Figure 4-3B**). Postprandial synthesis of total (**Figure 4-3C**) or individual FA from plasma-TG were not significantly different between groups at any timepoint, though synthesis of total FA and 16:0, 18:0, and 18:1 tended to be lower in the T1D group (data not shown). Plasma insulin levels were not correlated with fasting or postprandial whole-body DNFA in either group.

Figure 4-3: Whole-body de novo synthesized fatty acids (DNFA) of control and type 1 diabetes subjects. A) Fasting whole-body total DNFA; B) Fasting whole-body individual DNFA; C) Postprandial whole-body total DNFA.

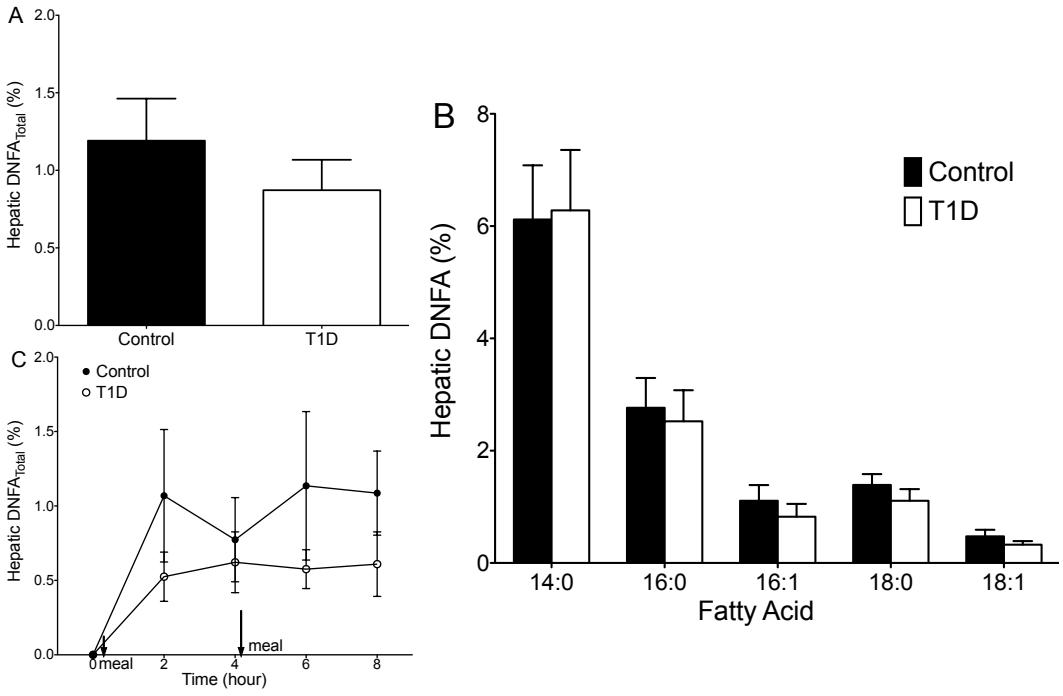


Data expressed as mean ± SEM; *p<0.05

Fasting hepatic DNFA estimated from VLDL-TG FA was not significantly different between T1D and Control groups (Figure 4-4A). Fasting hepatic DNFA ranged 0.43-2.82% in Control and 0.28-1.67% in T1D. Similarly, fasting synthesis of individual FA from VLDL-TG including 14:0, 16:0, 16:1, 18:0 and 18:1 were not significantly different between groups

(Figure 4-4B). Postprandial hepatic synthesis of total FA did not differ significantly between groups at any timepoint, though tended to be higher in Controls (Figure 4-4C). Hepatic postprandial synthesis of individual FA did not differ between groups at any timepoint (**data not shown**). Fasting hepatic total DNFA was significantly correlated to trunk (*r=0.89*), android (*r=0.82*), and gynoid (*r=0.96*) fat, as well as to dietary fiber intake (*r=-0.77*) in the Control group. Hepatic DNFA was not correlated to anthropometric or dietary measurements in the T1D subjects. Hepatic DNFA was significantly correlated with whole-body DNFA in both groups (Control *r=0.95*; T1D *r=0.81*) subjects. Plasma insulin levels were not correlated with fasting or postprandial hepatic DNFA in either group.

Figure 4-4: Hepatic de novo synthesized fatty acids (DNFA) of control and type 1 diabetes subjects. A) Fasting hepatic total DNFA; B) Fasting hepatic individual DNFA; C) Postprandial hepatic total DNFA.



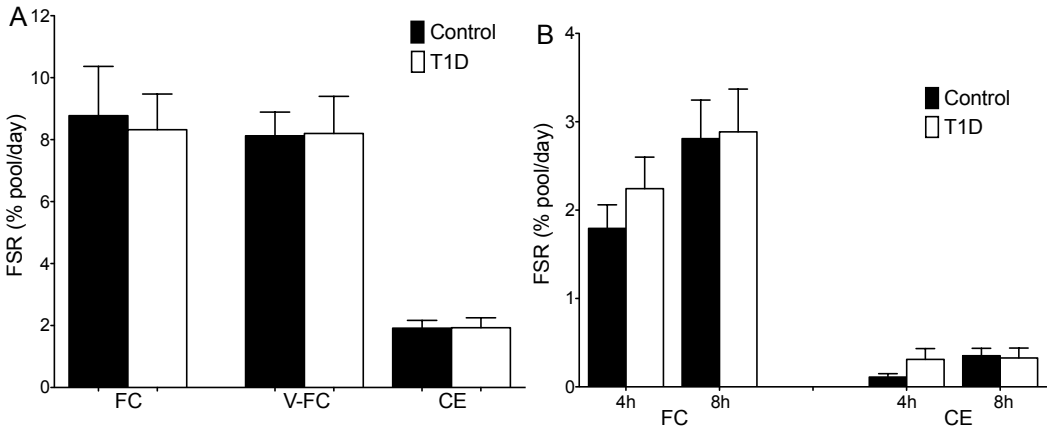
Data expressed as mean ± SEM; *p<0.05

4.2.4. Cholesterol synthesis

Fasting whole-body (FC) and hepatic (V-FC) synthesis of FC were not

significantly different between T1D and Control groups (**Figure 4-5A**). Rates of fasting whole-body cholesterol synthesis ranged from 3.47-20.24% in Control and 2.98-13.98% in T1D, whereas fasting hepatic FC synthesis ranged 3.72-11.50% in Control and 4.01-15.23% in T1D. Fasting whole-body synthesis of CE was not significantly different between T1D and Control groups (**Figure 4-5A**). Fasting synthesis of CE ranged 0.87-3.12% in Controls and 0.45-3.62% in T1D. Postprandial whole-body synthesis of FC and CE were not significantly different between T1D and Control groups (**Figure 4-5B**). Fasting whole-body synthesis of FC was significantly correlated with dietary carbohydrate (*r=0.78*), saturated fat (*r=-0.73*), and dietary cholesterol intake (*r=-0.75*) in T1D subjects, whereas FC-FSR was not correlated with anthropometric or dietary markers in Control subjects, except for plasma TC levels (*r=-0.72*). Fasting whole-body synthesis of CE was significantly correlated with FC-FSR in Controls (*r=0.75*) but not T1D, whereas CE-FSR was correlated with V-FC-FSR in both T1D (*r=0.93*) and Controls (*r=0.77*). Hepatic FC synthesis was correlated with dietary saturated fat intake (*r=-0.74*) in T1D, but was not correlated with dietary cholesterol intake or other anthropometric or dietary marks in Control or T1D subjects. Plasma insulin levels were not correlated with fasting or postprandial cholesterol synthesis in either group.

Figure 4-5: Fractional synthesis rate (FSR) of free cholesterol (FC), VLDL-FC (V-FC), cholesteryl ester (CE) of control and type 1 diabetes subjects. A) Fasting whole-body and hepatic FSR of FC and CE; B) Postprandial whole-body FC and CE FSR.



Data expressed as mean ± SEM

4.2.5. Comparison of matched control and diabetes subjects

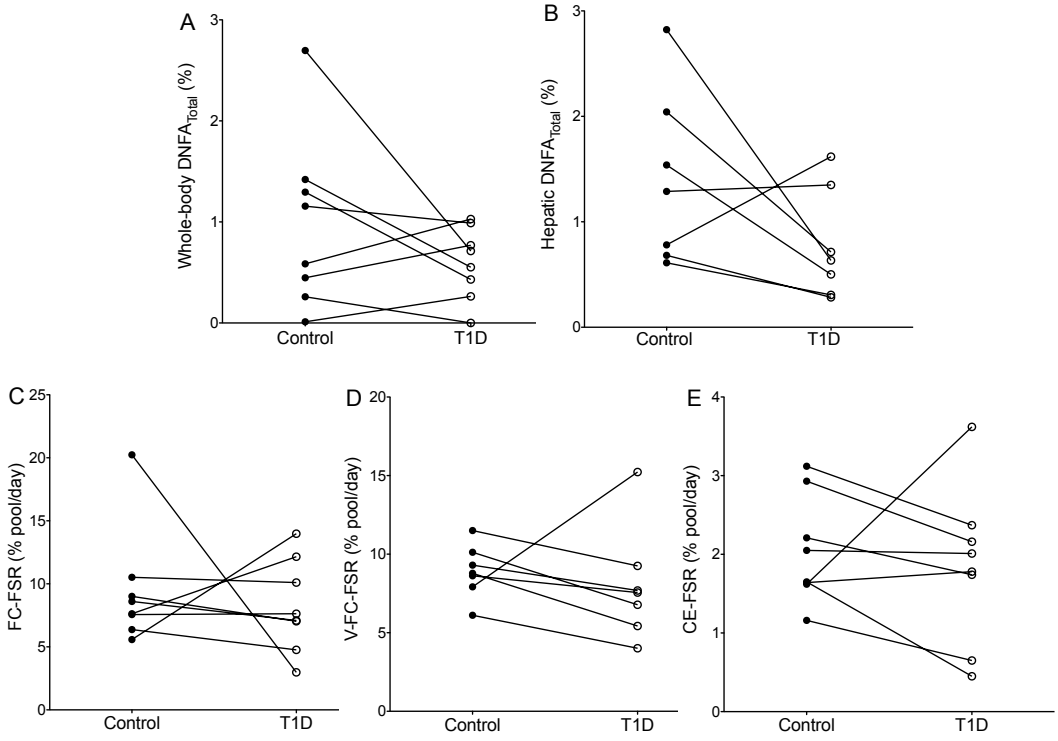
When T1D subjects were compared with their Control match (n=8 pairs) in paired tests, there were no significant differences found for blood lipids, glucose or insulin. These findings indicate that matching between groups was successful. However, comparing matched pairs there was a significantly higher BMI in T1D compared to Control subjects.

When T1D subjects were compared to their Control match (n=8 pairs) there were no significant differences in fasting whole-body DNFA (**Figure 4-6A**). When T1D subjects were compared to their Control match (n=7 pairs) there were no significant differences in fasting hepatic DNFA (**Figure 4-6B**). However, in five of seven pairs hepatic DNFA was lower in the T1D subjects compared to Controls (64 ± 5% lower, range 50-78%).

When T1D subjects were compared to their Control match (n=8 pairs) there were no significant differences in fasting whole-body or hepatic (n=7 pairs) synthesis of FC or CE (**Figure 4-6C-E**). One T1D subject had a V-FC-FSR of ~15% pool/day, while the rest had V-FC-FSR's <10% pool/day. When this

subject and matched control were removed, V-FC-FSR was significantly lower in the T1D compared to Control group (6.787 ± 0.753% vs. 9.074 ± 0.733% pool/day). The remaining T1D subject that was not matched to a Control had a fasting whole-body FC synthesis of 9.2% and hepatic FC synthesis of 9.6%.

Figure 4-6: Lipogenesis and cholesterol synthesis in individual control subjects and matched type 1 diabetes subjects. A) Fasting whole-body DNFA; B) Fasting hepatic DNFA; C) Fasting whole-body FC-FSR; D) Fasting hepatic FC-FSR; E) Fasting whole-body CE-FSR



4.3. Discussion and Conclusions

Results from the current work provide evidence that de novo lipogenesis does not differ in individuals with brittle type 1 diabetes compared to age-, sex-, and BMI-matched controls. Whole-body cholesterol synthesis was comparable between T1D and Control, but hepatic cholesterol synthesis tended to be lower in individuals with diabetes. Further research is

required to determine if this is a unique feature of brittle diabetes or if these results can be extended to well-controlled type 1 diabetes. The level of glycemic control estimated from HbA1c levels in the T1D subjects in the present study was ~8.9% (**Table 4-2**) indicating that perhaps results are generalizable to the larger diabetes population.

To the authors' knowledge this is the first investigation of de novo lipogenesis of total and individual FA in people with established type 1 diabetes. Higher intake of dietary fat tends to reduce lipogenesis; but despite higher fat intake, whole-body and hepatic de novo lipogenesis of total and individual FA were not found to be significantly different between T1D and Control groups. This finding is interesting given that emerging evidence suggests that a significant proportion of T1D subjects may have detectable fatty liver (*Leeds et al. 2009*). Insulin stimulates lipogenesis and regulates VLDL synthesis and secretion by targeting ApoB100 for degradation (*Chahil et al. 2006*). Development of fatty liver in type 1 diabetes may be due to hepatic accumulation of TG-rich particles due to impairments in remnant particle clearance, increased delivery of dietary fat in CM, or increased free FA from adipose tissue lipolysis combined with insufficient VLDL production due to ApoB100 degradation mediated by insulin excess. Recent evidence suggests a blunted suppression of free FA lipolysis by insulin in people with T1D (*Schauer et al. 2011*). Evidence from animal models of T1D suggests SREBP-1c may be downregulated leading to reduced FA synthesis, which is partially restored with insulin therapy (*Shimomura et al. 1999*; *Bloks et al. 2004*). Hyperinsulinemia that can occur in brittle diabetes due to exogenous insulin injections may stimulate lipogenesis, however this was not observed in the present study. One explanation could be that if hepatic lipogenesis is enhanced in brittle diabetes, newly synthesized FA may be shunted toward hepatic storage instead of transport in VLDL. A limitation of the present study was that insulin injections and levels in the T1D group were not controlled in

this study due to the brittle nature of diabetes in this group; however, insulin levels were not correlated with lipogenesis or cholesterol synthesis during fasting or postprandial measurements, and therefore are not expected to be a factor in the results obtained.

Cholesterol synthesis has been found to be upregulated in type 1 diabetes individuals beginning insulin therapy (*Feillet et al. 1994*), and during acute hyperinsulinemia (*Abrams et al. 1982*; *Stinson et al. 1993*). Long-term diabetes is associated with reduced synthesis and elevated absorption of cholesterol in a series of investigations by Miettinen and Gylling (*Miettinen et al. 2004*; *Gylling et al. 2004*; *Gylling et al. 2007*). The lower hepatic cholesterol synthesis found in the present study in most of the T1D subjects compared to their matched Control agrees with Gylling et al (2004) who found lower lathosterol, a precursor of cholesterol and marker of cholesterol synthesis, in VLDL of people with T1D. The present investigation is limited by a small sample size and presence of an outlier whose hepatic cholesterol synthesis was much higher than the rest of the group, therefore caution is emphasized when interpreting these results. Interestingly, in the present study whole-body cholesterol synthesis was not found to be different between T1D and Control groups when subjects were matched, and showed greater variation than hepatic synthesis between individuals with diabetes. In cases where whole-body appears higher than hepatic synthesis, newly synthesized cholesterol could arise from other organs. Cholesterol synthesized in the short-term that contributes to plasma lipoproteins is considered to occur primarily in liver and intestine (*Turley et al. 1981*; *Spady et al. 1983*; *Dietschy 1984*). Therefore in some individuals the intestine may contribute to body pools of newly synthesized cholesterol.

Hepatic cholesterol synthesis may have been reduced as compensation for increased delivery of cholesterol due to either enhanced intestinal synthesis or absorption. Animal models of T1D indicate greater

NPC1L1 and lower ABCG5/G8 expression as well as greater passive cholesterol transfer in the intestine, indicating enhanced intestinal active and passive absorption and retention of cholesterol (*Thomson 1983*; *Bloks et al. 2004*; *Lally et al. 2007*; *Tomkin 2008*). Cholesterol synthesis is reciprocally related to absorption in normal subjects across a range of absorption efficiencies; however, balance between cholesterol absorption and synthesis may not be well-maintained in type 1 diabetes (*Järvisalo et al. 2006*; *Gylling et al. 2007*). In the present study whole-body but not hepatic cholesterol synthesis was inversely related to dietary cholesterol in T1D subjects (*r=-0.75*). Testing day meals provided higher cholesterol content (240 mg/ 1000 kcal) than typical dietary intake in the T1D group (~180 mg), but Control subjects had comparable background cholesterol intakes (**Table 4-3**). If the relationship between absorption and synthesis is sustained it could be expected that there would have been similar reductions in synthesis for both groups, and therefore differences in hepatic synthesis would not be artifacts of dietary manipulation. Alternatively, if T1D subjects in fact have higher absorption efficiency than comparable Control subjects, the lower hepatic cholesterol synthesis observed in T1D may be in response to greater cholesterol intake on testing day.

Data from the current study and those of others suggests that cholesterol synthesis likely does not contribute to elevations in plasma cholesterol levels in type 1 diabetes. Other factors related to intestinal metabolism of cholesterol, including absorption and chylomicron production, may have a greater influence on overall plasma cholesterol levels and CVD risk in these subjects. In the present study, plasma TG levels were slightly elevated in the T1D subjects compared to Controls at the 6 h and 8 h postprandial measurements. In addition, fasting and postprandial ApoB48 levels have been previously shown to be elevated in this cohort of T1D subjects (*Su et al. 2009a*), indicating impaired lipoprotein clearance. Impaired

particle clearance may be due to reduced lipolysis or tissue uptake stemming from abnormal particle composition or clearance mechanisms (*Georgopoulos et al. 1991*). LPL activity may be normal in well-controlled type 1 diabetes, but may be impaired with poor glycemic control and insulin deficiency (*Nikkilä et al. 1977*; *Groop et al. 1996*; *Caixàs et al. 1998*; *Vergès 2009*). This would lead to slower clearance from the circulation and greater interaction of lipid-rich particles with arterial tissue (*Proctor et al. 2003*). This increased circulation time could be a promoting factor in atherosclerosis development and a potential contributing mechanism to the greater vascular disease and CVD mortality noted in individuals with T1D (*Weckbach et al. 2009*; *Maahs et al. 2010*). In addition, it has been shown in animal models that reduction of cholesterol synthesis in the liver is more sensitive to cholesterol delivery by CM compared to LDL and HDL (*Andersen et al. 1979*). Therefore if there are more CM particles, as suggested by Su et al (2009a), or greater cholesterol content in CM particles in type 1 diabetes due to enhanced cholesterol absorption, hepatic cholesterol synthesis may be lower compared to controls.

As has been shown by other groups, the plasma lipid levels of the T1D group were not significantly different from the non-diabetic control group, indicating that normolipidemic individuals with type 1 diabetes may not have significant alterations in lipid synthesis. Whether or not this holds true for type 1 diabetes individuals with hyperlipidemia cannot be concluded from the current work. It is interesting to note that the majority of subjects in the T1D group were taking statin medication to control plasma cholesterol levels. In the present study subjects were asked to refrain from this medication for a week to allow for washout of the effects of the drug. Therefore most of these subjects with type 1 diabetes had normal plasma cholesterol levels in absence of the statin medication. In addition, cholesterol synthesis was not found to be increased in these individuals and was comparable to healthy non-diabetic controls. If cholesterol synthesis is not elevated in type 1

diabetes and if instead absorption is elevated, as suggested by Miettenan and colleagues (*Miettinen et al. 2004*; *Gylling et al. 2004*; *Gylling et al. 2007*), then perhaps a different pharmaceutical agent should be utilized in these individuals for treatment or prophylaxis of hyperlipidemia.

A limitation of the present study is potential for contamination of the VLDL pool with chylomicron particles obtained with density ultracentrifugation. The density-gradient ultracentrifugation method used to separate VLDL from plasma and other lipoproteins does not guarantee that the fraction obtained does not contain other lipoproteins such as small, dense chylomicron remnants (*Terpstra 1985*; *Su et al. 2009b*). In the fasting state there should be low secretion of chylomicrons, therefore majority of particles will be VLDL. Postprandial measurements may require further consideration, as there could be contamination of the VLDL pool with chylomicron remnants. This is particularly true for the T1D group in whom it has been shown that ApoB48 levels are elevated in the postprandial period. Such contamination of the VLDL pool with chylomicron remnants may cause dilution of the VLDL-cholesterol pool by cholesterol contributed from chylomicron remnants; therefore conclusions about hepatic cholesterol synthesis may be influenced by the presence of newly synthesized cholesterol originating from the intestine and contributed by chylomicron remnants, as opposed to newly synthesized cholesterol originating from the liver and contributed by VLDL. Further, animal models suggest that cholesterol synthesis is enhanced in T1D; if this holds true in humans as well, newly synthesized cholesterol from the intestine may contribute to the newly synthesized cholesterol pool in the VLDL fraction that is assumed to originate from the liver, therefore diluting this pool and leading to a higher measured rate of cholesterol synthesis. This fact may be why hepatic cholesterol synthesis trended towards lower in the T1D compared to control group but did not reach statistical significance; in other words, a significantly reduced

hepatic cholesterol synthesis may be masked by a higher cholesterol synthesis in the intestine which would not be able to be evaluated from the VLDL fraction due to contamination from chylomicron remnants. A possible way to reconcile the rate of cholesterol synthesis in the intestine versus the liver would be to measure newly synthesized cholesterol in the chylomicron fraction at fasting and selected postprandial timepoints (within 2 hours after consuming a meal) to estimate intestinal cholesterol synthesis.

In conclusion, plasma lipid levels of the T1D group were not significantly different from the Control group, indicating normolipidemic individuals with T1D may not have significant alterations in lipid synthesis. Whether or not this holds true for individuals with hyperlipidemia cannot be concluded. The present work indicates that lipogenesis is not significantly different in individuals with brittle diabetes compared to control subjects. Cholesterol synthesis may be reduced in diabetes compared to healthy controls, however there may be large individual variation. In addition, there may be differences in regulation of lipid metabolism between the liver and other organs such as the intestine, which are not reflected in measurement of plasma cholesterol levels.

4.4. References for lipid synthesis in diabetes

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5. Lipid synthesis in liver failure

Liver damage that has progressed to cirrhosis and liver failure affects metabolism of carbohydrates, protein, and lipids. Lipid-related changes include alterations in lipoproteins as well as enzyme functions. Generally in advanced liver disease and cirrhosis, plasma levels of TG and cholesterol are reduced (*Miller 1990*; *Iglesias et al. 1996*; *Jiang et al. 2006*; *Varghese et al. 2007*). VLDL particles in cirrhotic patients may contain less cholesterol and TG which may stem from reduced hepatic synthesis or impairment in lipid packaging (*Rössner et al. 1979*; *Iglesias et al. 1996*; *Ferré et al. 2002*). However, increased hepatic lipid synthesis may contribute to development of steatosis, which is a pre-disposing factor for development of fibrosis and hepatocyte damage leading to cirrhosis. It has been proposed that HCV infection upregulates hepatic lipogenesis of FA by activation of SREBP-1c and fatty acid synthase in cellular models of HCV (*Waris et al. 2007*; *Yang et al. 2008*; *Negro et al. 2009*; *Syed et al. 2010*). Lipogenesis may also be upregulated in alcoholic steatosis and cirrhosis (*Tietge et al. 1998*; *Syn et al. 2009*), however, this effect has largely been inferred. Studies in which lipogenesis has been directly measured and found to be enhanced have predominantly only been carried out in patients with NAFLD, using deuterated water analyzed by mass isotopomer distribution analysis (*Diraison et al. 2003*), or labelled acetate to estimate lipogenesis (*Donnelly et al. 2005*). Therefore a direct evaluation of lipid synthesis in patients with liver failure due to causes other than NAFLD is warranted.

It has been proposed that hepatic cholesterol synthesis is reduced in HCV infection through viral-replication mechanisms (*Corey et al. 2009*). Hepatic cholesterol synthesis has also been suggested to be impaired in chronic liver disease, and cirrhosis, as evidenced from radioactive studies in animals and methods using sterol markers and ex vivo techniques in humans

(*Bissell et al. 1972*; *Miettinen 1972*; *Miller 1990*; *Zimmermann et al. 1992*; *Jiang et al. 2006*), but has not been confirmed using more direct methods in humans.

The objective of this study was to investigate if synthesis of FA is enhanced and cholesterol is impaired in individuals with liver failure compared to healthy subjects, using the deuterium incorporation method.

5.1. Methods - Subject recruitment

Individuals with liver failure from decompensated cirrhosis (Liver Failure) were recruited from waiting lists for the University of Alberta Clinical Liver Transplant program. Inclusion criteria for Liver Failure subjects was absence of cholestatic disorders, familial hypercholesterolemia and Type 2 diabetes, no prior myocardial infarction, and able to come to the testing unit at the University of Alberta. Of the eligible Liver Failure subjects, 50% declined to participate, for reasons including living outside the Edmonton area and difficultly travelling for testing, too ill to participate, being transplanted before a testing date could be scheduled, or disinterest. Seven subjects with Liver Failure completed testing. Clinical details of the Liver Failure patients are listed in **Table 5-1**. MELD scores were calculated according to Durand and Valla (2008). Creatinine and bilirubin levels <1 mg/dL were rounded to 1 before MELD calculation to prevent negative values after logarithmic transformation (*Durand et al. 2008*). Sodium levels <120 mEq/L were rounded to 120 and values >136 mEq/L rounded to 135 for calculation of MELD-Na (*Durand et al. 2008*). MELD-Na is provided as ascites is associated with poor prognosis and individuals with ascites and hyponatremia may have low MELD scores but a high risk of death (*Durand et al. 2008*).

Healthy subjects (Control; n=9) were recruited by advertisements at the University of Alberta and diabetes clinics in Edmonton as a control group.

Control subjects were not taking lipid-lowering or other medications, were non-smokers and normotensive, had fasting plasma LDL-cholesterol (LDL-c) <3.4 mmol/L, and HDL-cholesterol (HDL-c) >0.90 mmol/L, and did not have diagnosis or family history or premature CVD, diabetes, or other metabolic disorders.

For details regarding methods and procedures please refer to Chapter 3: Methods.

Table 5-1: Clinical characteristics of individual liver failure subjects.

Subject	**Sex**	**Reasons for Transplant**	**Age (y)**	**Creatinine (umol/L)#**	**Bilirubin (umol/L)#**	**INR**	**Calc. MELD §**	**Assigned MELD**	**Calc. MELD-Na§**
Liver-1	M	1) HCV; 2) HCC	57	81	26.5	1.15	7.8	22	7.8
Liver-2	M	1) HCC; 2) HCV	58	113	19	1.05	7.8	24	7.8
Liver-3	M	1) HCV; 2) HCC	56	67.5	15	1.25	7.5	22	7.5
Liver-4	M	1) HCV; 2) HCC	54	86.5	20.5	1.15	7.4	26	7.4
Liver-5	F	1) HCC; 2) HCV	56	70	24	1.2	7.8	22	7.8
Liver-6	F	Cirrhosis-methotrexate	59	80	12.5	1.2	7.3	n/a	7.3
Liver-7	M	Cirrhosis - Alcohol	48	92	45	2.2	12.0	22	15.2
Mean ± SEM			*55.4 ± 1.4*	*84.3 ± 5.8*	*23.2 ± 4.1*	*1.31 ± 0.15*	*8.2 ± 0.63*	*23 ± 0.7*	*8.7 ± 1.1*

Creatinine umol/L was converted to mg/dL by diving by 88.4 and bilirubin umol/L converted to mg/dL by dividing by 17.104 as per the AMA Manual of Style (10th ed.) (*Iverson et al. 2007*).

§ MELD = 9.6*log(creatinine mg/dL) + 3.8*log(bilirubin mg/dL) + 11.2*log(INR) + 6.4; MELD-Na = MELD + 1.59*(135-Na mEq/L); from Durand and Valla (2008).

5.2. Results

5.2.1. Subject characteristics

Control subjects were not taking any medications. Liver Failure subjects were taking hypertensive medications (n=2), diuretics (Furosemide and Spironolactone; n=5), proton pump inhibitors (n=5), bisphosphonate (n=1), and treatment for encephalopathy (lactulose; n=3).

There were no significant differences between Control and Liver Failure in major anthropometric markers, except for resting metabolic rate which was higher in the subjects with liver failure (**Table 5-2**). Total (TC) and LDL-cholesterol (LDL-c) were lower in Liver Failure compared to Control (**Table 5-3**), whereas levels of plasma HDL-c and TG were not significantly different. Fasting blood glucose levels were not significantly different between groups, but insulin and HOMA index were significantly higher in the Liver Failure group (**Table 5-3**). Inflammatory markers tended to be higher in the Liver Failure subjects compared to Control (**Table 5-3**) possibly indicating increased systemic inflammation in this group. Postprandial plasma levels of TC and incremental area under the curve were significantly lower in Liver Failure subjects compared to Control groups at 4, 6, and 8 h (**Appendix A**), whereas postprandial levels of TG and incremental area under the curve were not significantly different between groups (**Appendix A**).

Table 5-2: Anthropometric characteristics of liver failure subjects and control subjects.

Measurement	Control (n=9)	Liver Failure (n=7)	p-value
Males/Females	3/6	5/2	n/a
Age (years)	54 ± 4	55 ± 1	0.96
BMI (kg/m^2)	25 ± 0.8	28 ± 2	0.41
WC (cm)	87 ± 4	103 ± 7	0.07
WC (cm) - Males	94 ± 1	112 ± 5	>0.05
WC (cm) - Females	83 ± 6	83 ± 7	>0.05
Body fat (%)	33 ± 3	31 ± 3	0.47
Body fat (%) - Males	26 ± 4	33 ± 3	>0.05
Body fat (%) - Females	36 ± 3	24 ± 0.8	>0.05
RMR (kcal/day)	1366 ± 58	1692 ± 115 *	0.04
RMR (kcal/day) - Males	1383 ± 109	1803 ± 112	>0.05
RMR (kcal/day) - Females	1358 ± 76	1414 ± 211	>0.05
Systolic blood pressure (mmHg)	119 ± 7	119 ± 7	0.60
Diastolic blood pressure (mmHg)	74 ± 6	78 ± 5	0.87

Data expressed as mean ± SEM; *p<0.05; two-tailed

Table 5-3: Fasting blood lipid, glucose, insulin, and cytokine levels of control and liver failure subjects.

Fasting concentration	Control (n=9)	Liver Failure (n=7)	p-value
Total cholesterol (mmol/L)	5.1 ± 0.1	3.7 ± 0.3 *	0.002
LDL-c (mmol/L)	3.1 ± 0.2	1.9 ± 0.3 *	0.01
HDL-c (mmol/L)	1.5 ± 0.1	1.3 ± 0.1	0.08
TG (mmol/L)	0.8 ± 0.1	1.0 ± 0.1	0.09
Glucose (mmol/L)	5.0 ± 0.1	5.4 ± 0.6	0.71
Insulin (mU/L)	5.7 ± 2	18 ± 3 *	0.004
HOMA	1.3 ± 0.4	4.6 ± 1 *	0.002
TNF-a (pg/mL)	0.70 ± 0.2	1.6 ± 1	0.64
CRP (mg/L)	1.9 ± 0.7	10.5 ± 5	0.11
Fibrinogen (ng/mL)	1264 ± 166	2859 ± 473 *	0.001
IL-6 (pg/mL)	3.5 ± 2	50 ± 25 *	0.003
IL-10 (pg/mL)	0.31 ± 0.1	1.6 ± 0.6	0.06

Data expressed as mean ± SEM; *p<0.05; two-tailed

There were no significant differences in total caloric intake between groups or most dietary markers (**Table 5-4**). Percent of energy from fat and saturated fat, and cholesterol intake were significantly higher in the Liver Failure group compared to Control.

Table 5-4: Background dietary intake of control and liver failure subjects.

Background Dietary Intake	Control (n=9)	Liver Failure (n=7)	p-value
Total kcal	1607 ± 126	1844 ± 135	0.21
Total kcal - Males	1911 ± 330	1957 ± 121	>0.05
Total kcal - Females	1455 ± 52	1563 ± 358	>0.05
CHO (%)	55 ± 3	51 ± 3	0.41
Protein (%)	19 ± 1	19 ± 1	1.00
Fat (%)	24 ± 2	32 ± 2 *	0.005
SFA (%)	7.6 ± 0.8	12 ± 1 *	0.01
MUFA (%)	7.6 ± 0.9	8.9 ± 0.5	0.11
PUFA (%)	4.4 ± 0.6	3.7 ± 0.6	0.68
Cholesterol (mg)	201 ± 44	400 ± 59 *	0.01
Fiber (g)	22 ± 4	18 ± 3	0.76
Sugars (g)	90 ± 7	112 ± 10	0.055

Data expressed as mean ± SEM; *p<0.05; two-tailed

5.2.2. Fatty acid composition

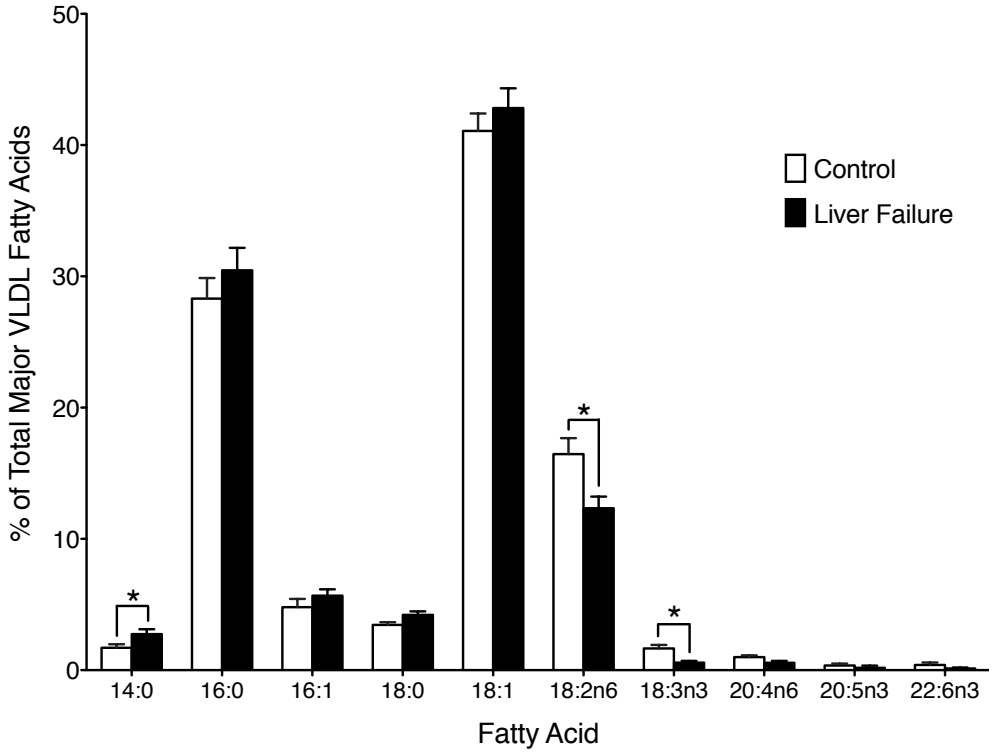
Concentration of fasting VLDL-TG FA was significantly lower in Liver Failure subjects compared to the Control group (88 ± 25 µg/mL vs. 151 ± 34 µg/mL respectively). Contribution of individual FA to overall fasting VLDL-TG was not different (**Figure 5-1**) except for 14:0 which was higher in the Liver Failure group compared to Control, and 18:2n6 and 18:3n3 which were lower in the Liver Failure group compared to Control.

Total major FA concentration of fasting plasma-TG showed a similar distribution pattern to fasting VLDL-TG FA composition. Contributions of individual FA to overall fasting plasma-TG levels were not significantly different between groups (**data not shown**) except for 16:0 which was

higher in the Liver Failure group compared to Control (32 ± 0.99% vs. 27 ± 1.3%, respectively) and 18:2n6 (11 ± 0.99% vs. 16 ± 1.2%, respectively) and 20:4n6 (0.66 ± 0.09 vs. 1.6 ± 0.38%, respectively) which were lower in the Liver Failure group compared to Control .

Concentration of fasting TPL-FA was significantly lower in Liver Failure subjects compared to Controls (372 ± 29 µg/mL and 732 ± 101 µg/ mL, respectively). Contributions of individual FA to fasting TPL-FA did not differ between groups (data not shown) except for 16:1 (0.94 ± 0.19% vs. 0.38 ± 0.14%, respectively) and 18:1 (17 ± 1.6% vs. 12 ± 0.81%, respectively) which were higher in Liver Failure compared to Control, and 18:0 (13 ± 2.0% vs. 19 ± 2.7%, respectively) which was lower in Liver Failure subjects compared to Controls.

Figure 5-1: Fasting VLDL TG fatty acid composition of control and liver failure subjects.



Data expressed as mean ± SEM; *p<0.05

5.2.3. Fatty acid synthesis

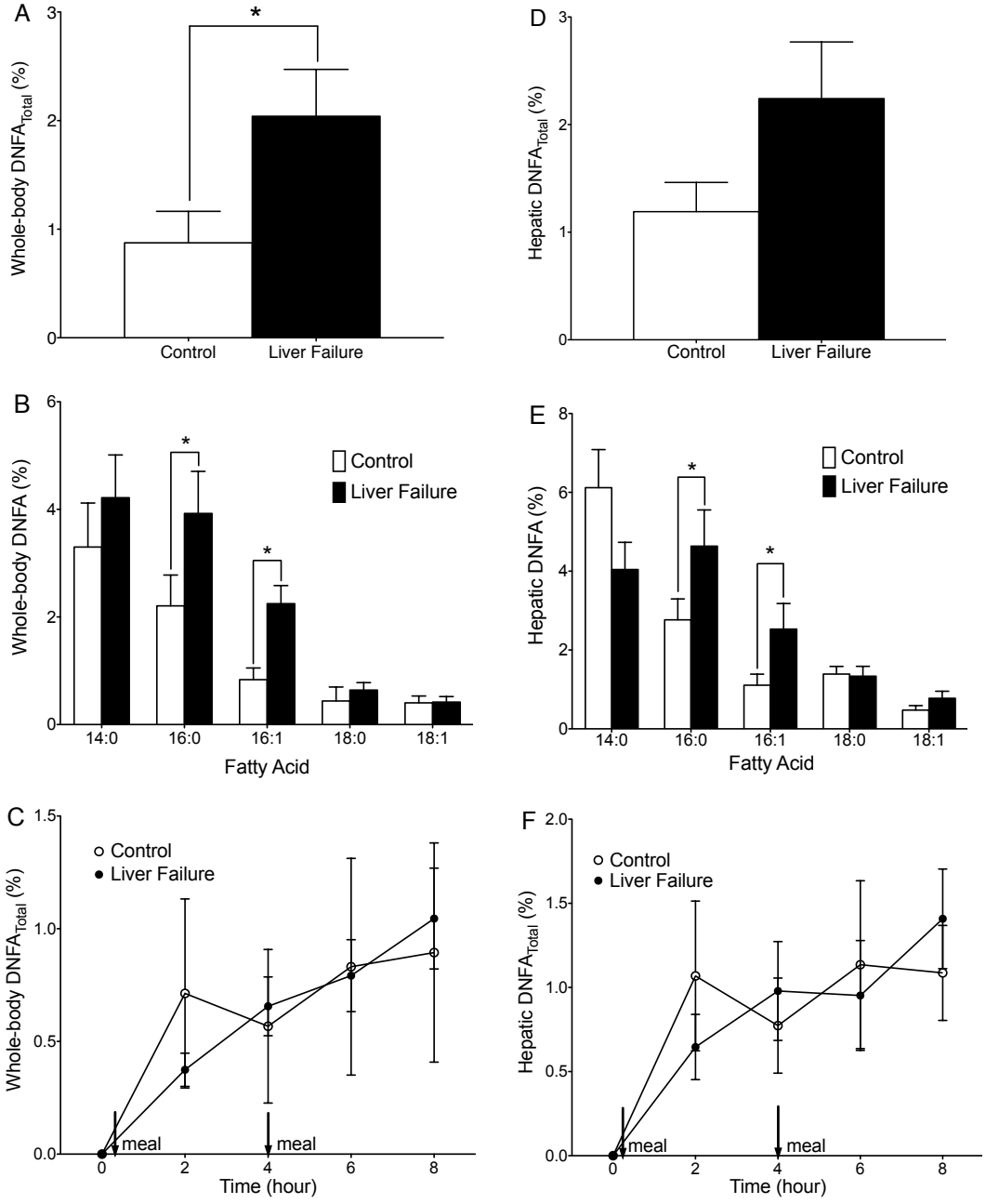
Fasting whole-body DNFA estimated from plasma-TG FA was significantly higher in the Liver Failure group compared to Controls (**Figure 5-2A**). Fasting whole-body DNFA ranged from 0-2.7% in Controls and 0.47-3.4% in Liver Failure. Similarly, fasting synthesis of individual FA including 14:0, 18:0 and 18:1 were not significantly different between groups, whereas synthesis of 16:0 and 16:1 were significantly higher in the Liver Failure group (**Figure 5-2B**). Postprandial synthesis of total (**Figure 5-2C**) or individual FA (data not shown) were not significantly different between groups at any timepoint.

Fasting hepatic DNFA estimated from VLDL-TG FA was not significantly different between Liver Failure and Control groups, but was higher in the Liver Failure group (**Figure 5-2D**). Fasting hepatic DNFA ranged from 0.43-2.82% in Control and 0.52-4.25% in Liver Failure. Similarly, fasting synthesis of individual FA including 14:0, 18:0 and 18:1 were not significantly different between groups, except for 16:0 and 16:1 which were significantly higher in the Liver Failure group (**Figure 5-2E**). Postprandial hepatic synthesis of total FA did not differ significantly between groups at any timepoint (**Figure 5-2F**). Hepatic postprandial synthesis of individual FA also did not differ between groups at any timepoint (data not shown). Hepatic DNFA was higher than whole-body DNFA for all Control subjects, whereas in the Liver Failure subjects whole-body DNFA was higher than hepatic DNFA in 4 of the 7 subjects (**Appendix D**), possibly indicating contribution of adipose lipogenesis. When subjects with (n=4) and without (n=3) presence of ascites were compared, there were no significant differences in fasting whole-body or hepatic total DNFA between groups (**Appendix D**).

Fasting hepatic DNFA was significantly correlated to dietary fiber intake (*r=-0.77*), and trunk (*r=0.89*), android (*r= 0.82*), and gynoid (*r=0.96*) body fat in the Control group. In the Liver Failure group, fasting hepatic DNFA

was significantly correlated to fasting plasma TG level (*r=0.79*), RMR (*r=0.82*), percent energy from dietary carbohydrate (*r=0.96*), fat (*r=-0.89*), and monounsaturated fat (*r=-0.86*), as well as CRP (*r=-0.79*), and IL-6 (*r=-0.79*). Fasting hepatic total DNFA was significantly correlated to fasting 16:0 DNFA in the Liver Failure (*r=0.89*) and Control group (*r=0.97*). Fasting hepatic DNFA was correlated to whole-body DNFA in the Control (*r=0.95*) but not Liver Failure (*r=0.54*) group.

Figure 5-2: Whole-body and hepatic de novo synthesized fatty acids (DNFA) of control and liver failure subjects. A) Fasting whole-body total DNFA; B) Fasting whole-body individual DNFA; C) Postprandial whole-body total DNFA; D) Fasting hepatic total DNFA; E) Fasting hepatic individual DNFA; F) Postprandial hepatic total DNFA.



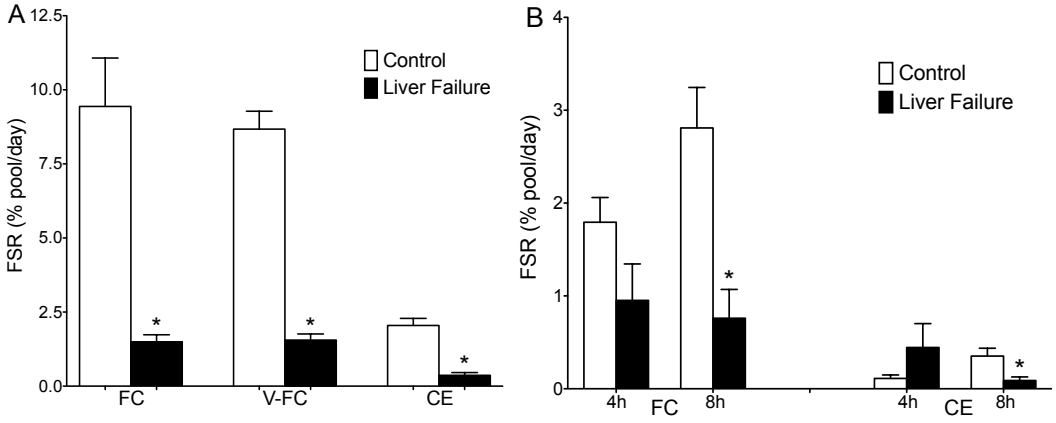
Data expressed as mean ± SEM; *p<0.05

5.2.4. Cholesterol synthesis

Fasting whole-body and hepatic FC synthesis were significantly lower in Liver Failure subjects compared to Controls (**Figure 5-3A**). Fasting whole-body FC synthesis ranged from 3.47-20.24% in Control and 0.67-2.42% in Liver Failure subjects, while hepatic FC synthesis ranged 3.72-11.5% in Control and 0.97-2.19% in Liver Failure. Fasting CE synthesis was significantly lower in Liver Failure subjects compared to Controls (**Figure 5-3A**). Fasting CE synthesis ranged 0.87-3.12% in Control subjects and 0.10-0.74% in Liver Failure subjects. Postprandial FC-FSR and CE-FSR were significantly lower in Liver Failure subjects at 8 h but not 4 h compared to Controls (**Figure 5-3B**). When subjects with (n=4) and without (n=3) presence of ascites were compared, there were no significant differences in fasting whole-body or hepatic FC-FSR or CE-FSR between groups (data not shown).

FC-FSR was significantly correlated to dietary n-6 FA intake in Liver Failure subjects ($r=-0.86$), as well as plasma creatinine ($r=-0.93$). FC-FSR in Control subjects was correlated with fasting TC ($r=-0.72$) levels. Hepatic cholesterol synthesis was not correlated with any anthropometric or dietary variables in Control subjects. In Liver Failure subjects, hepatic cholesterol synthesis was correlated with fasting HDL-c concentration ($r=0.79$), dietary protein intake ($r=0.79$), and ALP ($r=-0.82$).

Figure 5-3: Fractional synthesis rate (FSR) of free cholesterol (FC), VLDL-FC (V-FC), cholesteryl ester (CE) of control and liver failure subjects. A) Fasting whole-body and hepatic FSR of FC and CE; B) Postprandial whole-body FC and CE FSR.

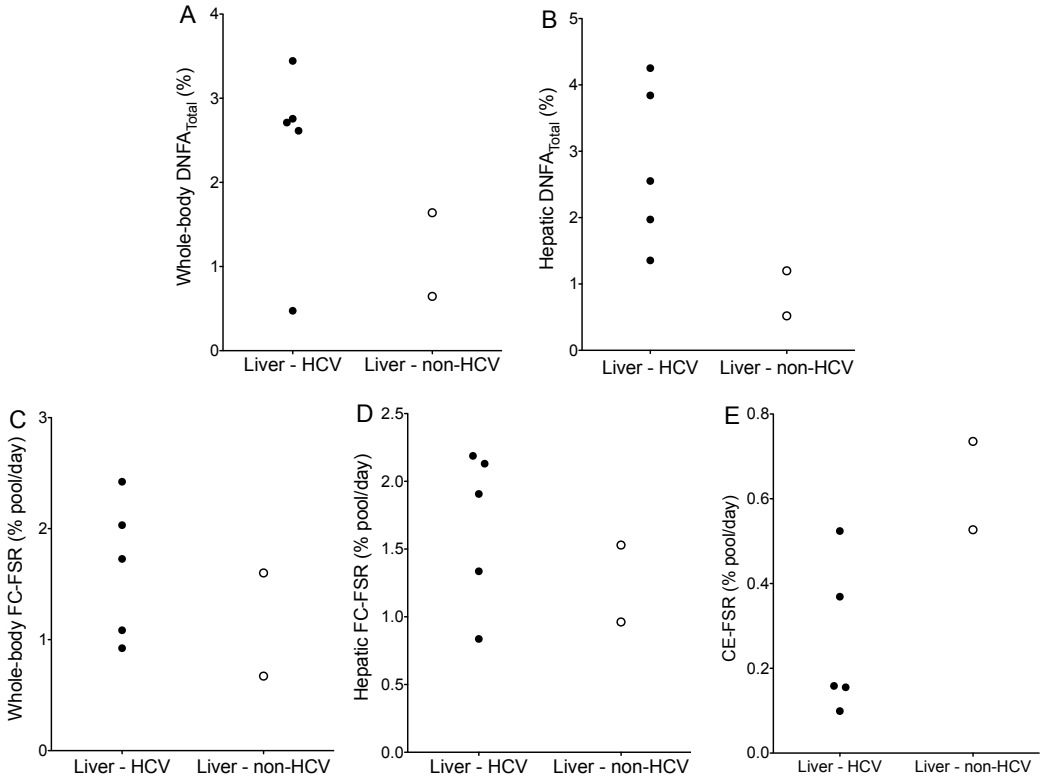


Data expressed as mean ± SEM; *p<0.05

5.2.5. Examination of effect of HCV

HCV may exert an influence on lipogenesis (*Syed et al. 2010*) and cholesterol synthesis (*Corey et al. 2009*), therefore subjects with (n=5) and without HCV (n=2) were compared. It was not possible to determine statistical differences between subjects with and without HCV in the Liver Failure group using nonparametric tests as there were only two subjects without HCV. However, it is tempting to suggest that whole-body and hepatic lipogenesis were higher in those subjects with HCV (**Figure 5-4A and B**). Whole-body and hepatic FC synthesis did not appear to be different in subjects with HCV compared to those without HCV (**Figure 5-4C and D**), but synthesis of CE appeared lower in subjects with HCV compared to those without (**Figure 5-4E**).

Figure 5-4: Lipogenesis and cholesterol synthesis in individual liver failure subjects with (n=5) and without (n=2) HCV. A) Fasting whole-body DNFA; B) Fasting hepatic DNFA; C) Fasting whole-body FC-FSR; D) Fasting hepatic FC-FSR; E) Fasting whole-body CE-FSR.



5.3. Discussion and Conclusions

Results from the current work provide evidence that cholesterol synthesis is impaired and de novo lipogenesis is elevated in liver failure subjects compared to control individuals. Generally in advanced liver disease and cirrhosis, plasma levels of TG and cholesterol are reduced (*Miller 1990*; *Iglesias et al. 1996*; *Jiang et al. 2006*; *Varghese et al. 2007*). Plasma lipid levels of the liver failure subjects in the current investigation agree with previous results. Subjects may also have low serum phospholipid levels, which may be due to reduced phospholipid synthesis (*Wilcox et al. 1978*; *Miller 1990*; *Burt et al. 2007*).

Incorporation of dietary FA into VLDL-TG may be lower in cirrhotic

patients, possibly due to reduced hepatic TG and VLDL synthesis (*Cabré et al. 2005*). Results from the present work are in agreement with previous findings, as FA quantities in plasma- and VLDL-TG were significantly lower in liver failure subjects despite greater dietary fat intake compared to control subjects. There were also differences between liver and control subjects in FA composition of plasma lipids noted in the present study, similar to previous investigations. It has been reported previously that there are low levels of PUFA in plasma lipid fractions of patients with advanced cirrhosis, including linoleic, linolenic, arachidonic, eicosapentanoic, and docosahexanoic acids (*Cabré et al. 1996*; *Burke et al. 2001*), and higher levels of palmitoleic, stearic and oleic (*Wilcox et al. 1978*; *González et al. 1992*). Low plasma lipid PUFA levels may arise from malnutrition or impaired activity of the Δ5 and Δ6 desaturases (*González et al. 1992*; *Cabré et al. 1996*). In the present study linoleic and linolenic acids were lower in plasma- and VLDL-TG fractions of liver failure subjects compared to control subjects, despite comparable total dietary PUFA intakes as percent of total energy.

Lipogenesis may also be upregulated in alcoholic steatosis and cirrhosis (*Tietge et al. 1998*; *Syn et al. 2009*), however, this effect has largely been inferred. The present data provides support that whole-body and hepatic lipogenesis is elevated in subjects with advanced liver failure. An ex vivo analysis of adipocytes from cirrhotic and healthy subjects found that adipocyte lipogenesis was higher in cirrhotic subjects than controls (*Harewood et al. 1982*). In the present study, hepatic lipogenesis was greater than whole-body lipogenesis in all of the control subjects but in only about half of the liver failure subjects. This finding may indicate that adipocyte lipogenesis is upregulated in individuals with liver failure. Adipose lipogenesis was not directly measured in the present study, but represents an interesting avenue for exploration.

Lipogenesis was elevated in subjects with liver failure despite higher

dietary fat intake, which normally suppresses lipogenesis (*Schwarz et al. 2003*). In addition, dietary carbohydrate was significantly and positively correlated with hepatic lipogenesis in the liver failure subjects (r=0.96). High intake of carbohydrate has been shown to increase lipogenesis in other individuals (*McDevitt et al. 2001*). Given the prevalence of hyperinsulinemia and insulin resistance in subjects with liver failure, prudent dietary advice may be to limit carbohydrate intake, particularly of foods with high levels of fructose, in cirrhotic subjects to prevent development or exacerbation of steatosis (*Parks et al. 2008*). Since the main products of lipogenesis are saturated FA, the elevated rate of lipogenesis could potentially cause an increase in cellular membrane content of saturated FA, particularly since dietary intake of saturated fat was also greater in the subjects with liver failure. Increased availability of saturated FA for incorporation into cellular plasma membranes may alter cellular function (*Clandinin et al. 1991*), and may exacerbate the effects of reduced cellular membrane PUFA levels which may already be low in subjects with liver failure.

HCV is associated with an upregulation of hepatic lipogenesis via activation of SREBP-1c and FAS as evidenced in cellular models and human liver samples (*Waris et al. 2007*; *Yang et al. 2008*; *Negro et al. 2009*; *Syed et al. 2010*; *Fujino et al. 2010*). Conclusions from the current work are complicated by the fact that many of the subjects with HCV in the current study also had HCC. HCC is typically a result of underlying liver disease and is a focal process as opposed to a primary cause of hepatic damage; therefore, HCC is unlikely to exert independent influences on lipogenic mechanisms. In the present work the sample size of subjects without HCV is too low to calculate significant differences. However, it is tempting to suggest that evidence from HCV cellular models indicating elevated lipogenesis are supported by the findings in human subjects presented here.

People with human immunodeficiency virus (HIV) are at a

significantly higher risk of CVD mortality when compared to similar control subjects (*Cockerham et al. 2010*). Like HCV-infected individuals, HIV is associated with insulin resistance and dyslipidemia (*Aragonès et al. 2010*). Similar to the present finding, an elevation in hepatic lipogenesis has been observed in HIV patients (*Hellerstein et al. 1993*), and circulating fatty acid synthase is higher in HIV patients (*Aragonès et al. 2010*). Plasma fatty acid synthase was lower in patients treated with antiretroviral agents compared to untreated patients, and was similar between patients with and without lipodystrophy (*Aragonès et al. 2010*). With highly active antiretroviral therapy, lipodystrophy and fatty liver appear to be mediated through insulin resistance (*Sutinen et al. 2002*). Given the similarities between HIV and HCV, there may be viral-related mechanisms contributing to dysregulation of hepatic lipid synthesis and metabolism in both of these conditions (*Slama et al. 2009*).

Insulin resistance and hyperinsulinemia are associated with elevated lipogenesis (*Hudgins et al. 2000*; *Diraison et al. 2003*), and stimulation with insulin has been shown to increase lipogenesis even in cirrhotic subjects (*Harewood et al. 1982*). The liver failure subjects in the current investigation had high fasting insulin levels and HOMA index scores compared to control subjects. Therefore, it is not possible to determine whether the elevated lipogenesis noted in these subjects is due to influence from elevated insulin levels, other aspects related to deteriorating liver function, or HCV-related mechanisms, or a combination of factors. Despite elevated lipogenesis, plasma TG was significantly lower in the liver failure compared to control subjects. It is emphasized that increased lipogenesis does not necessarily translate into increased TG synthesis or elevated plasma TG levels. In addition, increased lipogenesis could potentially promote development or worsening of hepatic lipid accumulation, which also may not be reflected by elevated plasma TG levels. In the present study, lipogenesis was related to

plasma TG levels in the liver failure group, even though plasma TG levels were still within recommended "desirable" levels (TG<1.7 mmol/L) for the general population (*Antonopoulos 2002*). Further study is required to determine if this relationship is maintained across a wider range of plasma TG concentrations, and also if either lipogenesis, plasma TG levels, or change in either of these markers over time are related to degree of steatosis in cirrhotic subjects. Unfortunately, presence and degree of steatosis was not measured in the present study. In NAFLD patients, there is a significant contribution from DNL to hepatic fat content, contributing 26% to liver TG by one estimation (*Donnelly et al. 2005*). Donnelly et al (2005) also found similarities in contribution of FA from various sources, including de novo synthesized, between hepatic and VLDL lipid content. Therefore, percent contribution of newly synthesized FA in the VLDL-TG were reflective of contribution of newly synthesized FA to hepatic lipid content. It is unknown whether this relationship extends to individuals with other forms of liver disease, but presents an avenue for exploration.

It would be interesting to examine the effect of dietary PUFA supplementation, specifically the n-3 PUFA EPA/DHA, in individuals with liver failure for several reasons. Firstly, PUFA levels in plasma lipids were shown in the present study, as well as others, to be lower in subjects with liver failure (*Cabré et al. 1996*; *Burke et al. 2001*). The metabolic effects of reduced cellular PUFA content in these subjects are unknown, but it could be expected that the imbalance of PUFA to saturated FA may have metabolic consequences. For example, low cellular PUFA content may impair binding of insulin to the insulin receptor (*Clandinin et al. 1991*), which in liver failure patients may increase the insulin resistance already observed in these subjects. Secondly, PUFA have been shown to inhibit FA synthesis (*Davidson 2006*) and may discourage development or progression of steatosis in patients with NAFLD. PUFA treatment was recently shown to reduce steatosis

in rats after 2 weeks, as well as de novo lipogenesis estimated from ratios of saturated to monounsaturated FA (*Marsman et al. 2011*); however, the type of PUFA supplemented, beyond "omega-3 FA", was not described by the authors of this study. In another investigation, human subjects with NAFLD given PUFA for 6 months showed reduction and complete regression of fatty liver in ~50% and 20-30% of patients, respectively (*Spadaro et al. 2008*; *Zhu et al. 2008*). It would be of interest to know if PUFA supplementation has promising effects on steatosis in humans with steatosis due to hepatic disorders other than NAFLD. The n-3 PUFA EPA/DHA are also considered anti-inflammatory due to production of prostaglandins and leukotrienes that are less inflammatory than those produced by omega-6 FA (*Calder 2010*). Reduction in inflammatory cells may further reduce hepatic damage associated with cirrhosis. Thirdly, by inhibiting lipogenesis, PUFA supplementation may be useful as a treatment for HCV as a way to inhibit viral replication and progression. A cell culture study by Kapadia et al (2005) showed that treatment with PUFA including arachidonic acid, EPA, and DHA each individually inhibited HCV replication by 3- to 6-fold (*Kapadia et al. 2005*). Therefore, it would be interesting to see if PUFA supplementation in humans with HCV may be effective in reducing HCV progression. There is currently an ongoing study investigating supplementation of fish oil in subjects infected with HCV to investigate if PUFA improves response to interferon treatment, which may address some of these questions (ClinicalTrials.gov identifier NCT00547716).

The present study suggests that cholesterol synthesis is significantly impaired in subjects with liver failure. Hepatic cholesterol synthesis has been suggested to be impaired in chronic liver disease and cirrhosis, as evidenced from radioactive studies in animals and methods using sterol markers and ex vivo techniques in humans (*Bissell et al. 1972*; *Miettinen 1972*; *Zimmermann et al. 1992*). The results of the present study support similar findings from

others using different techniques such as sterol balance and ratios of labelled acetate and mevalonate (*Miettinen 1972*) and ex vivo analysis of liver tissue (*Bissell et al. 1972*). This is in contrast to a report by Yoshida et al (1999) in which cholesterol synthesis was not found to be significantly different in subjects with hepatitis or cirrhosis compared to control subjects (*Yoshida et al. 1999*). In this study cholesterol synthesis was inferred from plasma mevalonate levels, indicating that perhaps mevalonate may not be a reliable measure of cholesterol synthesis in liver disease. For example, Miettinen (1972) reported difficulty accurately measuring cholesterol synthesis using the acetate-mevalonate test and serum methyl sterols (*Miettinen 1972*). However, plasma cholesterol levels in the study by Yoshida et al (1999) were not significantly different between control and liver disease groups, suggesting that degree of hepatic impairment may not have been as advanced as the subjects presented herein.

It has been proposed that hepatic cholesterol synthesis is reduced in HCV infection through viral-replication mechanisms (*Corey et al. 2009*). HCV may interrupt cholesterol synthesis by diverting the intermediate geranylpyrophosphate from the cholesterol synthesis pathway towards viral replication, or by directing cholesterol towards synthesis of membranes necessary for the virus to continue replication (*Corey et al. 2009*). A recent study in HCV-infected human liver samples found upregulation of cholesterol synthetic genes including HMGCoA-r in HCV and an inverse relationship between serum LDL and synthetic gene expression (*Fujino et al. 2010*). Furthermore, statin therapy appears to reduce HCV replication (*Ye et al. 2003*; *Bader et al. 2008*), and greater HCV sustained virological response is associated with statin use (*Harrison et al. 2010*). It is unclear whether greater HCV sustained virological response with statin therapy is related to changes in plasma cholesterol levels, and is complicated by the finding that higher LDL is related to greater sustained virological response (*Harrison et al. 2010*).

These results provide support that the cholesterol synthetic pathway may be upregulated in HCV but that the intermediates are likely diverted toward viral-specific purposes; therefore synthesis of cholesterol specifically could be impaired which may contribute to lower plasma cholesterol levels. The results found in the present study do not appear to support the hypothesis that cholesterol synthesis is reduced primarily due to HCV infection, but the sample size of non-HCV-infected individuals is too small to make unequivocal comparisons.

Dietary cholesterol intake was higher in the liver failure subjects compared to control subjects. The feedback mechanisms normally regulating cholesterol synthesis in response to dietary cholesterol has been suggested to be impaired in HCC but preserved in other forms of liver disease (*Bissell et al. 1972*). This suggestion is not supported by the current findings, in which cholesterol synthesis was not found to be correlated with dietary cholesterol intake in the liver failure group. However, cholesterol synthesis was not found to be correlated with dietary cholesterol intake in the control group either. The lack of relationship between synthesis and dietary intake may be due to limitations in sample size and range of dietary cholesterol intakes necessary to tease out this relationship.

In conclusion, FA synthesis is elevated but cholesterol synthesis is reduced in subjects with liver failure in the absence of cholestatic disorders. Elevated lipogenesis may arise from HCV-induced mechanisms, however this is not conclusive. Increased lipogenesis may not translate into higher TG synthesis and plasma TG levels, and further study is required to determine if enhanced lipogenesis is related to hepatic damage and steatosis.

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6. Lipid synthesis in islet and liver transplant

Organ transplantation is a life-saving procedure, however the trade-off is lifelong immunosuppressive medication to prevent graft loss. Predicted 10-year risk of CVD is higher in transplant recipients compared to the general population (*Neal et al. 2004*). Hypertension, hypercholesterolemia, and hypertriglyceridemia are associated with vascular events in the general population as well as in transplant recipients (*Laryea et al. 2007*; *Borg et al. 2008*; *Pfitzmann et al. 2008*). Immunosuppressive medications potently prevent graft rejection, however these medications target intermediates that are not specific to inflammatory cells. Due to the relatively non-specific nature of action, these drugs are associated with a variety of side-effects, including weight gain, diabetes, and hyperlipidemia. Post-transplant hyperlipidemia is a common occurrence across a variety of organ transplant groups (*Moore et al. 2001*).

Despite the prevalence of hyperlipidemia across transplant populations, there have been very few assessments of lipid synthesis as a possible mediator of hyperlipidemia in post-transplant human subjects. Elevations in plasma lipid levels could arise due to increases in lipid absorption or synthesis, reduction in plasma clearance, or a combination of these mechanisms. Reports indicate that approximately half of islet and liver transplant patients require lipid-lowering medications to treat hyperlipidemia post-transplant (*Ryan et al. 2001*; *Adam et al. 2009*). Changes occurring post-transplant also due to immunosuppressive medication may exacerbate alterations in lipid metabolism leading to hyperlipidemia in the post-transplant period, such as weight gain leading to obesity and derangements in insulin metabolism leading to hyperinsulinemia and insulin resistance (*Adam et al. 2009*). Insulin influences lipid metabolism by increasing transcription of key enzymes involved in lipogenesis, including

fatty acid synthase and acetyl CoA carboxylase, through transcription of SREBP-1c (*Saltiel et al. 2001*). Immunosuppressive medications may exert independent influences on lipid metabolic mediators. For example, Sirolimus may inhibit activity of the LDL-receptor (*Ma et al. 2007b*; *Ma et al. 2007a*) while Tacrolimus may reduce activity of lipoprotein lipase (*Tory et al. 2008*; *Tory et al. 2009*). Inhibition of the LDL-receptor or lipoprotein lipase can impair lipoprotein particle clearance and contribute to increased plasma lipid levels. Elevations in plasma lipid levels can also occur due to disturbances in absorption or synthesis. To date there have been no direct investigations of fatty acid or cholesterol synthesis in post-transplant patients under the influence of Sirolimus or Tacrolimus without concomitant corticosteroid therapy.

Islet transplant (ITx) is a relatively novel treatment for type 1 diabetes that can restore insulin independence (*Shapiro et al. 2000*). ITx is unique in organ transplantation compared to traditional transplants such as liver transplant (LTx), in which the damaged organ is replaced by a donor organ into the same anatomical location. In ITx, by comparison, islets are isolated from cadaveric donors and infused into the portal vein of the recipient, adhering to hepatic tissue and secreting insulin (*Kobayashi et al. 2004*). Therefore, ITx patients are a unique population in which to study the effects of immunosuppressive medications, as the major lipid-regulating organ, the liver, is not replaced but insulin regulation is restored.

The objective of this study was to determine if FA and cholesterol synthesis are elevated in islet and liver transplant patients compared to non-transplant control subjects.

6.1. Methods - Subject recruitment

The original study design was to follow individuals with type 1 diabetes or liver failure before and after islet or liver transplant, respectively.

Due to location logistics, length of time required for transplant, protocol changes within the islet transplant program, and health issues following liver transplant, it became unrealistic to follow subjects over time. Therefore, separate groups of post-transplant subjects were recruited for analysis.

ITx subjects were recruited from the University of Alberta Clinical Islet Transplant program. Admission to the islet transplant program required absence of unstable coronary artery disease, active proliferative retinopathy or severe macroproteinuria (*Ryan et al. 2005*). Inclusion criteria for ITx subjects included stable medication levels, and oral insulin, if required, below 0.2 units/kg and stable, defined as no increase in insulin requirements over 0.1 unit/kg over the previous 6 months. Due to the small number of ITx recipients available to participate, ITx patients were included if they had been transplanted within the previous 5 years. Subjects often required two islet transplants to obtain insulin independence, therefore subjects were only recruited and tested after the second transplant. Of the eligible ITx subjects, 50% declined participation due to conflict with other follow-up appointments scheduled, lack of time, or disinterest. Eight ITx subjects completed testing.

LTx subjects were recruited from the University of Alberta Clinical Liver Transplant program. Exclusion criteria for LTx subjects included transplantation >2 years ago, not local to the Edmonton area, unstable or changing medication levels, too ill to participate, presence of Type 2 diabetes, and current Hepatitis C treatment. Of the eligible LTx subjects, 40% declined to participate due to living out of town, still ill, lack of time, or disinterest. Seven LTx subjects completed testing. Indications for LTx were HCV with HCC (n=3), hepatitis B (n=1), alcohol-induced cirrhosis (n=1), Wilson's disease (n=1), and NASH (n=1).

ITx and LTx subjects taking lipid-lowering medications such as statins and fibrates were included. Subjects were instructed to stop medications 1

week before the testing day to allow a wash-out period before testing. A one-week period was determined as adequate due to the relatively short half-life of most statin medications, with the longest half-life being estimated at up to 30 hours for Atorvastatin (*Bellosta et al. 2004*), and fenofibrate (the only fibric acid medication used by patients included in the study) having a half-life of 20 hours (*Najib 2002*).

Healthy subjects (Control; n=9) were recruited by advertisements at the University of Alberta and diabetes clinics in Edmonton as a control group. Control subjects were not taking lipid-lowering or other medications, were non-smokers and normotensive, had fasting plasma LDL-c <3.4 mmol/L, and HDL-c >0.90 mmol/L, and did not have diagnosis or family history or premature CVD, diabetes, or other metabolic disorders. This study was approved by the Health Research Ethics Board of the University of Alberta.

For details regarding methods and procedures please refer to Chapter 3: Methods.

6.2. Results

6.2.1. Subject characteristics

All ITx patients and 6 of 7 LTx patients were taking Tacrolimus immunosuppression; the remaining LTx patient was taking Sirolimus. All ITx patients and 4 of 7 LTx patients were also taking mycophenolate mofetil. All LTx patients had received only one transplant, where ITx patients had received 2.0 ± 0.38 transplants (median: 2.0; range: 1-4). LTx patients were tested at 16.86 ± 2.8 months after transplant (median: 21; range: 4-22) and ITx patients were tested at 30.13 ± 8.8 months after transplant (median: 26.5; range: 2-78), due to the extended transplant date criteria allowed for the ITx subjects. No significant difference was found between groups in number of months since transplant.

Almost all ITx patients (7 of 8) were taking statin therapy, compared

to none of the LTx patients. Two of the LTx patients were taking fibrate therapy. Most of the ITx (63%) and LTx (57%) patients were taking at least one anti-hypertensive medication. One of the ITx patients was taking hypothyroid therapy, and two were taking anti-diabetic medication (one taking metformin and one taking a DPP-IV inhibitor) as part of other islet transplant-related investigative protocols. There were no significant differences between transplant groups in Tacrolimus dose (ITx patients 6.56 ± 1.5 mg/day and LTx patients 4.50 ± 0.89 mg/day), but fasting Tacrolimus blood level was greater in ITx patients (9.41 ± 0.60 μg/L) compared to LTx patients (6.53 ± 0.63 μg/L). Fasting blood Tacrolimus level was not correlated with Tacrolimus dosage, weight, BMI, or percent body fat in ITx or LTx patients.

LTx subjects exhibited higher BMI and body fat than the Control and ITx groups, but groups were similar in age and other anthropometric indices (**Table 6-1**). ITx patients exhibited lower fasting TC and LDL-c levels compared to Control and LTx, whereas LTx patients exhibited higher TG and lower HDL-c fasting levels as well as higher fasting insulin levels compared to Control and ITx (**Table 6-2**). No subjects in the ITx group exhibited hyperlipidemia, while 2 of the control subjects and 4 of the LTx subjects were hyperlipidemic (defined as TC>5.2 or TG>1.7 mmol/L) (*Antonopoulos 2002*). Inflammatory markers were higher in the LTx subjects compared to Control and ITx (**Table 6-2**). Intake of dietary total and saturated fat was higher in ITx and LTx compared to Control subjects, but there were no other differences between groups in background dietary intake (**Table 6-3**).

Table 6-1: Anthropometric characteristics of control, islet, and liver transplant subjects.

Measurement	**Control (n=9)**	**ITx (n=8)**	**LTx (n=7)**	**p-value**
Males/Females	3/6	5/3	5/2	n/a
Age (years)	54 ± 4	56 ± 2	56 ± 2	0.99
BMI (kg/m^2)	25 ± 0.8 ***ab***	22 ± 0.8 ***a***	30 ± 3 ***b***	0.01
WC (cm)	87 ± 4	82 ± 2	106 ± 10	0.052
WC (cm) - Males	94 ± 1 ***ab***	85 ± 3 ***a***	114 ± 9 ***b***	<0.05
WC (cm) - Females	83 ± 6	79 ± 3	85 ± 25	>0.05
Body fat (%)	33 ± 3 ***a***	17 ± 5 ***b***	34 ± 5 ***ab***	0.048
Body fat (%) - Males	26 ± 4 ***ab***	11 ± 4 ***a***	31 ± 6 ***b***	0.04
Body fat (%) - Females	36 ± 3	30 ± 6	41 ± 8	>0.05
RMR (kcal/day)	1366 ± 58 ***ab***	1239 ± 54 ***a***	1711 ± 177 ***b***	0.01
RMR (kcal/day) - Males	1383 ± 109 ***ab***	1328 ± 33 ***a***	1845 ± 245 ***b***	<0.05
RMR (kcal/day) - Females	1358 ± 76	1148 ± 71	1442 ± 27	>0.05
Systolic blood pressure (mmHg)	119 ± 7	123 ± 4	119 ± 6	0.88
Diastolic blood pressure (mmHg)	74 ± 6	72 ± 3	84 ± 5	0.15

Data expressed as mean ± SEM; different letters indicate significant difference p<0.05

Table 6-2: Fasting blood lipid, glucose, insulin, and cytokine levels of control, islet, and liver transplant subjects.

Fasting concentration	**Control (n=9)**	**ITx (n=8)**	**LTx (n=7)**	**p-value**
Total cholesterol (mmol/L)	5.0 ± 0.1 ***a***	3.8 ± 0.2 ***b***	4.5 ± 0.6 ***ab***	0.008
LDL-c (mmol/L)	3.1 ± 0.2 ***a***	2.2 ± 0.1 ***b***	2.5 ± 0.5 ***ab***	0.03
HDL-c (mmol/L)	1.5 ± 0.1 ***a***	1.4 ± 0.1 ***a***	0.9 ± 0.1 ***b***	0.002
TG (mmol/L)	0.80 ± 0.1 ***a***	0.75 ± 0.1 ***a***	2.4 ± 0.5 ***b***	0.003
Glucose (mmol/L)	5.0 ± 0.1	5.5 ± 0.3	5.1 ± 0.2	0.36
Insulin (mU/L)	5.7 ± 2 ***a***	12 ± 4 ***ab***	17 ± 7 ***b***	0.047
HOMA	1.3 ± 0.4	2.9 ± 1	3.7 ± 1	0.055
TNF-a (pg/mL)	0.70 ± 0.2 ***ab***	0.67 ± 0.08 ***a***	0.43 ± 0.05 ***b***	0.056
CRP (mg/L)	1.9 ± 0.7 ***ab***	0.5 ± 0.3 ***a***	3.0 ± 1.2 ***b***	0.03
Fibrinogen (ng/mL)	1264 ± 166	2130 ± 471	2875 ± 1916	0.57
IL-6 (pg/mL)	3.4 ± 2 ***ab***	1.3 ± 0.2 ***a***	4.3 ± 1 ***b***	0.03
IL-10 (pg/mL)	0.31 ± 0.08	0.25 ± 0.06	0.67 ± 0.4	0.77

Data expressed as mean ± SEM; different letters indicate significant difference $p<0.05$

Table 6-3: Background dietary intake of control, islet, and liver transplant subjects.

Background Dietary Intake	Control (n=9)	ITx (n=8)	LTx (n=7)	p-value
Total kcal	1607 ± 126	1673 ± 143	1917 ± 254	0.61
Total kcal - Males	1911 ± 330	1751 ± 192	2182 ± 267	>0.05
Total kcal - Females	1455 ± 52	1542 ± 232	1253 ± 148	>0.05
CHO (%)	55 ± 3	46 ± 2	50 ± 2	0.096
Protein (%)	19 ± 1	21 ± 2	19 ± 1	0.59
Fat (%)	24 ± 2 ***a***	35 ± 2 ***b***	33 ± 2 ***ab***	0.005
SFA(%)	7.6 ± 0.8 ***a***	12 ± 0.9 ***b***	12 ± 1 ***ab***	0.02
MUFA (%)	7.6 ± 0.9	8.3 ± 1.1	8.9 ± 0.6	0.29
PUFA (%)	4.4 ± 0.6	4.6 ± 0.7	3.8 ± 0.4	0.77
Cholesterol (mg)	201 ± 44	285 ± 59	368 ± 58	0.077
Fiber (g)	22 ± 4	21 ± 3	18 ± 2	0.67

Data expressed as mean ± SEM; different letters indicate significant difference $p<0.05$

6.2.2. Fatty acid composition

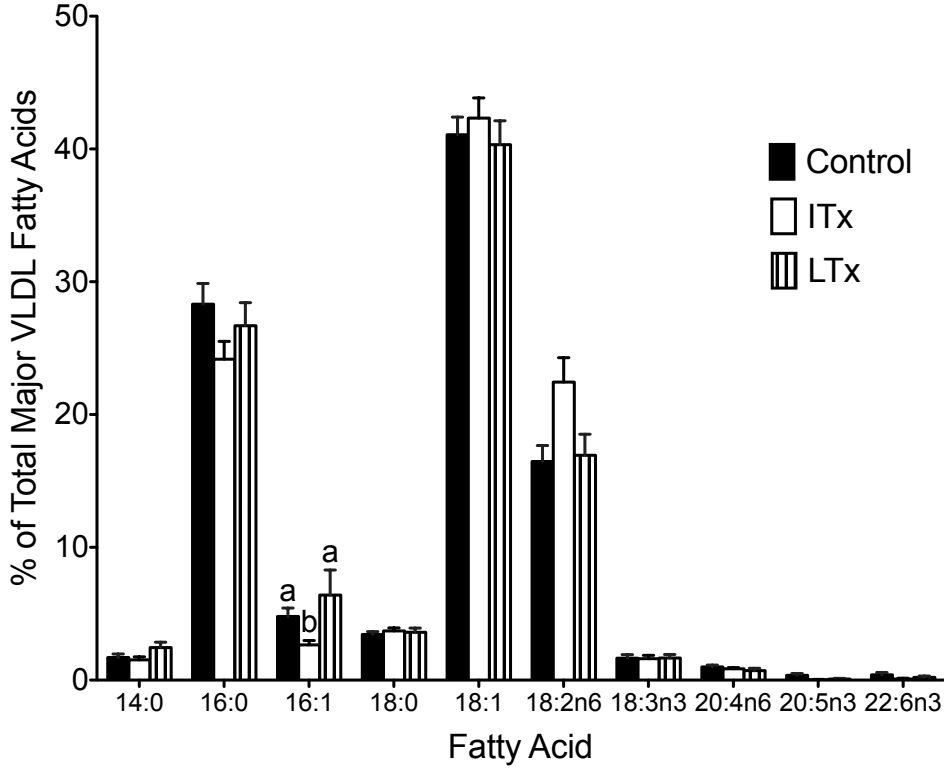
Percent contribution of individual FA to total fasting plasma-TG FA composition was not different between groups (data not shown), except for 14:0 (Control 2.0 ± 0.3%; ITx 1.5 ± 0.2%; LTx 3.0 ± 0.4 %), 16:1 (Control 3.4 ± 0.5%; ITx 1.2 ± 0.6%; LTx 3.0 ± 0.3%), and 20:5n3 (Control 1.2 ± 0.9%; ITx 0 ± 0%; LTx 0.04 ± 0.03%).

Fasting total VLDL-TG FA was significantly higher in LTx compared to Control and ITx (LTx 425 ± 111 μg/mL, Control 151 ± 34 μg/mL, and ITx 163 ± 56 μg/mL, respectively). Contribution of individual FA to fasting VLDL-TG FA levels were not significantly different between groups except for 16:1 which was higher in Control and LTx compared to ITx (**Figure 6-1**).

Fasting total TPL-FA concentration was not significantly different

between groups (Control 732 ± 101 μg/mL; ITx 546 ± 99 μg/mL; LTx 637 ± 48 μg/mL). Individual FA contribution to fasting TPL-FA composition did not differ between groups (data not shown), except for 18:2n6 which was higher in ITx compared to Control but not LTx (25.4 ± 1.0%, 20.9 ± 1.1%, and 28.8 ± 5.4 %, respectively).

Figure 6-1: Fasting VLDL-TG fatty acid composition in control, islet, and liver transplant subjects.



Data expressed as mean ± SEM; different letters indicate significant difference $p<0.05$

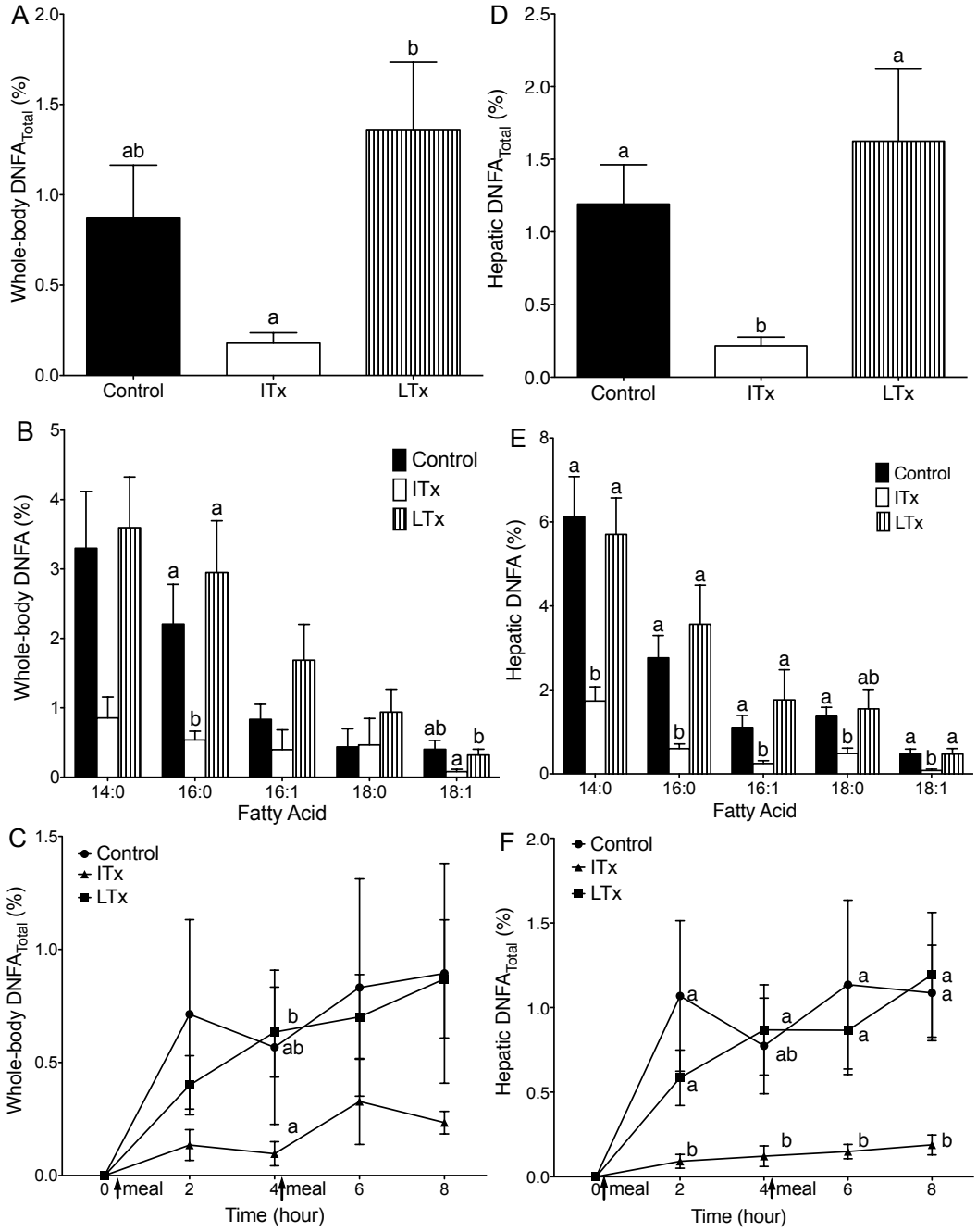
6.2.3. Fatty acid synthesis

Fasting whole-body DNFA was significantly higher in LTx than ITx subjects, whereas Control were similar to the transplant groups (**Figure 6-2A**). Fasting whole-body synthesis ranged from 0.0-2.70 % in Control, 0.0-0.39% in ITx, and 0.59 -3.22% in LTx. Fasting whole-body synthesis of individual FA were not different between groups except for 16:0 and 18:1

(**Figure 6-2B**). Postprandial whole-body total DNFA was not different between groups at any timepoint except at 4 h (**Figure 6-2C**). Whole-body DNFA was significantly correlated with trunk (*r=0.79*) and gynoid (*r=0.96*) body fat in the Control group. Fasting total whole-body DNFA was not significantly correlated with dietary intake of carbohydrates or fat in any group except for in ITx subjects in which whole-body lipogenesis was inversely related to carbohydrate intake (*r=-0.78*). Whole-body synthesis of 16:0 was positively correlated to total whole-body lipogenesis in Control (*r=0.98*), ITx (*r=0.78*), and LTx (*r=0.89*) subjects. Total whole-body lipogenesis was not significantly correlated with Tacrolimus dose or fasting blood level in either transplant group.

Fasting hepatic total DNFA was significantly higher in Control and LTx subjects compared to ITx (**Figure 6-2D**). Fasting hepatic lipogenesis ranged from 0.51-2.83% in Control, 0.01-0.57% in ITx, and 0.57-3.87% in LTx. A similar pattern was found for fasting synthesis rates of all individual FA except for 18:0, which was higher in Control compared to ITx, but similar between ITx and LTx (**Figure 6-2E**). Postprandial hepatic synthesis of total FA was significantly higher in Control and LTx subjects compared to ITx at all timepoints, except for 4 h in which LTx was greater than ITx and Control (**Figure 6-2F**). Hepatic DNFA was correlated with trunk (*r=0.89*), android (*r=0.82*), and gynoid (*r=0.96*) body fat as well as with dietary fiber intake (r=-0.77) in Control subjects. Hepatic synthesis of 16:0 was highly correlated with total hepatic lipogenesis in Control (*r=0.97*) and LTx (*r=0.96*) subjects, but not in ITx subjects. Total hepatic lipogenesis was not significantly correlated with dietary intake of carbohydrates or fat in any group, or with Tacrolimus dose or fasting blood level in either transplant group. Whole-body and hepatic lipogenesis were significantly correlated in control subjects (*r=0.95*), but not in the transplant groups.

Figure 6-2: Whole-body and hepatic de novo lipogenesis in control, islet, and liver transplant subjects. A) Fasting whole-body total DNFA; B) Fasting whole-body individual DNFA; C) Postprandial whole-body total DNFA; D) Fasting hepatic total DNFA; E) Fasting hepatic individual DNFA; F) Postprandial hepatic total DNFA.

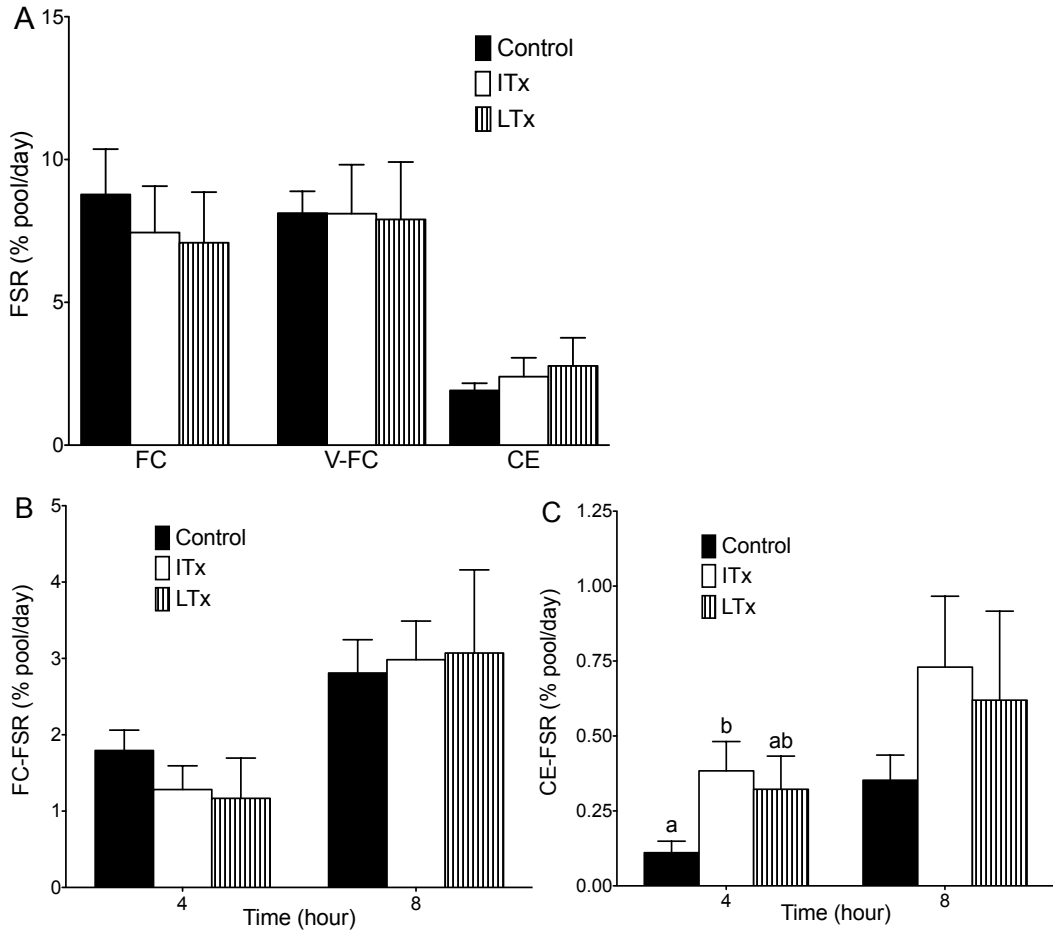


Data expressed as mean ± SEM; different letters indicate significant difference $p<0.05$

6.2.4. Cholesterol synthesis

Fasting whole-body and hepatic synthesis of FC and whole-body synthesis of CE were not significantly different between groups (**Figure 6-3A**). Whole-body FC-FSR ranged from 3.5-20.2% in Control, 1.6-17.2% in ITx, and 2.7-16.6% in LTx. Hepatic FC-FSR ranged from 3.72-11.5% in Control, 2.27-18.1% in ITx, and 2.98-18.6% in LTx subjects. CE-FSR ranged from 0.9-2.9% in Control, 0.5-6.6% in ITx, and 1.2-8.2% in LTx. Postprandial synthesis of FC was not significantly different between groups at any timepoint (**Figure 6-3B**), whereas postprandial synthesis of CE was significantly different between Control and ITx subjects at 4 h (**Figure 6-3C**). CE-FSR was less than FC-FSR at all timepoints, and CE-FSR and FC-FSR were significantly correlated in all groups (Control *r=0.75*; ITx *r=0.93*; and LTx *r=0.96*). Hepatic FC synthesis was correlated to whole-body total FC synthesis in ITx (*r=0.98*) and LTx (*r=1.00*) subjects but not Controls. Synthesis of CE was also significantly correlated to hepatic and whole-body lipogenesis in Controls (*r=0.83* and *r=0.77*, respectively), but not in transplant groups. Cholesterol synthesis was not significantly correlated with dietary intake of carbohydrates, fat, or cholesterol in any group, except for ITx subjects in which FC-FSR and V-FC-FSR were inversely correlated with dietary carbohydrate intake (*r=-0.76* and *r=-0.79*, respectively). Cholesterol synthesis was not correlated with Tacrolimus dose or fasting blood level in either transplant group.

Figure 6-3: Whole-body and hepatic synthesis of free cholesterol (FC and V-FC), and cholesteryl ester (CE) of control, islet, and liver transplant subjects. A) Fasting whole-body and hepatic synthesis of FC, V-FC, and CE; B) Post-prandial whole-body synthesis of FC; C) Postprandial whole-body synthesis of CE.



Data expressed as mean ± SEM; different letters indicate significant difference $p<0.05$

6.2.5. Pooling of Transplant Groups and Division based on BMI

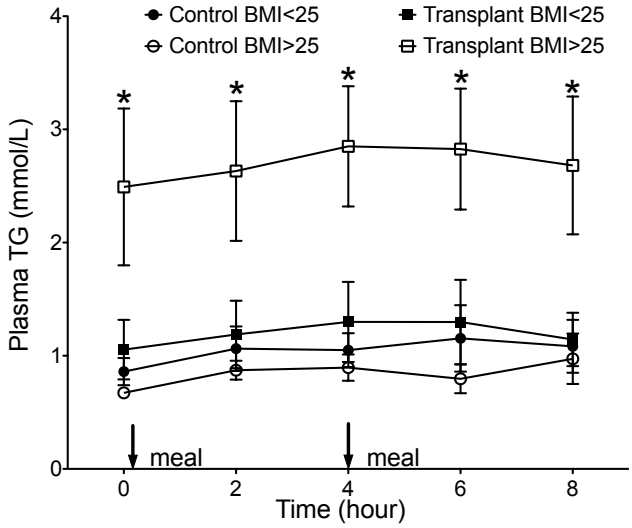
There may be differences in lipogenesis and cholesterol synthesis between normal weight and overweight individuals. Overweight individuals are more likely to have greater metabolic aberrations such as insulin resistance and dyslipidemia. Both excess adiposity and insulin resistance have been previously shown to be associated with greater rates of lipogenesis and cholesterol synthesis (*Guo et al. 2000*; *Diraison et al. 2002*), and weight

loss is associated with reduction in cholesterol synthesis (*Di Buono et al. 1999*; *Santosa et al. 2007*). Therefore differences in lipid synthetic rates between groups may be complicated by presence of varying adiposities. In the present study, whole-body lipogenesis was correlated with fasting plasma TG levels in ITx (*r=0.87*) and LTx (*r=0.86*) groups, and negatively correlated with fasting plasma HDL-c levels in the LTx group (*r=-0.96*).

It was not possible to match individual transplant subjects with controls due to limited numbers of subjects. Therefore ITx and LTx patients were pooled into normal weight (defined as BMI<25 kg/m^2; n=10) or higher weight (defined as BMI>25 kg/m^2; n=5) groups and compared to control subjects of similar weight status (BMI<25 kg/m^2, n=4; BMI>25 kg/m^2, n=4). One control subject was omitted from these comparisons due to much younger age than the rest of the subjects (age 28 compared to age 48-68 for the rest of the group). Groups in the same weight category were similar in levels of BMI, age, and glucose. Plasma TC and LDL-c were significantly lower in the BMI<25 transplant group compared to the similar control group (TC 3.9 ± 0.3 mmol/L vs. 5.0 ± 0.1 mmol/L, respectively; LDL-c 2.2 ± 0.3 mmol/L vs. 3.1 ± 0.1 mmol/L, respectively). Plasma TG was significantly higher (2.5 ± 0.7 mmol/L vs. 0.67 ± 0.03 mmol/L, respectively) and HDL-c significantly lower (0.88 ± 0.2 mmol/L vs. 1.6 ± 0.03, respectively) in the BMI>25 transplant group compared to the similar control group. Fasting insulin levels were also significantly higher in the BMI>25 transplant group compared to the similar control group (25 ± 10 mU/L vs. 5.3 ± 1 mU/L, respectively). Plasma TG levels were higher at every timepoint during postprandial measurements in the BMI>25 transplant group compared to the BMI<25 transplant group and both control groups (**Figure 6-4**). In addition, incremental area under the curve for changes in plasma TG during the postprandial timepoints were significantly higher at 4, 6, and 8 hours in the heavier weight transplant group compared to the rest of the groups, possibly

indicating impairment in postprandial lipoprotein clearance.

Figure 6-4: Postprandial plasma TG levels in control and pooled transplant subjects divided by BMI.



Data expressed as mean ± SEM; * indicates significant difference between BMI>25 transplant group and all other groups $p<0.05$

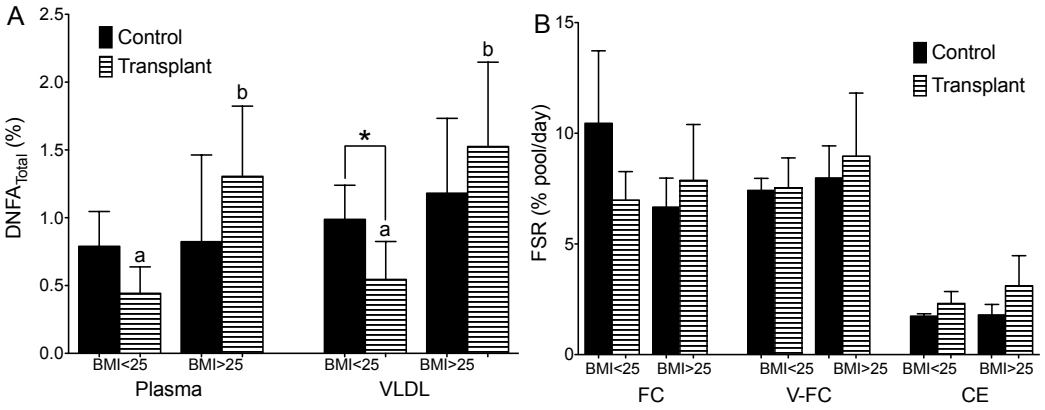
Fasting whole-body and hepatic total DNFA were lower in BMI<25 transplant subjects compared to the similar control group, but only hepatic DNFA was statistically significantly lower (**Figure 6-5A**). Conversely, fasting whole-body and hepatic DNFA were higher in the BMI>25 transplant group compared to the control group, but this was not statistically significant. Within the transplant groups, fasting whole-body and hepatic DNFA were both significantly higher in the BMI>25 transplant group compared to the BMI<25 transplant group (**Figure 6-5A**). One of the transplant patients with BMI<25 had high fasting insulin, plasma TC and TG levels which were more similar to the BMI>25 transplant subjects. When this subject was moved into the BMI>25 category, fasting whole-body DNFA was 67% lower in the BMI<25 transplant group compared to control group (0.26 ± 0.08% vs. 0.79 ± 0.3%, respectively; NS), whereas fasting hepatic DNFA was 73% lower in the BMI<25 transplant group compared to controls (0.27 ± 0.08% vs. 0.99 ± 0.3%, respectively; $p<0.05$). When the BMI>25 groups were compared, there

were no significant differences between control and transplant groups in whole-body DNFA (0.82 ± 0.6% vs. 1.4 ± 0.4%, respectively) and hepatic DNFA (1.2 ± 0.6% vs. 1.8 ± 0.6%, respectively).

In the pooled transplant subjects fasting whole-body DNFA was significantly correlated with fasting plasma TG (*r=0.94*) and HDL-c (*r=-0.74*) levels, weight (*r=0.75*), BMI (*r=0.71*), and waist circumference (*r=0.68*). Fasting hepatic DNFA was significant correlated with fasting plasma TG levels (*r=0.80*), weight (*r=0.76*), BMI (*r=0.73*), waist circumference (*r=0.63*), and blood Tacrolimus level (*r=-0.62*).

Fasting whole-body FC-FSR tended to be lower in the BMI<25 transplant subjects compared to controls (**Figure 6-5B**; NS). Conversely, FC-FSR was not significantly different in the BMI>25 transplant group compared to the similar control group. Fasting hepatic FC-FSR and whole-body CE-FSR were not significantly different between groups in either BMI category. Moving the hyperinsulinemic transplant subject from the BMI<25 to the BMI>25 group did not have an effect on differences in synthesis of FC or CE between groups. In the pooled transplant subjects fasting whole-body and hepatic FC-FSR were significantly correlated with fasting plasma LDL-c (*r=-0.51* and *r=-0.55*, respectively) and with each other (*r=0.99*).

Figure 6-5: Fasting fatty acid and cholesterol synthesis in control and pooled transplant subjects divided by BMI. A) Whole-body and hepatic total fatty acid synthesis; B) Whole-body and hepatic synthesis of free cholesterol (FC and V-FC) and cholesteryl ester (CE).



Data expressed as mean ± SEM; different letters indicate significant difference $p<0.05$

6.3. Discussion and Conclusions

Results from the present investigation suggest that lipid synthesis may be suppressed by up to 50% by immunosuppressive medications following transplantation. Transplant subjects with BMI and plasma lipid levels similar to control subjects had significantly reduced lipogenesis, while transplant subjects with elevated BMI and plasma lipid levels had similar rates of lipogenesis to the control subjects. The present data suggests that FA and cholesterol synthesis are not elevated in transplant subjects and are unlikely to contribute to post-transplant hyperlipidemia induced by Tacrolimus immunosuppression.

DNL has been regarded to make a small contribution towards overall plasma lipid levels. For example, Parks et al (2008) found a fasting hepatic DNL of 5-7% in healthy males, with individual rates ranging from 0-15.3% (*Parks et al. 2008*). Barrows et al (2006) reported an average hepatic DNL rate of 4%, whereas Vogt et al (2006) reported 10-12%, and Jones et al (1995) reported 6-8% (*Jones et al. 1995*; *Barrows et al. 2006*; *Vogt et al. 2006*). In contrast, control subjects in the present study exhibited lower fasting

hepatic lipogenic rates ranging from 0.51-2.83%. However, Marques-Lopez et al (2001) also reported low rates of hepatic DNL in lean men of 1-2%, Guo et al (2000) reported lipogenesis of 1.8 ± 0.4% in lean women measured from plasma-TG, and Siler et al (1999) reported 2.1 ± 1% in normal males measured from VLDL-TG palmitate (*Siler et al. 1999*; *Guo et al. 2000*; *Marques-Lopes et al. 2001*). Values obtained from measurements of cholesterol synthesis appear to agree with previously reported values from the literature. Control subjects in the present study exhibited FC-FSR rates ranging from 3.5-20.2%, with the majority of subjects within the range of 5-10%. This is similar to Diraison et al (2002) who reported 3.3 ± 0.5% in lean subjects, Di Buono et al (2000) who reported 7.8 ± 2.5% in normal subjects, and Jones et al (1992) who reported 7.5 ± 0.5% in normal subjects (*Jones et al. 1992*; *Di Buono et al. 2000*; *Diraison et al. 2002*).

Despite the different nature of the two transplant groups investigated in the current study, similar results were found for each. When taken as a group, the LTx patients would appear to have similar and the ITx patients lower lipogenic rates compared to the control subjects (**Figures 6-2A and 6-2D**). The LTx group was complicated by the presence of two groups differing in metabolic characteristics; half of the group had normal weight, plasma lipids and insulin, whereas the other half of the group had elevated weight, lipids, and insulin levels. This is representative of the broader liver transplant population, in which upwards of half of patients post-transplant experience weight gain and increase in plasma lipid levels (*Sheiner et al. 2000*; *Adam et al. 2009*; *Pagadala et al. 2009*; *Watt et al. 2010*), as well as disruptions in glucose metabolism that can lead to impaired glucose tolerance and diabetes (*Heisel et al. 2004*; *Tietge et al. 2004*; *Kuo et al. 2010*). When these two types of patients are considered independently, the normal weight transplant patients appear to have reduced rates of lipogenesis relative to comparable controls (**Figure 6-5A**).

FC-FSR rates for overweight and obese individuals have been reported at 5-14% (*Di Buono et al. 1999*; *Guo et al. 2000*; *Santosa et al. 2007*), which are not drastically different from those reported in healthy subjects. However, DNFA has been reported to be as high as 30% in individuals with obesity, type 2 diabetes or non-alcoholic fatty liver disease (*Hudgins et al. 2000*; *Diraison et al. 2003*; *Wilke et al. 2009*). In comparison, rates of hepatic DNFA in the present study ranged from 0.01-3.87% in the transplant groups, with a range of 0.57-3.87% in the transplant patients who were overweight or obese. In a study of individuals with type 2 diabetes and matched control subjects, Wilke et al (2009) found lipogenesis rates of 0-33% per day (*Wilke et al. 2009*). The control subjects in the investigation by Wilke et al (2009) did not have diabetes, but were matched with subjects with type 2 diabetes and thus had elevated weight and likely impaired glucose tolerance. Comparing subjects in the study by Wilke et al (2009) to the transplant subjects in the present investigation with similar metabolic phenotypes (elevated weight, insulin, and plasma TG levels), it could be reasonably predicted that these transplant subjects would have a much greater lipogenic rate than was observed. Interestingly, fasting lipogenesis and cholesterol synthesis were not correlated with fasting plasma insulin levels in any group; in the control group this could be due to the low range of fasting insulin levels, however, other reports have not shown a significant correlation of either cholesterol synthesis or lipogenesis with insulin (*Jones et al. 1995*; *Cachefo et al. 2003*). Given the significantly lower rate of lipogenesis found in the normal weight transplant subjects compared to the controls, it could be proposed that Tacrolimus exerts a suppressive effect on lipogenesis. Furthermore, it was found that when the transplant groups were pooled together, hepatic lipogenesis was inversely correlated with plasma Tacrolimus level ($r=-0.62$).

The transplant patients in the current study were receiving Tacrolimus immunosuppression in conjunction with mycophenolate mofetil,

except for one LTx patient who received Sirolimus and mycophenolate mofetil. This patient was included in all analyses because measured levels of whole-body and hepatic DNFA as well as cholesterol synthesis were similar to other patients included in the study, and removing this data from the analyses did not change statistical findings. This individual may be considered a case study on effects of Sirolimus on lipid synthesis; however lipogenesis and cholesterol synthesis were comparable between this individual and the rest of the group which suggests Sirolimus may also have suppressive effects on lipogenesis.

This is the first investigation to the authors' knowledge on the effect of Tacrolimus on lipogenic and cholesterol synthetic rates in transplant patients. A previous study on lipogenesis in transplant patients was performed by Minehira et al (2001) in which the authors concluded that lipogenesis in transplant patients was not significantly different from non-transplant patients (*Minehira et al. 2001*). Prednisone was used by half the transplant patients in the analysis by Minehira et al (2001), which may exert independent effects on lipid metabolism (*Kobashigawa et al. 1997*). The goal of many transplant centers currently is to taper steroid therapy within the first few months post-transplant (*Adam et al. 2009*), meaning that long-term lipid abnormalities will not be due to steroid therapy but rather to the remaining immunosuppressive cocktail. In addition the primary immunosuppressive medication in the study by Minehira et al (2001) was Cyclosporine. Cyclosporine is a calcineurin inhibitor like Tacrolimus, but the two may have different effects on metabolism (*Brown et al. 2007*). For example, Cyclosporine interferes with bile acid metabolism, whereas this effect has not been reported for Tacrolimus (*McCashland et al. 1994*). Cyclosporine has been associated with increased FA and cholesterol synthesis (*Ballantyne et al. 1989*; *Gueguen et al. 2007*; *Brown et al. 2007*), whereas the present study suggests Tacrolimus may be associated with

reduced lipogenesis. Administration of Tacrolimus in male rats showed elevated hepatic cholesterol synthesis (*Rosenblum et al. 1990*), however this study has not been replicated. In animal and cellular models, cyclosporine has also been shown to reduce cholesterol absorption, impair esterification of cholesterol, and suppress LDL-receptor and lipoprotein lipase activity (*Dias et al. 1994*; *Winegar et al. 1996*; *Kockx et al. 2010*).

Suppression of lipogenesis could be due to a direct effect by Tacrolimus on genes regulating lipogenesis, such as SREBP-1c, fatty acid synthase and acetyl CoA carboxylase. Sirolimus has been suggested to reduce expression of SREBP-1-related genes such as acetyl CoA carboxylate, fatty acid synthase, and stearoyl-CoA desaturase-1 in cell culture models, thereby impairing de novo lipogenesis (*Brown et al. 2007*; *Porstmann et al. 2008*; *Düvel et al. 2010*). Inhibition of lipogenic gene expression by Sirolimus may dominate even in the presence of insulin, which is normally a strong inducer of SREBP-1c expression (*Porstmann et al. 2008*; *Li et al. 2010*). Sirolimus may inhibit cholesterol synthesis through reduced expression of SREBP-2 and HMGCoA-reductase, however it has also been shown to upregulate HMGCoA-reductase gene expression concomitant with impairment of bile acid synthesis (*Gueguen et al. 2007*; *Ma et al. 2010*; *Düvel et al. 2010*). These effects on transcription mediators have not been investigated for Tacrolimus.

Alternatively, lipogenesis suppression could be a compensatory mechanism for other metabolic perturbations induced by immunosuppression, such as an elevation in circulating plasma lipids resulting from increased lipoprotein production, reduced clearance, or disturbances in insulin regulation. Tacrolimus has been shown to reduce lipoprotein lipase activity (*Tory et al. 2008*; *Tory et al. 2009*). Reduction in lipoprotein lipase activity may impair clearance of lipoprotein particles and contribute to increased plasma lipid levels. In the present study, postprandial plasma TG levels and change from baseline were significantly higher in the

BMI>25 transplant subjects compared to the BMI<25 transplant and control subjects (**Figure 6-4**). This elevation in postprandial plasma TG levels could indicate impairment in postprandial lipoprotein clearance. Renal transplant patients treated with Tacrolimus have been shown to have elevated levels of ApoB (*Tur et al. 2000*; *Ichimaru et al. 2001*). It is unknown whether the elevated ApoB-containing lipoprotein particles observed with Tacrolimus is due to impaired clearance by LDL-receptors or impaired lipoprotein lipase activity as has been shown in patients receiving Cyclosporine (*Superko et al. 1990*; *Rayyes et al. 1996*; *Ruiu et al. 2005*; *Subramanian et al. 2007*). Greater cholesterol content in VLDL particles has also been observed in renal transplant patients receiving Tacrolimus (*Ichimaru et al. 2001*). Enhanced cholesterol or fatty acid delivery to the liver, in turn, could induce a compensatory reduction in hepatic cholesterol or fatty acid synthesis in order to preserve hepatic stores at homeostasis.

Whole-body lipogenesis was inversely related to carbohydrate intake in ITx subjects ($r=-0.78$), which is interesting because previous work has shown that carbohydrate feeding tends to increase lipogenesis (*Schwarz et al. 2003*). Conversely, feeding dietary fat tends to reduce lipogenesis (*Schwarz et al. 2003*). It is unlikely that the higher habitual fat intake of both the transplant groups was the sole reason for the reduced lipogenesis found, because rates of lipogenesis were not related to total dietary fat intake in any of the subject groups. In addition, the amount of fat fed in the meals on testing day (35%) was similar to the habitual fat intakes of the transplant groups (35% in the ITx group and 33% in the LTx group; **Table 6-3**), compared to the control group (24%). If anything, the testing day meals would be expected to have induced a suppression in lipogenesis in the control subjects due to the higher fat content; this would not be expected to occur in the transplant groups. Furthermore, Schwarz et al (2003) found that obese hyperinsulinemic subjects actually have higher rates of lipogenesis in

response to high-fat diet compared to lean and obese normoinsulinemic subjects (*Schwarz et al. 2003*). Therefore, the low lipogenesis noted in the transplant groups is likely to be a real effect of immunosuppressive medication and not an artifact of dietary manipulation. Dietary cholesterol content was also greater in the testing day meals compared to habitual consumption by the control and ITx subjects, though the difference was less for the LTx subjects. Rates of cholesterol synthesis were not correlated to habitual dietary cholesterol intakes for any of the groups, and were similar between the ITx and LTx subjects, suggesting that dietary cholesterol changes are unlikely to be responsible for the rates of cholesterol synthesis observed in the present study.

A limitation of the present study was that a larger number of transplant patients with hyperlipidemia were not recruited. However, approximately half the LTx patients in the present study had plasma total cholesterol levels >5.2 mmol/L and/or plasma TG levels >1.7 mmol/L. This is representative of the broader liver transplant population in which ~50% of liver transplant patients experience hyperlipidemia 6 months post-transplant, with hypercholesterolemia occurring in ~15-65% and hypertriglyceridemia occurring in ~30-70% of LTx patients (*Gisbert et al. 1997*; *Sheiner et al. 2000*; *Guckelberger et al. 2005*; *Laryea et al. 2007*; *Bianchi et al. 2008*; *Benten et al. 2009*; *Adam et al. 2009*). In contrast, none of the ITx patients recruited had elevated cholesterol or TG, yet almost all were treated with statin therapy. In the present study participants were asked to refrain from statin medication for a week's time to allow for washout of the effects of the drug. Therefore most of the ITx patients had normal plasma cholesterol levels in absence of the statin medication. Cholesterol synthesis was not found to be increased in these individuals and was comparable to healthy non-transplanted control subjects. If cholesterol synthesis is not elevated in these patients perhaps a different pharmaceutical agent should be utilized in

these individuals for treatment or prophylaxis of hyperlipidemia.

In conclusion, this study indicates that Tacrolimus-based immunosuppression may be associated with reduced lipid synthesis. Therefore lipid synthesis is not likely to contribute as a mechanism for the hyperlipidemia induced by this medication post-transplant. Alterations in lipid absorption and lipoprotein production or clearance may instead be driving forces leading to elevations in plasma lipid levels, and should be the targets for dietary or pharmaceutical treatments.

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7. Effect of dietary intervention on plasma lipids and lipid synthesis

Organ transplantation is a life-saving procedure, but the trade-off is lifelong immunosuppressive medication to prevent graft loss. Relative risk of cardiovascular disease in transplanted patients has been estimated to be as high as 3, and risk of death is 2.5 compared to matched subjects (*Johnston et al. 2002*). Hypertension and hyperlipidemia are associated with vascular events in the general population as well as in transplant patients (*Laryea et al. 2007*; *Borg et al. 2008*; *Pfitzmann et al. 2008*). Immunosuppressive medications potently prevent graft rejection, but are relatively non-specific in nature; therefore, they are associated with a variety of side-effects, including weight gain, diabetes, and hyperlipidemia. As such, post-transplant hyperlipidemia is a common occurrence across a variety of organ transplant groups (*Moore et al. 2001*).

Reports indicate that up to half of islet and liver transplant (ITx and LTx, respectively) patients require lipid-lowering medications to treat hyperlipidemia post-transplant (*Ryan et al. 2001*; *Adam et al. 2009*). Changes occurring post-transplant due to immunosuppressive medication, such as weight gain and derangements in insulin metabolism leading to hyperinsulinemia and insulin resistance, may also exacerbate alterations in lipid metabolism leading to hyperlipidemia (*Adam et al. 2009*). Elevations in plasma lipid levels could arise due to increases in lipid absorption or synthesis, reduction in plasma clearance, or a combination of these mechanisms.

Pharmacological agents can be used to treat hyperlipidemia in transplant patients, but there are concerns of drug interactions due to similar degradation and metabolic pathways between these drugs and immunosuppressive medications (*Moore et al. 2001*; *Zimmerman 2004*;

Koshman et al. 2005; *Bergman et al. 2006*). In addition, the mechanisms contributing to the increase in plasma lipids with immunosuppressive therapy are not yet elucidated, therefore there is the potential that other lipid-related mechanisms could be targeted for more effective treatment. Dietary therapy is considered the safest option for treating elevated lipid levels and should be the first line of treatment (*Kobashigawa et al. 1997*; *Kasiske et al. 2004*; *Wenke 2004*). Dietary therapy in post-transplant patients has been met with mixed results. Some interventions based on whole-diet approaches have shown reduction in plasma TC, LDL-c, and TG levels (*Guida et al. 2007*; *Guida et al. 2009*). While dietary interventions may lower plasma lipid levels, the level achieved may not be enough for most patients to reach target lipid levels (*Hines 2000*). Single nutrient interventions have met with some success. For example, fish oil has been found to effectively lower plasma TG levels by ≥20% in renal transplant patients (*Tatsioni et al. 2005*). Phytosterols in combination with statin therapy effectively lowered plasma TC levels in renal and heart transplant patients 10-20% (*Vorlat et al. 2003*; *Sutton et al. 2009*). Dietary components have the potential to target multiple mechanisms when used in conjunction, and may be more effective when used in combination as opposed to single-nutrient interventions. Jenkins and colleagues have shown that a dietary "portfolio" can be used to effectively lower plasma lipid levels in at-risk hyperlipidemic individuals and is comparable to statin therapy (*Jenkins et al. 2005*; *Jenkins et al. 2006*).

Most of the dietary investigations in transplant patients have been primarily in patients taking Cyclosporine and steroid therapy. Corticosteroids are particularly hyperlipidemic and may reduce effectiveness of dietary interventions (*McPartland et al. 2007*). Present immunosuppressive protocols favour cessation of steroid use shortly after transplant (*Taylor et al. 2005*; *McPartland et al. 2007*), meaning that results from previous trials may not necessarily translate to present-day populations.

Cyclosporine is a popular immunosuppressive drug and is a calcineurin inhibitor. Tacrolimus is also a calcineurin inhibitor and a relatively newer immunosuppressive drug that is being increasingly utilized in transplant patients. Cyclosporine and Tacrolimus have similar mechanisms of immunosuppressive action (*Allison 2000*; *Aw 2003*), but may have different effects on aspects of lipid metabolism (*Tory et al. 2008*; *Tory et al. 2009*). Therefore, the possibility exists that type of immunosuppressive medication may also influence response to dietary therapy, and that evidence for one medication may not necessarily translate to other immunosuppressive medications. The majority of dietary trials have been in patients receiving Cyclosporine therapy, whereas currently Tacrolimus therapy gaining popularity and may be less hyperlipidemic.

Despite the prevalence of hyperlipidemia in transplant patients, there have been very few assessments of lipid synthesis as a possible mediator of hyperlipidemia in post-transplant human subjects. The objective of this study was to investigate whether a multi-nutrient dietary intervention can lower plasma lipid levels in ITx and LTx subjects, and to investigate the effects of diet intervention on FA and cholesterol synthesis using the deuterium incorporation method in these patients compared to control subjects.

7.1. Methods - Subject recruitment

ITx subjects (n=4) were recruited from the University of Alberta Clinical Islet Transplant program. Inclusion criteria has previously been discussed (please see Chapter 6). Of eligible ITx subjects recruited that were available to participate in the dietary intervention, 20% declined due to lack of time or interest. All ITx subjects completed the full 4-week protocol (i.e. there were no drop-outs for the dietary intervention).

LTx subjects were recruited from the University of Alberta Liver Transplant program. Inclusion criteria has previously been discussed (please

see Chapter 6). Of the 7 LTx subjects recruited and approached for participation in the dietary intervention, only 1 declined. However, this subject consumed a traditional Indian diet and therefore food consumption on the dietary plan would have been quite different from usual dietary patterns and likely would have impacted compliance. All LTx subjects completed the full 4-week protocol (i.e. there were no drop-outs for the dietary intervention). One LTx subject was excluded from final analysis due to extremely poor compliance during the dietary phase (30% compliance to the supplement bar and overall dietary plan). Therefore analysis was completed on 5 LTx patients for the dietary intervention.

ITx and LTx subjects taking lipid-lowering medications such as statins and fibrates were included. Subjects were instructed to stop medications 1 week before the testing day to allow a wash-out period before testing, and for the last two weeks of the diet intervention. A one-week period was determined as adequate due to the short half-life of most statin medications, the longest half-life estimated at 30 hours for Atorvastatin (*Bellosta et al. 2004*), and fenofibrate (the only fibrate medication used by patients in the study) having a half-life of 20 hours (*Najib 2002*).

Healthy (Control; n=9) were recruited by advertisements at the University of Alberta and diabetes clinics in Edmonton as a control group. Inclusion criteria has previously been discussed (please see Chapter 6). All Control subjects completed the full 4-week protocol (i.e. there were no drop-outs for the dietary intervention).

Subjects were tested before and after a 4-week diet intervention. Details regarding methods, laboratory procedures, and statistics are detailed in Chapter 3: Methods.

7.1.1. Additional Methods for Dietary Intervention

7.1.1.1. Menu Plan

The dietary intervention was a 4-week plan consisting of a 7-day rotating menu including food items and the specially prepared supplement bar and margarine-like spread. The menu plan provided approximately 21% energy from protein, 46% from carbohydrate, and moderate 33% energy from fat, a polyunsaturated to saturated fat ratio of approximately 0.8, and total cholesterol <200 mg. Total caloric intake was adjusted to account for energy contributed by the supplements. Due to the high number of subjects travelling from out of town, meals were kept simple and required minimal preparation such as sandwiches, cereals, and Lean Cuisine entrees (Nestlé, Glendale, CA, USA) in order to simplify and add macronutrient composition consistency to the menu plan.

The total gram quantity of each food for all meals on the menu plan as well as the total caloric value of the menu plan was scaled for each individual subject to mimic normal total caloric intake, as the goal of the menu plan was to be for weight maintenance only. An individual's calculated normal caloric intake was converted into the base menu plan caloric quantities, and the amounts adjusted for the contribution from the dietary supplements. Quantities of the other food items were then adjusted in order to closely match each day's goal calories within ~10 kcal of the goal, and the calculations compared with data from Food Processor. Subjects were given a menu log book which detailed for each day the type and quantity of each food to be consumed, with space for actual quantity consumed and any food left over. There were also spaces available for the subject to list any extra foods or substitutions consumed. The log book therefore served as a record of compliance for both the menu plan and the supplements, as well as a 4-week food intake record. Subjects were provided with food scales for the duration of the intervention to assist in accurate recording. Weekly phone calls were

used to assess compliance and troubleshoot issues with menu plan as well as address any questions or concerns from the subject. Subjects were encouraged not to change their usual physical activity patterns over the course of the intervention. Physical activity patterns were estimated and monitored by 3-day physical activity records completed before and at the end of the dietary intervention.

7.1.1.2. Supplements

The supplements designed and produced for the dietary intervention trial were a supplement bar and a fortified margarine. The bars were made in batches by hand in the Human Nutrition Research Unit kitchen. A list of ingredients, amounts, and source of products for each 30 bar batch and per bar are illustrated in **Table 7-1**.

Ingredients were mixed thoroughly in separate bowls, combined by hand, and put into a food processor for thorough mixing. Dough was weighed into 32g portions and formed into bars using molds. Bars were robed in chocolate (Richmond SemiSweet chocolate wafers; J&S Foods; Edmonton, AB) melted using a double-boiler and hot dryer to blow off excess chocolate and produce a thin film. Bars were cooled until the chocolate hardened, and then wrapped first in plastic film and then in aluminum foil. Bars were kept in the research unit refrigerator in the dark at 4°C. For transport, bars were packaged into a closed box for direct short duration transport to homes or into a cooler with ice packs for longer trips outside the Edmonton area. Subjects were instructed to keep the bars in the fridge, but not freeze them. The red palm oil was comprised mainly of 18:1 (43%), 16:0 (41%), and 18:2n6 (10%), with trace amounts of 18:0 (<5%). The fish oil powder provided a minimum 150 mg EPA and DHA per 1 g powder. The FA in the bar were primarily 18:1 (37%), 16:0 (33%), 18:0 (13%) and 18:2n6 (9%), with trace amounts of 14:0 (6%) and 18:3n3 (2%).

The margarine was a commercially prepared margarine base (Lactantia Healthy Attitude Omega 3 non-hydrogenated; Parmalat Canada) and was comprised of primarily 18:1 (58%), 18:2n6 (20%), and 16:0 (13%), with trace amounts of 18:3n3 and 18:0 (<5% each). To make the fortified margarine, 100 g portions of margarine were mixed with 12.6 g of fish oil powder and 2 g of phytosterols. Subjects were instructed to keep the margarine in the fridge, not cook with it (e.g. in frying), but to add it to cooked foods if desired.

Supplements were dosed based on subject body weight. The goal of supplementation was to provide 15 mg EPA/DHA per kg body weight, which translated into at least 2 bars per day for each subject, and 9 servings of margarine per week contributing a minimum of 1.0 g EPA/DHA per day. Subjects weighing over 200 lb required 3-4 bars per day, depending on body weight.

Donation of food products

Microencapsulated fish oil powder was donated by Ocean Nutrition Canada (Dartmouth, NS). Soy protein was donated by Blends Inc. (Edmonton, AB). Oat beta-glucan was donated by Cevena Bioproducts Inc. (Edmonton, AB). Chicory inulin was donated by Quadra Chemicals Ltd (Vaudreuil-Dorion, QC). Lean Cuisine entrees were donated by Nestle Nutrition (North York, ON). Red palm oil was donated by the Palm Oil Research Institute of Malaysia. All other items were purchased though local or national vendors. Companies that donated food products had no role in the design, collection, analysis, or data interpretation.

Table 7-1: Ingredients, amounts, and sources for dietary supplements.

Ingredient	**Source/Supplier**	**Amount per bar (g)**
Xanthan gum (H645)	Blends, Inc. (Edmonton, AB)	0.3
Salt (Windsor iodized table salt)	Retail	0.15
Oat beta-glucan (Viscofiber)	Cevena Bioproducts (Edmonton, AB)	1.0
Almonds (pieces)	Retail	0.5
Chicory inulin (Beneo Raftilene or Orafti ST-Gel)	Quadra Chemicals Ltd. (Vaudreuil-Dorion, QC)	1.25
Phytosterols (Chol SAP-15)	Nutritional Fundamentals for Health (Vaudreuil, QC)	1.0
Soy protein (Pro-Fam 891 Isolated Soy Protein)	Archer Daniels Midland Company (Decatur, IL)	8.5
Microencapsulated fish oil powder (MEG-3; MC601812TG-NG)*	Ocean Nutrition Canada (Dartmouth, NS)	3.0
Flax oil (Gold Top Organics; Edmonton, AB)	Retail	0.5
Red palm oil (Carotino Superolein NVRSO)	Palm Oil Research Institute of Malaysia	3.5
Lemon juice (ReaLemon)	Retail	0.25
Maple flavour (F085)	Blends Inc. (Edmonton, AB)	0.5
Vanilla flavour	Retail	0.3
Sunflower oil (100% pure)	Retail	0.75
Brown rice syrup (Lundberg Sweet Dreams; Lundberg Family Farms, Richvale, CA)	Retail	0.75
Buckwheat honey (Golden Acres Honey Products Ltd.; Three Hills, AB)	Retail	5.0
Bottled water	Retail	3.0
Almond paste (Nuts to You Nut Butter Inc.; Paris, ON)	Retail	2.2

7.2. Results

7.2.1. Baseline characteristics

7.2.1.1. Subject characteristics

All ITx patients and 4 of 5 LTx patients were taking Tacrolimus immunosuppression; the remaining LTx patient was taking Sirolimus. All ITx patients and 3 of 5 LTx patients were also taking mycophenolate mofetil as adjunct therapy. All LTx patients had received only one transplant, where ITx patients had received 1.8 ± 0.5 transplants (median: 1.5; range: 1-3). LTx patients were tested at 15 ± 4 months after transplant (median: 18; range: 4-22) and ITx patients were tested at 41 ± 16 months after transplant (median: 42; range: 2-78). No significant difference was found between groups in number of months since transplant. Almost all ITx patients (3 of 4) were taking statins, and 2 of 5 LTx patients were taking fibrates. Most of the ITx (3 of 4) and LTx (3 of 5) patients were taking at least one anti-hypertensive medication including ACE inhibitor, beta blocker, or calcium channel blocker. One of the ITx patients was taking hypothyroid therapy, and one was taking metformin as part of another transplant investigation protocol. There were no significant differences between transplant groups in Tacrolimus dose (ITx 5.0 ± 0.7 mg/day and LTx 4.8 ± 1 mg/day) or fasting Tacrolimus blood level (ITx 8.7 ± 0.5 μg/L and LTx 6.8 ± 0.8 μg/L). Fasting blood Tacrolimus level was not correlated with Tacrolimus dosage, weight, BMI, or percent body fat in ITx or LTx patients.

Pre-intervention, LTx subjects were heavier and had higher body fat than the Control and ITx groups (**Table 7-2**). Intake of dietary fat was higher in ITx and LTx compared to Control subjects, but there were no other significant differences in dietary intake between groups (**Table 7-3**). ITx patients had lower fasting TC and LDL-c levels compared to Control and LTx (**Table 7-4**). LTx patients had higher TG and lower HDL-c levels as well as

higher fasting insulin levels compared to Control and ITx (**Table 7-4**). No subjects in the ITx group had hyperlipidemia, while 2 of the control subjects and 2 of the LTx subjects were hyperlipidemic defined as fasting TC>5.2 or TG>1.7 mmol/L.

Table 7-2: Baseline anthropometric characteristics of control, islet, and liver transplant subjects.

Measurement	**Control (n=9)**	**ITx (n=4)**	**LTx (n=5)**	**p-value**
Males/Females	3/6	3/1	4/1	n/a
Age (years)	54 ± 4	54 ± 2	56 ± 3	0.83
BMI (kg/m^2)	25 ± 0.8 ***ab***	22 ± 0.8 ***a***	31 ± 4 ***b***	0.035
WC (cm)	87 ± 4	83 ± 3	107 ± 14	0.24
WC (cm) - Males	94 ± 1 ***ab***	84 ± 3 ***a***	119 ± 10 ***b***	<0.05
WC (cm) - Females	83 ± 6	81 ± 0	60 ± 0	>0.05
Body fat (%)	33 ± 3	18 ± 7	35 ± 5	0.13
Body fat (%) - Males	26 ± 4 ***ab***	12 ± 6 ***a***	35 ± 6 ***b***	<0.05
Body fat (%) - Females	36 ± 3	35 ± 0	33 ± 0	>0.05
RMR (kcal/day)	1366 ± 58	1245 ± 116	1787 ± 266	0.06
RMR (kcal/day) - Males	1383 ± 109	1360 ± 19	1911 ± 333	>0.05
RMR (kcal/day) - Females	1358 ± 76	1014 ± 0	1415 ± 0	>0.05
Systolic blood pressure (mmHg)	119 ± 7	126 ± 4	119 ± 7	0.75
Diastolic blood pressure (mmHg)	74 ± 6	73 ± 5	84 ± 6	0.44

Data expressed as mean ± SEM; different letters indicate significant difference p<0.05

Table 7-3: Background dietary intake of control, islet, and liver transplant subjects.

Background Intake	**Control (n=9)**	**ITx (n=4)**	**LTx (n=5)**	**p-value**
Total kcal	1607 ± 126	1686 ± 228	1868 ± 304	0.58
Total kcal - Males	1911 ± 330	1774 ± 297	2059 ± 305	>0.05
Total kcal - Females	1455 ± 52	1420 ± 0	1105 ± 0	>0.05
CHO (%)	55 ± 3	46 ± 4	50 ± 2	0.22
Protein (%)	19 ± 1	20 ± 3	19 ± 2	0.98
Fat (%)	24 ± 2 ***a***	35 ± 4 ***b***	32 ± 3 ***ab***	0.04
SFA(%)	7.6 ± 0.8	12 ± 1	10 ± 2	0.056
MUFA (%)	7.6 ± 0.9	8.7 ± 0.7	9.6 ± 0.6	0.12
PUFA (%)	4.4 ± 0.6	4.9 ± 1	4.3 ± 0.4	0.92
Cholesterol (mg)	201 ± 44	284 ± 86	385 ± 54	0.066
Fiber (g)	22 ± 4	16 ± 3	17 ± 2	0.620

Data expressed as mean ± SEM; different letters indicate significant difference $p<0.05$

Table 7-4: Changes in blood lipid, glucose, insulin, and cytokine levels of control, islet, and liver transplant subjects after diet intervention.

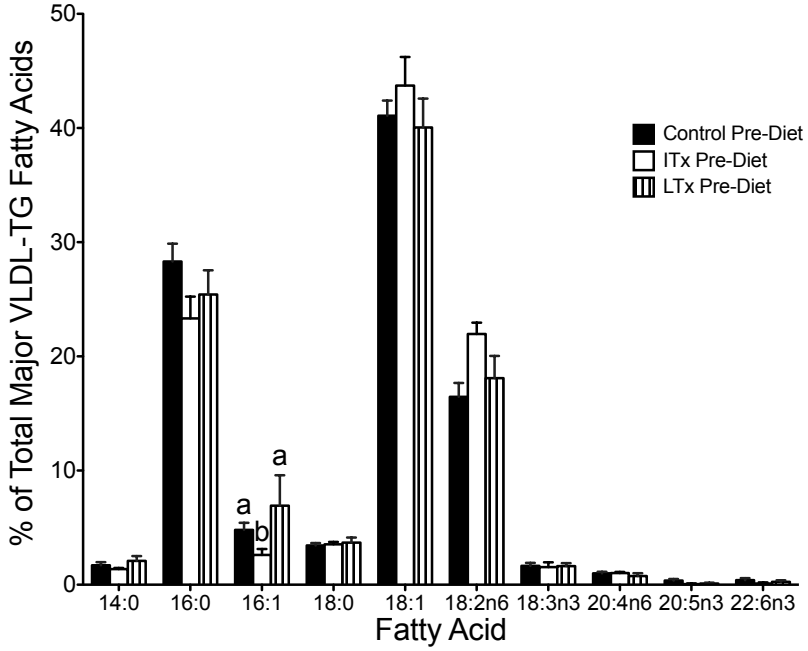
Fasting concentration	**Control pre**	**Control post**	**ITx pre**	**ITx post**	**LTx pre**	**LTx post**
TC (mmol/L)	5.0 ± 0.1 ***a***	4.7 ± 0.2 * p=0.02	4.0 ± 0.2 ***b***	4.6 ± 0.2 * p=0.04	4.2 ± 0.7 ***ab***	3.9 ± 0.4 p=0.34
LDL-c (mmol/L)	3.1 ± 0.2 ***a***	2.9 ± 0.2 * p=0.006	2.0 ± 0.1 ***b***	2.7 ± 0.1 * p=0.04	2.2 ± 0.5 ***ab***	2.4 ± 0.5 p=0.54
HDL-c (mmol/L)	1.5 ± 0.1 ***a***	1.6 ± 0.1 p=0.79	1.6 ± 0.1 ***a***	1.4 ± 0.1 p=0.36	0.9 ± 0.1 ***b***	0.9 ± 0.1 p=0.35
TG (mmol/L)	0.80 ± 0.1 ***a***	0.63 ± 0.04 p=0.11	0.84 ± 0.2 ***ab***	0.93 ± 0.2 p=0.34	2.3 ± 0.7 ***b***	2.0 ± 0.7 p=0.60
Glucose (mmol/L)	5.0 ± 0.1	4.9 ± 0.1 p=0.31	5.6 ± 0.4	5.8 ± 0.3 p=0.66	5.1 ± 0.1	4.8 ± 0.2 p=0.28
Insulin (mU/L)	5.7 ± 2	3.6 ± 0.7 p=0.29	5.6 ± 1	4.3 ± 2 p=0.52	19 ± 9	20 ± 11 p=0.60
HOMA	1.3 ± 0.4	0.8 ± 0.2 p=0.19	1.4 ± 0.4	1.1 ± 0.3 p=0.56	4.1 ± 2	4.5 ± 3 p=0.62
TNF-a (pg/mL)	0.70 ± 0.2	0.96 ± 0.3 p=0.10	0.78 ± 0.1	0.56 ± 0.2 p=0.19	0.40 ± 0.1	0.48 ± 0.1 p=0.37
CRP (mg/L)	1.9 ± 0.7	1.0 ± 0.3 * p=0.049	0.14 ± 0.06	0.15 ± 0.05 p=0.46	3.8 ± 1.6	3.8 ± 2.2 p=0.99
Fibrinogen (ng/mL)	1264 ± 166	946 ± 104 p=0.13	1485 ± 393	3309 ± 2083 p=0.37	925 ± 81	1476 ± 506 p=0.36
IL-6 (pg/mL)	3.4 ± 1.7	3.2 ± 2.0 p=0.72	1.1 ± 0.2	1.2 ± 0.3 p=0.39	3.8 ± 1.2	3.4 ± 0.7 p=0.51
IL-10 (pg/mL)	0.31 ± 0.08	0.92 ± 0.5 p=0.29	0.34 ± 0.1	0.39 ± 0.1 p=0.40	0.90 ± 0.5	1.2 ± 0.9 p=0.62

Data expressed as mean ± SEM; different letters indicate significant difference between groups; * indicates significant difference within groups after intervention; $p<0.05$; two-tailed

7.2.1.2. Baseline Fatty acid composition

Fasting plasma-TG FA composition was similar between groups (data not shown) except for 16:1 which was significantly higher in Control (3.4 ± 0.5%) and LTx (2.9 ± 0.2%) compared to ITx (0.67 ± 0.6%) subjects. Fasting total VLDL-TG FA was not significantly different between groups but tended to be higher in LTx (441 ± 160 μg/mL) compared to ITx (228 ± 108 μg/mL) and Control (151 ± 33.7 μg/mL). Fasting VLDL-TG FA composition was also similar between groups except for 16:1 which was higher in Control and LTx compared to ITx (**Figure 7-1**). Fasting total TPL-FA concentration was not significantly different between groups (Control 732 ± 101 μg/mL; ITx 541 ± 136 μg/mL; and LTx 678 ± 48 μg/mL) before diet intervention. Individual FA contribution to fasting TPL-FA and concentrations of individual TPL-FA did not differ between groups (data not shown).

Figure 7-1: Fasting VLDL-TG fatty acid composition in control, islet, and liver transplant subjects before diet intervention.



Data expressed as mean ± SEM; different letters indicate significant difference $p<0.05$

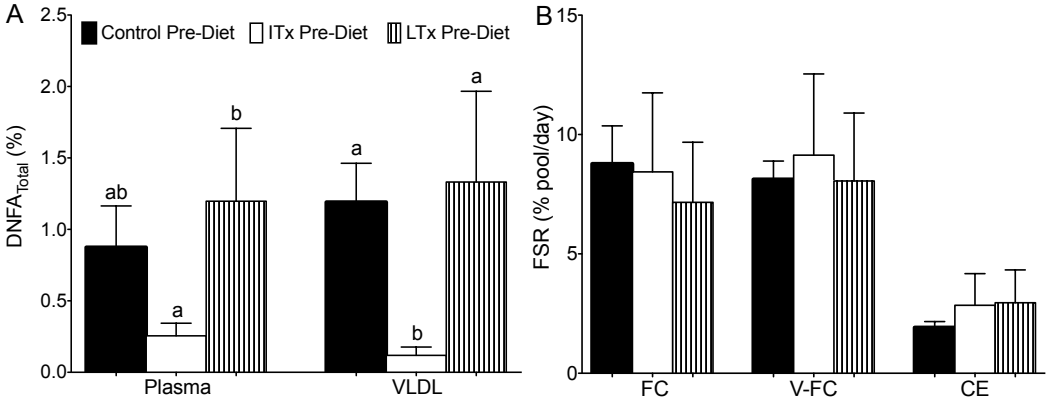
7.2.1.3. Baseline fatty acid and cholesterol synthesis

Prior to diet intervention, whole-body and hepatic DNFA were significantly lower in ITx compared to Control and LTx subjects (**Figure 7-2A**), whereas whole-body synthesis of FC and CE and hepatic synthesis of FC were similar between groups (**Figure 7-2B**). There may be differences in lipogenesis and cholesterol synthesis between normal weight and overweight individuals. Overweight individuals are more likely to have more metabolic aberrations such as insulin resistance and dyslipidemia. Both excess adiposity and insulin resistance have been previously shown to be associated with greater rates of lipogenesis and cholesterol synthesis (*Guo et al. 2000*; *Diraison et al. 2002*), and weight loss is associated with reduction in cholesterol synthesis (*Di Buono et al. 1999*; *Santosa et al. 2007a*). Therefore differences in lipid synthetic rates may be complicated by presence of varying adiposities within the transplant groups. It was not possible to match individual transplant subjects with control subjects due to limited numbers of subjects. Therefore ITx and LTx patients were pooled into normal weight (defined as BMI <25 kg/m^2; n=6) or higher weight (defined as BMI <25 kg/m^2; n=3) groups and compared to control subjects of similar weight status (BMI <25 kg/m^2, n=4; BMI <25 kg/m^2, n=4). One control subject was omitted from these comparisons due to much younger age than the rest of the subjects (age 28 compared to age 48-68 for the rest of the group).

Groups in the same weight category were similar in age and plasma glucose levels. BMI was higher in the higher BMI transplant group than the higher BMI control group (35 ± 4 kg/m^2 vs 27 ± 0.5 kg/m^2; p=0.057). Plasma TC and LDL-c were significantly lower in the normal BMI transplant group compared to the control group (TC 3.8 ± 0.2 mmol/L vs. 5.0 ± 0.1 mmol/L; LDL-c 1.9 ± 0.1 mmol/L vs. 3.1 ± 0.1 mmol/L). In the higher BMI transplant group plasma TG was significantly higher (3.1 ± 1 mmol/L vs. 0.7 ± 0.03

mmol/L) and HDL-c was significantly lower (0.8 ± 0.2 mmol/L vs. 1.6 ± 0.03 mmol/L) compared to the higher BMI control group. Fasting plasma insulin levels were comparable between the normal BMI transplant and control groups but were higher in the higher BMI transplant group (25 ± 15 mU/L) compared to the higher BMI control group (5.3 ± 1 mU/L).

Figure 7-2: Lipogenesis and cholesterol synthesis in control, islet, and liver transplant subjects before diet intervention. A) Fasting whole-body and hepatic de novo fatty acid (DNFA) synthesis; B) Fasting whole-body and hepatic fractional synthesis rate (FSR) of free cholesterol (FC) and cholesteryl ester (CE).

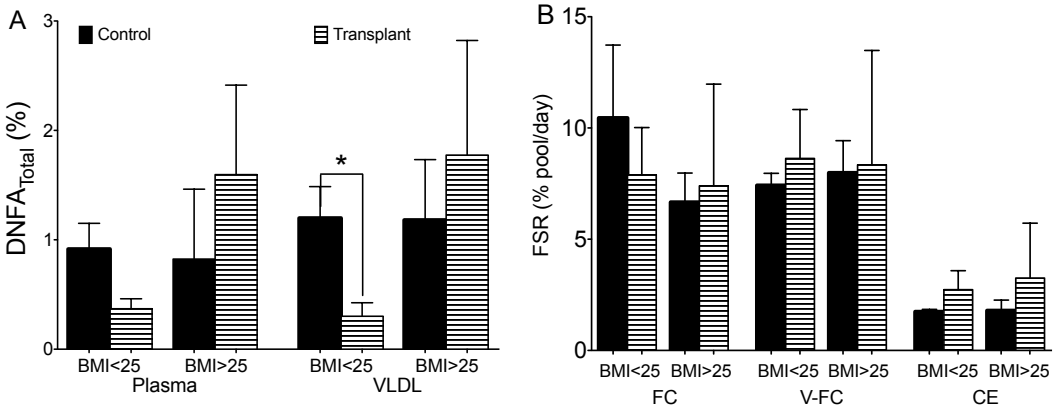


Data expressed as mean ± SEM; different letters indicate significant difference $p<0.05$

Whole-body and hepatic DNFA were lower in the BMI<25 transplant group compared to the control group, whereas DNFA was slightly higher in the BMI>25 transplant group compared to controls (**Figure 7-3A**). Whole-body DNFA was inversely correlated with fasting HDL-c (*r=-0.91*) and positively associated with fasting plasma TG (*r=0.83*), weight (*r=0.87*), BMI (*r=0.83*) and waist circumference (*r=0.77*) in the pooled transplant subjects. Hepatic DNFA was inversely associated with fasting plasma HDL-c (*r=-0.78*) and positively associated with fasting plasma TG (*r=0.73*), weight (*r=0.82*), and BMI (*r=0.78*) in the pooled transplant subjects. Whole-body and hepatic synthesis of FC and whole-body synthesis of CE were not different between comparable BMI groups (**Figure 7-3B**). Fasting LDL-c concentration was

inversely correlated to whole-body synthesis of FC (*r=-0.70*) and CE (*r=-0.83*) and hepatic synthesis of FC (*r=-0.75*) in the pooled transplant subjects.

Figure 7-3: Lipogenesis and cholesterol synthesis in control and pooled transplant subjects divided by BMI before diet intervention. A) Fasting whole-body and hepatic de novo fatty acid (DNFA) synthesis; B) Fasting whole-body and hepatic fractional synthesis rate (FSR) of free cholesterol (FC) and cholesteryl ester (CE).



Data expressed as mean ± SEM; *p<0.05

7.2.2. Effect of diet intervention

7.2.2.1. Compliance to intervention and supplements

Compliance to the supplement bars and spread was assessed by counting the number of bar and spread servings consumed over the dietary intervention period and comparing these to the number of bars and spread prescribed. The values were then averaged over the period of intervention days to determine an average intake per day of bars and spread. Compliance to the supplement bars was higher in the Control (94 ± 3%; median 96%; range 74-100%) and LTx (95 ± 4%; median 93%; range 88-109%) subjects compared to ITx (66 ± 6%; median 63%; range 54-81%). Compliance to the supplement spread was not different between Control (77 ± 8%; median 84%; range 15-98%), ITx (71 ± 23%; median 64%; range 23-132%) and LTx (86 ± 6%; median 92%; range 67-100%). Compliance to the base menu plan

was assessed by comparing the number of foods items prescribed and the number consumed by the subject per day. Foods including vegetables, fruit, and low-fat dairy that were substituted for other food items of similar composition on the menu plan or consumed in addition to the daily prescribed menu items were not counted as infractions against menu plan compliance. Compliance to the dietary menu plan was similar between Control (69 ± 8%; median 77%; range 35-97%), ITx (82 ± 16%; median 98%; range 33-100%) and LTx (82 ± 11%; median 100%; range 50-100%). Intake of external high sugar or high fat foods was similar between groups and was <1.0 servings per day (data not shown). High sugar or high fat foods were defined as foods not included in the menu plan that would add extra calories and nutrients, such as pizza, fast food, soda, ice cream, potato chips, cookies, and candy. Intake of alcohol was higher in the Control (0.24 ± 0.08 servings/ day) compared to ITx (0.05 ± 0.05) and LTx (0.01 ± 0.01) (NS).

7.2.2.2. Anthropometric measurements and plasma lipid levels after diet intervention

There was a significant reduction in weight (Pre 70 ± 2 kg vs. Post 68 ± 2 kg), BMI (Pre 25 ± 0.8 kg/m^2 vs. Post 24 ± 0.6 kg/m^2), and systolic blood pressure (Pre 119 ± 7 mmHg vs. Post 112 ± 6 mmHg) in control subjects after diet intervention. In ITx subjects there were no significant changes in weight or BMI, but a significant reduction in waist circumference (Pre 83 ± 3 cm vs. Post 79 ± 2 cm). There were no significant changes in any anthropometric measurement in LTx subjects.

Fasting plasma TC were significantly reduced 5.1% and fasting LDL-c levels were reduced 8.7% after diet intervention in Control subjects (**Table 7-4**). Percent change in fasting plasma TC levels ranged from +4% to -13% (median -2%), and percent change in fasting plasma LDL-c levels ranged from -0.7% to -23% (median -6%) in control subjects after diet intervention. Fasting plasma HDL-c level did not change in Control subjects. Fasting plasma

TG levels were reduced 16% in the Control group after diet intervention but did not reach significance which may have been due to the low baseline plasma TG levels (**Table 7-4**). Range in percent change fasting TG levels after intervention was +19% to -58% (median 6%). When Control subjects with baseline fasting plasma TG>1 mmol/L (n=2; plasma TG 1.1 ± 0.08 mmol/L) were compared to subjects with baseline plasma TG<1 mmol/L (n=7; plasma TG 0.70 ± 0.02 mmol/L), plasma TG levels in those with higher baseline levels were reduced 50% after diet intervention (Pre 1.1 ± 0.08 mmol/L vs. Post 0.5 ± 0.01 mmol/L). Postprandial plasma TG levels were not significantly lower after dietary intervention in Control subjects, but incremental area under the curve was significantly lower at 8h after dietary intervention (**Appendix A**). Percent change in plasma TC and LDL-c levels in Control subjects was moderately related to compliance to the diet (TC *r=-0.60*; LDL-c *r=-0.70*) and consumption of alcohol (TC *r=0.67*; LDL-c *r=0.63*), indicating that greater reductions in plasma TC and LDL-c were related to higher menu plan compliance and lower alcohol consumption. Changes in plasma TC and LDL-c were not related to compliance to the bars or spread, consumption of high sugar or fat foods, or weight change.

Contrary to the Control group, fasting plasma TC and LDL-c levels were increased after diet intervention in the ITx subjects (**Table 7-4**). Change in fasting plasma TC levels ranged from +5 to +27%, (median +13%) and changes in fasting LDL-c ranged from +17 to +72% (median +25%) in ITx subjects after diet intervention. Changes in fasting plasma TG levels after diet intervention ranged from +34 to -8% (median +7%) in ITx subjects. There were no significant changes in fasting plasma HDL-c, TG, or cytokine levels in the ITx group after diet intervention. In the LTx group there were no significant changes in fasting blood lipid or cytokine levels after diet intervention (**Table 7-4**). Changes in fasting plasma TC levels ranged +12 to -20% (median 0%), changes in fasting LDL-c levels ranged +19 to -24%

(median -2%), and changes in fasting plasma TG levels ranged +24 to -50% (median +5%) in LTx subjects after diet intervention. There were no significant changes in postprandial plasma TG levels or incremental area under the curve in either ITx or LTx subjects after dietary intervention (**Appendix A**).

When transplant subjects were pooled into normal (BMI<25 kg/m^2) and higher (BMI>25 kg/m^2) weight categories, fasting plasma TC levels in the normal BMI transplant group were significantly higher after diet intervention (Pre 3.8 ± 0.2 mmol/L vs. Post 4.1 ± 0.3 mmol/L). Percent increase in fasting plasma TC levels ranged 0-26% and in plasma TG ranged 1-34% in the normal BMI transplant group. Conversely, fasting plasma TC levels decreased slightly in the higher BMI transplant group after diet intervention (Pre 4.8 ± 1.0 mmol/L vs. Post 4.3 ± 0.6 mmol/L) though this did not reach statistical significance. In a similar fashion, fasting plasma LDL-c levels were significantly higher following diet intervention in the normal BMI transplant group (Pre 1.9 ± 0.1 mmol/L vs. Post 2.4 ± 0.2 mmol/L) but were reduced slightly in the higher BMI transplant group (Pre 3.4 ± 0.6 mmol/L vs. Post 3.0 ± 0.9 mmol/L; NS). Fasting plasma TG levels did not change in normal BMI transplant subjects after diet intervention (Pre 0.9 ± 0.1 mmol/L vs. Post 1.0 ± 0.2 mmol/L), and were reduced slightly in higher BMI transplant patients (Pre 3.1 ± 1.0 mmol/L vs. Post 2.5 ± 1.1 mmol/L; NS). In pooled transplant subjects, percent change in fasting TC and LDL-c was moderately but significantly related to compliance to supplement bars (TC *r=-0.68*; LDL-c *r=-0.88*) and to baseline BMI (TC *r=-0.62*; LDL-c *r=-0.71*). Change in fasting plasma TG levels was not related to dietary compliance or baseline BMI.

There were only 3 subjects in the heavier BMI transplant group, who were characterized by higher BMI, insulin, and lipid levels compared to normal BMI transplant group (subjects Tx-A, Tx-B, and Tx-C). When these subjects are considered, Tx-B and Tx-C experienced reductions in fasting

plasma TC of 17% and 20% respectively, and in fasting plasma TG of 50% and 31%, respectively. Tx-A had no change in fasting plasma TC (0.3% increase) and actually had an increase in fasting plasma TG levels of 24%. This increase in fasting plasma TG was despite having a very high baseline fasting plasma TG level of 3.7 mmol/L, which is comparable to Tx-B (baseline plasma TG 4.4 mmol/L) who experienced a 50% reduction in fasting plasma TG after diet intervention. In addition, compliance to the bars and overall diet was similar between these two individuals (90-100% compliance for the bars and 100% compliance to the overall diet). The fish oil powder used in this study guaranteed a minimum of 150 mg EPA/DHA per 1 g of powder, with a range up to 180 mg EPA/DHA per 1 g powder. It was not possible to calculate the exact EPA/DHA content for each subject due to use of different batches of fish oil powder for the bars, but a minimum and maximum EPA/DHA consumption for each subject was calculated based on intake from the bars and spread. Based on these estimations, Tx-A received a minimum of 2.2 g EPA/DHA per day and a maximum of 2.7 g/day. Tx-B received a minimum of 1.7 g EPA/DHA per day and a maximum of 2 g/day. These amounts of EPA/DHA are in contrast to the other subjects in the trial which received a minimum of ~1 g EPA/DHA per day and a maximum of ~1.4 g/day (**Table 7-5**). Therefore, despite comparable and even higher intake of EPA/DHA per day compared to Tx-B, fasting plasma TG levels were not reduced in Tx-B and actually increased 23%. In addition, Tx-A consumed 4.4 g phytosterols per day and had no reduction in fasting plasma TC, compared to Tx-B who consumed 3.2 g phytosterols per day and had a 20% reduction in fasting plasma TC levels. However, Tx-A had low baseline TC (3.5 mmol/L), indicating TG-specific hypertriglyceridemia. In comparison, the remaining subjects in the trial consumed ~1.7-2.2 g phytosterols per day (**Table 7-5**).

Table 7-5: Consumption of functional nutrients contained in the supplements estimated from reported supplement bar and spread intake in control and pooled transplant subjects divided by BMI.

Functional Nutrient (g/ day)	**Control (BMI<25)**	**Control (BMI>25)**	**Transplant (BMI<25)**	**Transplant (BMI>BMI)**
EPA/DHA (min)	1.1 ± 0.1 ***a***	1.1 ± 0.1	0.9 ± 0.1 ***b***	1.8 ± 0.2 *
EPA/DHA (max)	1.3 ± 0.1 ***a***	1.4 ± 0.1	1.1 ± 0.1 ***b***	2.2 ± 0.2 *
Phytosterols	2.1 ± 0.1 ***a***	2.2 ± 0.1	1.7 ± 0.1 ***b***	3.5 ± 0.4 *
Almonds	0.9 ± 0.04 ***a***	0.98 ± 0.01 ***a***	0.73 ± 0.1 ***b***	1.6 ± 0.2 ***b*** *
Almond paste	4.0 ± 0.2 ***a***	4.3 ± 0.1 ***a***	3.2 ± 0.3 ***b***	6.8 ± 0.9 ***b*** *
Chicory inulin	2.3 ± 0.1 ***a***	2.5 ± 0.03 ***a***	1.8 ± 0.2 ***b***	3.9 ± 0.5 ***b*** *
Oat beta-glucan	1.8 ± 0.1 ***a***	2.0 ± 0.03 ***a***	1.5 ± 0.1 ***b***	3.1 ± 0.4 ***b*** *
Soy protein	15 ± 0.7 ***a***	17 ± 0.2 ***a***	12 ± 1 ***b***	26 ± 3 ***b*** *
Flax oil	0.91 ± 0.04 ***a***	0.98 ± 0.01 ***a***	0.73 ± 0.1 ***b***	1.6 ± 0.2 ***b*** *
Red palm oil	6.4 ± 0.3 ***a***	6.9 ± 0.1 ***a***	5.1 ± 0.4 ***b***	11 ± 1 ***b*** *
Sunflower oil	1.4 ± 0.1 ***a***	1.5 ± 0.02 ***a***	1.1 ± 0.1 ***b***	2.3 ± 0.3 ***b*** *

Data expressed as mean ± SEM; different letters indicate significant difference between groups within BMI class; * indicates significant difference between BMI class within control or transplant groups; $p<0.05$

7.2.2.3. Fatty acid composition after diet intervention

Table 7-6 depicts changes in plasma lipid long-chain FA composition before and after dietary intervention in control and transplant groups. After diet intervention there was no change in percent contribution of individual FA to total fasting plasma-TG FA concentration in Controls except for 18:2n6 which increased (**Table 7-6**). There was no change in percent contribution of individual FA to total fasting plasma-TG FA concentration in ITx subjects after the diet except for 16:1 (Pre 0.67 ± 0.6% vs. Post 1.8 ± 0.3%, respectively) and 20:5n3 (**Table 7-6**) which increased and 18:1 (Pre 47 ± 2% vs. Post 41 ±

2%) which decreased. There was no change in percent contribution of individual FA to total fasting plasma-TG FA in LTx subjects after diet intervention except for 18:1 which decreased (Pre 43 ± 2% vs. Post 40 ± 2%).

After diet intervention, there was no significant change in total fasting VLDL-TG FA concentration in Control (Pre 151 ± 34 μg/mL vs. Post 141 ± 64 μg/mL; p=0.83), ITx (Pre 228 ± 108 μg/mL vs. Post 149 ± 47 μg/mL; p=0.31), and LTx (Pre 441 ± 160 μg/mL vs. Post 228 ± 62 μg/mL; p=0.14), though fasting VLDL-TG FA concentration tended to be lower after diet intervention in all groups. After diet intervention there was no change in percent contribution of individual FA to total fasting VLDL-TG FA in Control subjects except for 18:2n6 and 22:6n3 which increased (**Table 7-6**), and 16:0 (Pre 28 ± 2% vs. Post 24 ± 2%) and 18:0 (Pre 3.5 ± 0.2% vs. Post 3.2 ± 0.3%) which decreased. There was no significant change in percent contribution of individual FA to total fasting VLDL-TG FA concentration in ITx subjects after diet intervention except for 16:0 (Pre 23 ± 2% vs. Post 25 ± 2%) and 18:3n3 (**Table 7-6**) which increased. There was no change in percent contribution of individual FA to total fasting VLDL-TG FA concentration in LTx subjects after diet intervention except for 20:5n3 which increased (**Table 7-6**).

When concentrations of fasting VLDL-TG FA were considered, there were no significant changes in FA concentrations except for 20:4n6 which was lower in Control subjects after intervention (**Table 7-6**). Concentration of 20:4n6 also tended to be lower in ITx and LTx subjects (**Table 7-6**; NS). Concentration of 20:5n3 increased in Control and LTx subjects but not ITx subjects (**Table 7-6**; NS). Concentration of 22:6n3 increased in Control subjects and slightly in ITx subjects but not in LTx (**Table 7-6**; NS).

After diet intervention, there was a significant reduction in fasting total TPL-FA concentration in Control subjects (Pre 732 ± 101 μg/mL vs. Post 510 ± 58.3 μg/mL), but not ITx (Pre 541 ± 136 μg/mL vs. Post 988 ± 231 μg/mL) or LTx (Pre 678 ± 47.3 μg/mL vs. Post 589 ± 57.0 μg/mL). After diet

intervention there was no change in percent contribution of individual FA to fasting total TPL-FA concentration in Control subjects except for 20:5n3 which increased (**Table 7-6**). However, concentrations of 18:2n6 and 20:4n6 were reduced after diet intervention in Control subjects and concentration of 20:5n3 doubled (**Table 7-6**). There was no change in percent contribution of individual FA to fasting total TPL-FA concentration or concentrations of TPL-FA in ITx subjects after diet intervention (**Table 7-6**). Concentration of TPL 20:5n3 was not changed in ITx subjects, but concentration of TPL 22:6n3 was higher after diet intervention (**Table 7-6**). There was no change in percent contribution of individual FA to fasting total TPL-FA concentration in LTx subjects after diet intervention (**Table 7-6**). Concentration of TPL 22:6n3 was significantly higher after the intervention, whereas there were no significant differences in concentrations of 20:4n6 or 20:5n3 in LTx subjects after diet intervention (**Table 7-6**). Correlates of changes in VLDL-TG and TPL FA are listed in **Table 7-7**.

Table 7-6: Plasma-TG, VLDL-TG, and TPL fatty acid composition and concentration before and after diet intervention in control, islet, and liver transplant subjects.

Lipid fraction and FA	Control pre	Control post	ITx pre	ITx post	LTx pre	LTx post
Plasma TG						
18:2n6 (%)	16 ± 1.2	20 ± 2 *	19 ± 2	20 ± 2	18 ± 2	18 ± 0.6
18:3n3 (%)	1.2 ± 0.2	1.1 ± 0.1	0.76 ± 0.5	0.99 ± 0.4	1.5 ± 0.3	1.4 ± 0.2
20:4n6 (%)	1.6 ± 0.4	1.3 ± 0.3	0.76 ± 0.1	1.2 ± 0.2	0.90 ± 0.2	0.72 ± 0.2
20:5n3 (%)	1.2 ± 0.9	0.99 ± 0.4	not detectable	0.23 ± 0.1 *	0.05 ± 0.04	0.20 ± 0.1
22:6n3 (%)	0.48 ± 0.2	0.73 ± 0.3	0.04 ± 0.04	0.04 ± 0.04	0.10 ± 0.1	0.20 ± 0.1
VLDL-TG						
18:2n6 (%)	17 ± 1	20 ± 0.9 *	22 ± 1	21 ± 2	18 ± 2	19 ± 0.8
18:3n3 (%)	1.7 ± 0.3	1.6 ± 0.1	1.5 ± 0.4	1.7 ± 0.4 *	1.6 ± 0.3	1.4 ± 0.4
20:4n6 (%)	0.99 ± 0.1	0.87 ± 0.1	1.0 ± 0.1	1.1 ± 0.1	0.76 ± 0.3	0.66 ± 0.2
20:5n3 (%)	0.36 ± 0.1	0.49 ± 0.1	0.06 ± 0.1	not detectable	0.11 ± 0.1	0.31 ± 0.1 *
22:6n3 (%)	0.40 ± 0.2	0.85 ± 0.3 *	0.10 ± 0.1	0.48 ± 0.1	0.25 ± 0.1	0.57 ± 0.2
18:2n6 (µg/mL)	27 ± 4	37 ± 9	62 ± 24	41 ± 5	85 ± 31	59 ± 8.9
18:3n3 (µg/mL)	3.1 ± 0.6	3.3 ± 0.9	4.9 ± 2	3.4 ± 0.5	8.5 ± 3	5.9 ± 1
20:4n6 (µg/mL)	2.1 ± 0.7	1.2 ± 0.3 *	3.2 ± 0.9	1.8 ± 0.2	3.9 ± 2	1.8 ± 0.6
20:5n3 (µg/mL)	0.15 ± 0.1	0.47 ± 0.2	0.19 ± 0.2	not detectable	0.67 ± 0.4	0.71 ± 0.3

Table 7-6 (cont'd)

Lipid fraction and FA	Control pre	Control post	ITx pre	ITx post	LTx pre	LTx post
22:6n3 (µg/mL)	0.38 ± 0.1	1.1 ± 0.4	0.60 ± 0.4	0.66 ± 0.2	1.4 ± 0.9	1.3 ± 0.4
TPL						
18:2n6 (%)	21 ± 1	21 ± 1	24 ± 1	20 ± 5	30 ± 8	22 ± 1
18:3n3 (%)	0.38 ± 0.1	0.81 ± 0.5	0.40 ± 0.1	0.37 ± 0.1	0.55 ± 0.1	0.57 ± 0.1
20:4n6 (%)	8.3 ± 0.7	7.6 ± 1	9.8 ± 0.9	9.0 ± 0.7	7.8 ± 1	7.2 ± 0.4
20:5n3 (%)	0.92 ± 0.2	2.7 ± 0.4 *	0.93 ± 0.4	0.79 ± 0.5	0.75 ± 0.1	1.2 ± 0.4
22:6n3 (%)	2.2 ± 0.3	3.0 ± 0.2	1.9 ± 0.2	2.2 ± 0.3	2.4 ± 0.4	3.3 ± 0.3
18:2n6 (µg/mL)	149 ± 16	100 ± 7 *	131 ± 35	190 ± 70	209 ± 61	125 ± 9
18:3n3 (µg/mL)	2.7 ± 0.5	4.7 ± 3	2.7 ± 1	2.8 ± 0.6	3.7 ± 0.6	3.4 ± 0.7
20:4n6 (µg/mL)	59 ± 8	35 ± 4 *	53 ± 13	87 ± 19	53 ± 11	43 ± 6
20:5n3 (µg/mL)	6.0 ± 1	13 ± 2 *	5.9 ± 2	5.9 ± 3	5.1 ± 0.9	7.7 ± 3
22:6n3 (µg/mL)	17 ± 4	15 ± 2	11 ± 3	21 ± 6	17 ± 3	20 ± 3 *

Table 7-7: Correlations of VLDL-TG and TPL FA levels after diet intervention in control and pooled transplant subjects.

Correlates		r value	p-value
Control			
Post VLDL-TG 20:5n3 (µg/mL)	spread compliance	0.77	0.02
Post VLDL-TG 20:4n6 (%)	spread compliance	0.60	0.09
Post VLDL-TG total FA (µg/mL)	high sugar & high fat foods/day	0.62	0.09
Post VLDL-TG 20:5n3 (%)	spread compliance	0.77	0.02
% change TPL 22:6n3	% change TG	-0.75	0.03
Pooled Transplant			
Post VLDL-TG 18:2n6 (µg/mL)	spread compliance	0.62	0.07
Post VLDL-TG 18:2n6 (µg/mL)	high sugar & high fat foods/day	-0.61	0.08
Post VLDL-TG 18:2n6 (µg/mL)	EPA/DHA per day	0.58	0.09
Post VLDL-TG 18:3n3 (µg/mL)	high sugar & high fat foods/day	-0.59	0.09
Post VLDL-TG 20:5n3 (µg/mL)	bar compliance	0.74	0.02
Post VLDL-TG 20:5n3 (µg/mL)	EPA/DHA per day	0.93	0.00
Post VLDL-TG 22:6n3 (µg/mL)	EPA/DHA per day	0.82	0.01
Post VLDL-TG 18:2n6 (%)	spread compliance	0.68	0.04
Post VLDL-TG 18:3n3 (%)	spread compliance	0.59	0.09
Post VLDL-TG 18:3n3 (%)	high sugar & high fat foods/day	-0.61	0.08
Post VLDL-TG 20:5n3 (%)	bar compliance	0.71	0.02
Post VLDL-TG 20:5n3 (%)	EPA/DHA per day	0.86	0.003
Post TPL 22:6n3 (%)	% change TC	-0.74	0.02
Post TPL Total FA (µg/mL)	bar compliance	-0.73	0.03
% change TPL 20:5n3	% change TC	-0.61	0.08
% change TPL 20:5n3	EPA/DHA per day	0.66	0.054
% change TPL Total FA	bar compliance	-0.65	0.059
% change TPL Total FA	% change LDL	0.81	0.02

7.2.2.4. Fatty acid synthesis after diet intervention

Fasting whole-body DNFA was reduced after diet intervention in Control subjects, though this did not reach statistical significance (**Figure 7-4A**). Fasting whole-body synthesis of individual FA was not different after diet intervention (data not shown) except for 16:0 which was lower (Pre 2.2 ± 0.6% vs. Post 0.83 ±0.1%). Whole-body postprandial synthesis of total (**Appendix A**) or individual FA (data not shown) were not significantly different at any timepoint in Controls, but tended to be lower after diet intervention. Percent change in whole-body DNFA was not related to compliance to the bar, spread, or diet, or with quantity intake of EPA/DHA or other supplement ingredients.

Fasting hepatic total DNFA was significantly reduced by diet intervention in control subjects (**Figure 7-4B**). Fasting hepatic synthesis of 14:0, 16:0, 16:1, 18:0 and 18:1 were lower after diet intervention in control subjects (**Figure 7-4C**). Hepatic postprandial synthesis of total VLDL-TG FA was significantly lower at 4 h (Pre 0.77 ± 0.3% vs. Post 0.19 ± 0.1%) and 6 h (Pre 1.1 ± 0.5% vs. Post 0.20 ± 0.1%) after diet intervention (**Appendix A**). Hepatic postprandial synthesis of individual VLDL-TG FA (data not shown) was not different at any timepoint for any FA except for 14:0 which was lower at 2h (Pre 4.1 ± 0.9% vs. Post 1.9 ± 0.7%) and 6 h (Pre 3.8 ± 1% vs. Post 2.6 ± 0.9%), 16:0 which was lower at 4 h (Pre 1.7 ± 0.6% vs. Post 0.55 ± 0.2%), 6 h (Pre 2.3 ± 0.9% vs. Post 0.51 ± 0.2%) and 8 h (Pre 2.8 ± 0.7% vs. Post 1.3 ± 0.4%), and 18:1 which was lower at 4 h (Pre 0.35 ± 0.1% vs. Post 0.04 ± 0.02%). Percent change in plasma TG levels was significantly related to baseline whole-body (*r=-0.77*) and hepatic (*r=-0.70*) DNFA, indicating those with higher baseline lipogenesis experienced greater reductions in plasma TG levels. Percent change in hepatic DNFA was not related to compliance to the bar, spread, or diet, or with quantity intake of EPA/DHA or other

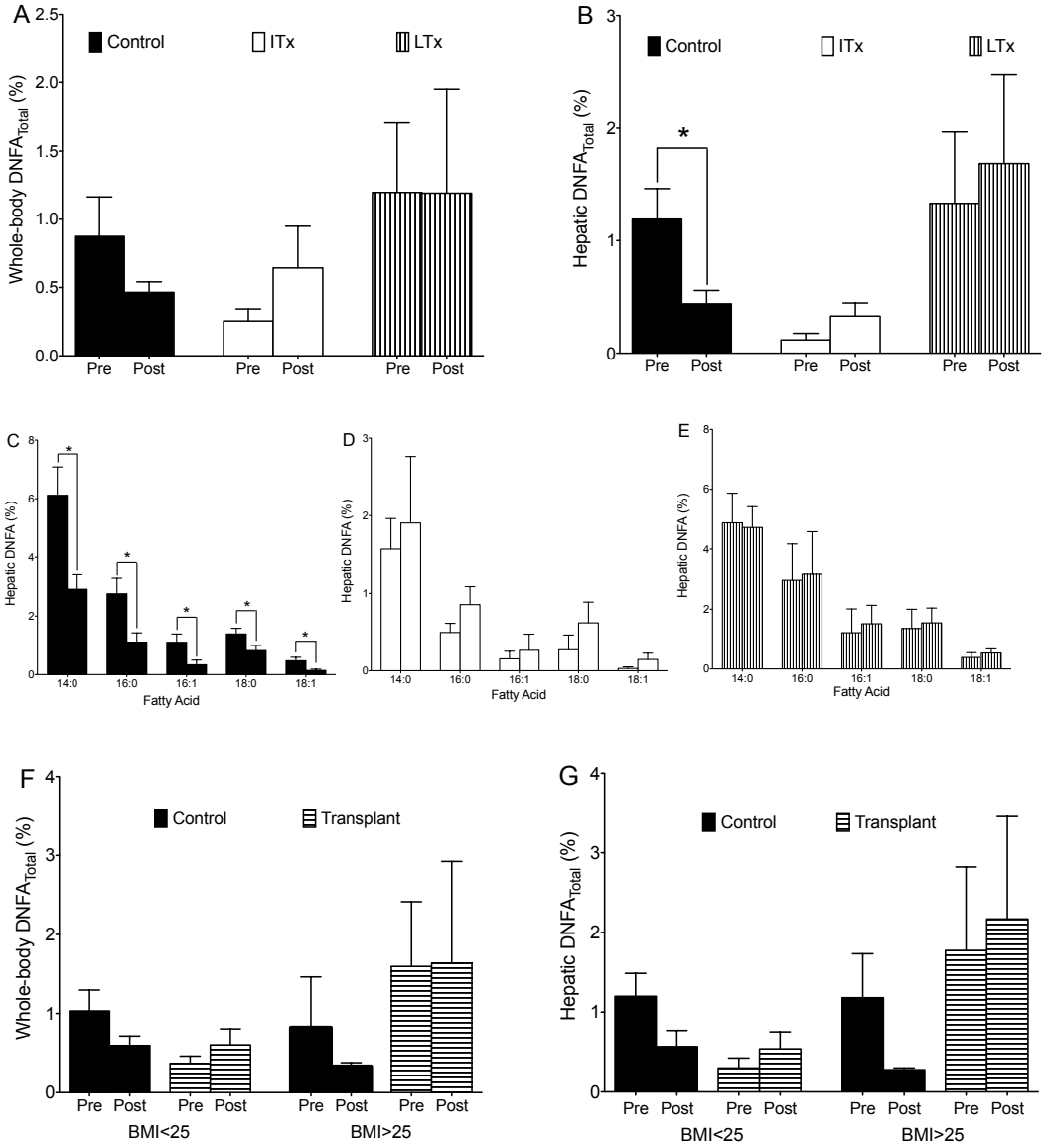
supplement ingredients.

Whole-body DNFA was not different after diet intervention in ITx or LTx subjects (**Figure 7-4A**). Similarly, fasting whole-body synthesis of individual FA was not significantly different after diet intervention in ITx or LTx subjects (data not shown). Postprandial whole-body synthesis of total plasma-TG FA (**Appendix A**) was significantly higher in ITx subjects at 2h (Pre 0.16 ± 0.1% vs. Post 0.20 ± 0.1%) but synthesis of individual FA (data not shown) were not different at any postprandial timepoint. Whole-body synthesis of total plasma-TG FA (**Appendix A**) was not different at any postprandial timepoint in LTx subjects after diet intervention, however postprandial synthesis of 16:0 was lower at 2 h (Pre 0.82 ± 0.4% vs. Post 0.58 ± 0.3%) and 4 h (Pre 1.0 ± 0.5% vs. Post 0.78 ± 0.5 %), and synthesis of 18:0 was higher at 2 h (Pre 0.32 ± 0.2% vs. Post 0.83 ± 0.3%) after diet intervention. Percent change in whole-body DNFA was not related to compliance to the bar, spread, or diet, or with quantity intake of EPA/DHA or other supplement ingredients in ITx, LTx, or pooled transplant groups.

There was no significant difference in rate of fasting hepatic total DNFA in ITx subjects after diet intervention, though DNFA tended to be higher compared to pre-intervention (**Figure 7-4B**). Fasting hepatic synthesis of individual FA were not different after diet intervention in ITx subjects (**Figure 7-4D**). Hepatic postprandial synthesis of total VLDL-TG FA was not significantly different at any timepoint after diet intervention in ITx subjects (**Appendix A**) or in individual FA except for 14:0 which was higher at 6 h (Pre 0.37 ± 0.1% vs. Post 0.98 ± 0.1%), and 16:1 which was lower at 8 h (Pre 0.59 ± 0.1% vs. Post 0.07 ± 0.1%). There was no significant difference in rate of fasting hepatic total DNFA synthesis in LTx subjects after diet intervention (**Figure 7-4B**). Fasting hepatic synthesis of individual FA were not different after diet intervention in LTx subjects (**Figure 7-4E**). Hepatic postprandial synthesis of total (**Appendix A**) and individual VLDL-TG FA

(data not shown) were not significantly different at any timepoint and for any FA after diet intervention in LTx subjects. When the transplant groups were pooled and divided based on BMI (as above), there were no significant changes in whole-body or hepatic DNFA synthesis in either transplant group (**Figure 7-4F and 4G**). Percent change in hepatic DNFA was not related to compliance to the bar, spread, or diet, or with quantity intake of EPA/DHA or other supplement ingredients in ITx, LTx, or pooled transplant groups.

Figure 7-4: Change in fatty acid synthesis in control, islet, and liver transplant subjects after diet intervention. A) Fasting whole-body de novo total fatty acid (DNFA) synthesis; B) Fasting hepatic total DNFA; C) Fasting hepatic DNFA of individual FA in control subjects; D) Fasting hepatic DNFA of individual FA in islet transplant subjects; E) Fasting hepatic DNFA of individual FA in liver transplant subjects; F) Fasting whole-body DNFA in pooled groups; G) Fasting hepatic total DNFA in pooled groups.



Data expressed as mean ± SEM; * indicates significant difference within group $p<0.05$

7.2.2.5. Cholesterol synthesis after diet intervention

Fasting whole-body and hepatic synthesis of FC and whole-body CE

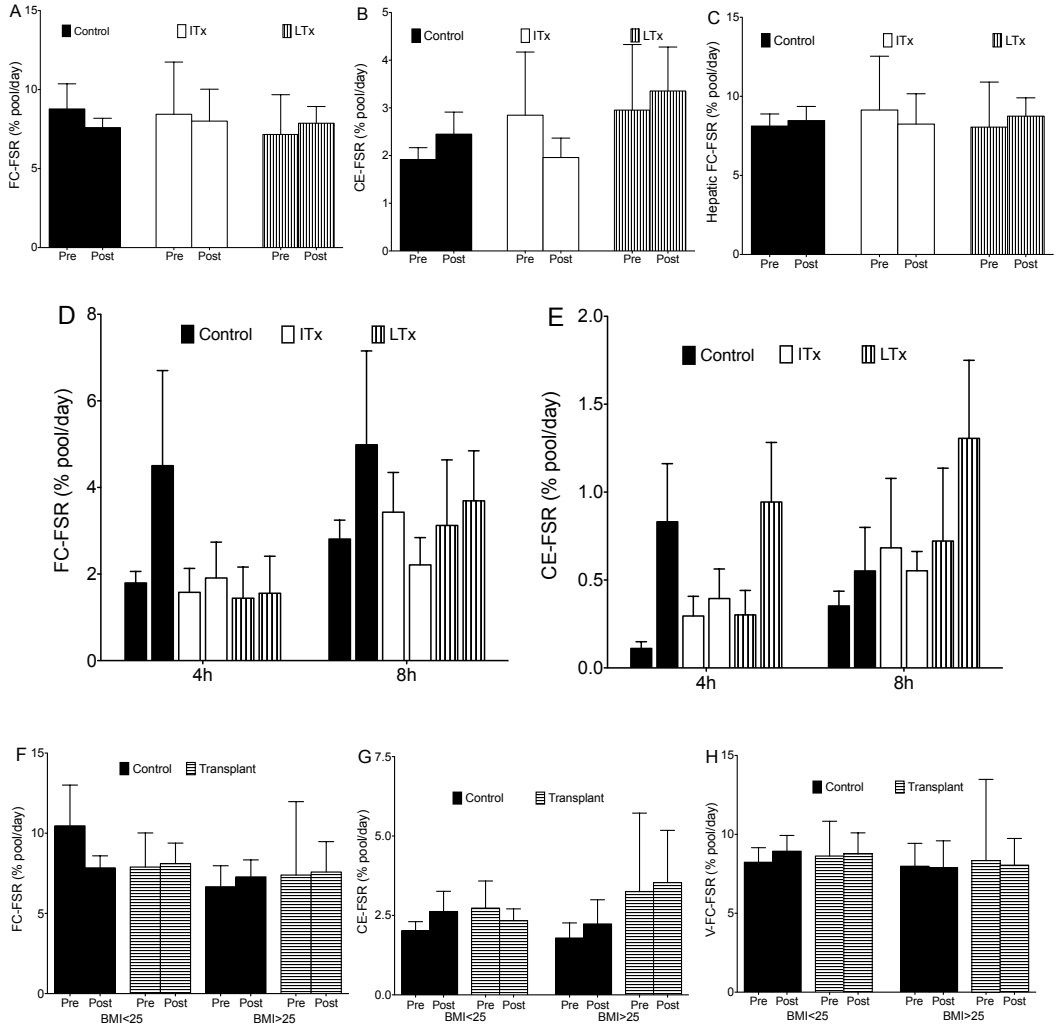
synthesis did not change after diet intervention in any group (**Figure 7-5A-C**). Likewise, postprandial synthesis of FC and CE did not differ after diet intervention in any group at either 4 h or 8 h (**Figure 7-5D and 5E**). When the transplant groups were pooled and divided based on BMI (as above), there were no significant changes in whole-body or hepatic FC synthesis, or CE synthesis in either transplant group (**Figure 7-5F-G**). Percent change in whole-body or hepatic FC-FSR was not related to compliance to the bar, spread, or diet, or with quantity intake of EPA/DHA or other supplement ingredients in control, ITx, LTx, or pooled transplant groups.

Percent change in plasma TC levels was positively correlated with baseline whole-body and hepatic synthesis of FC and CE (*r=1.00* for each) in ITx patients. In LTx patients percent change in plasma TC levels was positively correlated with CE synthesis only (*r=0.90*), while FC-FSR was correlated (*r=0.80*) though this did not reach significance. When transplant groups were combined, percent change in plasma TC levels was positively correlated with FC-FSR only (*r=0.63*), though to a lesser degree than in ITx subjects. This data suggests that those with higher baseline fractional synthesis of cholesterol experienced greater increases in plasma cholesterol levels in response to diet intervention. Percent change in plasma cholesterol levels was also negatively correlated with percent change in whole-body (*r=-0.68*) and hepatic (*r=-0.68*) synthesis, and percent change in LDL-c was also correlated with percent change in whole-body (*r=-0.81*) and hepatic (*r=-0.76*) FC synthesis. Percent change in whole-body FC-FSR was negatively correlated with baseline FC-FSR in the pooled transplant group (*r=-0.72*). Percent change in hepatic FC-FSR was also negatively correlated with baseline hepatic FC-FSR (*r=-0.80*) in the pooled transplant group, indicating that the higher the baseline cholesterol synthesis the lower the change in FC-FSR with diet intervention.

Percent change in plasma TG levels was positively related to baseline

CE-FSR in ITx (*r=0.90*) and LTx (*r=1.00*) subjects, and to whole-body (*r=0.90*) and hepatic FC (*r=0.90*) synthesis in LTx subjects. These correlations indicate those with lower cholesterol synthesis had greater reductions in plasma TG levels. In the pooled transplant group, change in plasma TG level was significantly related to baseline whole-body (*r=0.78*) and hepatic (*r=0.77*) FC synthesis and baseline CE-FSR (*r=0.82*), as well as to percent change whole-body and hepatic FC-FSR (*r=-0.83* for both). This finding indicates that individuals with lower cholesterol synthesis had a greater reduction (or, conversely, lesser increase) in plasma TG levels. Percent change in plasma TG was also correlated with percent change in LDL-c level (*r=0.86*), indicating those subjects with lower change in LDL-c level also had lower change in plasma TG level. Percent change in plasma TG was not related to baseline whole-body or hepatic DNFA. Percent change in plasma TG level was related to percent change in whole-body (*r=0.75*) but not hepatic DNFA in the pooled transplant patients.

Figure 7-5: Change in cholesterol synthesis in control, islet, and liver transplant subjects after diet intervention. A) Fasting whole-body free cholesterol (FC) synthesis (FSR); B) Fasting whole-body cholesteryl ester (CE) synthesis; C) Fasting hepatic FC-FSR; D) Postprandial FC-FSR; E) Postprandial CE-FSR; F) Fasting whole-body FC-FSR in pooled groups; G) Fasting whole-body CE-FSR in pooled groups; H) Fasting hepatic FC-FSR in pooled groups.



Data expressed as mean ± SEM

7.3. Discussion and Conclusions

The present study provides evidence that a dietary intervention utilizing multiple functional ingredients and provided in an efficient and accessible manner is effective in healthy subjects but has mixed results in

post-transplant patients. This type of diet intervention may have promise for hyperlipidemic post-transplant patients, however the current analysis is limited by low sample size of these types of subjects. Therefore, a further investigation of the efficacy of this diet intervention in a greater number of hyperlipidemic subjects is required to make definitive conclusions. Response to dietary therapy in post-transplant patients may be complicated by underlying lipid synthetic regulation.

In the present study diet intervention reduced plasma TC and LDL-c levels in healthy normolipidemic individuals. Though absolute changes in TC and LDL-c were small (5-10% average reduction) there was a consistent reduction across individuals in the Control group. Plasma TG levels were not significantly reduced after diet intervention, however this may be due to the relatively low baseline plasma TG levels in these subjects (**Table 7-3**). In subjects with plasma TG levels >1 mmol/L, the diet intervention reduced plasma TG levels by approximately 50% . Overall this data indicates that the diet was effective in reducing plasma lipid levels in normal subjects without metabolic disturbances. In addition, the fact that the diet intervention was able to achieve a significant and consistent lipid-lowering effect is a clinically significant finding, considering that these were normolipidemic subjects and lowering plasma lipid levels in individuals who already have normal lipid levels is considerably difficult. The diet may have also improved postprandial metabolism. This data indicates that this type of intervention may be successful in other groups of patients who may have or be at-risk for hyperlipidemia, but this remains to be determined. Efficacy of the supplements used in this intervention may also depend on the rest of the dietary pattern.

The diet intervention had mixed results in the transplant subjects. Contrary to reasonable predictions, transplant subjects with normal lipid levels and weight actually had an increase in plasma cholesterol levels after

diet intervention. However, the plasma TC and LDL-c levels still fall under "normal" recommendations (TC<5.2 mmol/L; LDL-c<3.4 mmol/L; TG <1.7 mmol/L) (*Antonopoulos 2002*). There were only 3 subjects in the heavier BMI transplant group, which may be considered case studies. When these subjects are considered, Tx-B and Tx-C experienced reductions in plasma TC and TG. Therefore it could be suggested that the dietary intervention has promise for some transplant patients with hyperlipidemia, however this will need confirmation with larger studies in these patients. Of these subjects, Tx-C was taking Tacrolimus and Tx-B was taking Sirolimus; therefore it is not possible to say from these results if type of immunosuppressive medication influences response to diet intervention. A third subject, Tx-A, had no change in plasma TC and actually had an increase in plasma TG, despite a very high baseline plasma TG level and excellent compliance to the bars and overall diet. This subject may have other metabolic disturbances that were not able to be characterized by this study, because this subject had isolated elevated TG with low plasma TC; therefore, this subject might represent an anomaly.

There are a few reasons why the diet intervention did not lower plasma lipid levels in the majority of the transplant subjects. First, it could be due to the fact that most of the transplant subjects had normal lipid levels to begin with, and that reducing them further is very difficult. Second, it could be suggested that the amounts of functional ingredients provided by the supplements were not high enough to effectively reduce plasma TG levels in these subjects. Bars were dosed based on weight to ensure a minimum 15 mg EPA/DHA/day per kg body weight of the subject. For most subjects in the trial this equated to a minimum of ~1 g EPA/DHA per day, up to ~1.2 g/day, while the heavier subjects consumed ~2 g EPA/DHA per day. It is emphasized that amounts of EPA/DHA discussed herein are solely EPA/DHA and do not refer to dose of fish oil as a whole, which is not always distinguished in other studies. Fish oil is considered safe in transplant

patients and does not appear to affect graft rejection (*van der Heide et al. 1993*; *Kooijmans-Coutinho et al. 1996*; *Santos et al. 2000*). Available data suggests that lower doses of EPA/DHA <2 g/day may improve plasma TG levels to a small degree (10%) while larger doses (~3 g/day) may be more effective, particularly when combined with statin therapy (*Castro et al. 1997*; *Santos et al. 2000*; *Grekas et al. 2001*; *Hernández et al. 2002*; *Celik et al. 2008*). Phytosterol intake for most subjects in the trial was~1.5-2.3 g phytosterols per day, and for the heavier subjects was 3-4.5 g phytosterols per day. These levels are similar to that recommended by the American Heart Association National Cholesterol Education Program (*Antonopoulos 2002*). Phytosterol-containing foods provided to renal transplant patients on Cyclosporine therapy providing 2 g/day of phytosterols reduced plasma TC levels by ~9% in patients taking statins and ~7% in patients not taking statins (*Sutton et al. 2009*), whereas consumption of phytosterol-enriched margarine providing 2.5 g/day phytosterols lowered plasma TC and LDL-c 20% in cardiac transplant patients also maintained on statin therapy and steroid and Cyclosporine-based immunosuppression (*Vorlat et al. 2003*).

The heavier transplant patients were those who received greater amounts of functional ingredients and showed reductions in plasma lipid levels in response to dietary therapy; however these subjects also tended to be more compliant to the supplements and diet plan in general, therefore conclusions about adequacy of functional ingredient amount are limited. The normal weight transplant patients had functional nutrient intake similar to but significantly lower than the control subjects (**Table 7-6**). The dietary portfolio by Jenkins and colleagues emphasizes similar functional nutrients to the present study (*Esfahani et al. 2010*). This dietary portfolio achieved TG reduction of 9% and TC reduction of 25% in hyperlipidemic subjects (*Jenkins et al. 2005*; *Esfahani et al. 2010*). The dietary portfolio provides 1-1.2 g/day phytosterols, 16-23 g/day soy protein, and 8-10 g/day viscous fiber per 1000

kcal (*Esfahani et al. 2010*), which are higher amounts of soy and fiber than in the current study. Therefore the possibility remains that even higher levels are needed for reduction in plasma lipid levels in transplant subjects, or to maintain lipid levels without need for lipid-lowering drugs. However, the levels administered in the present study achieved cholesterol and TG reduction in some transplant subjects with elevated baseline lipid levels, thereby providing support that a lipid-lowering effect can be achieved with moderate doses of these ingredients.

Finally, it could be argued that the diet intervention was not long enough to see meaningful changes in plasma lipid levels. Most dietary interventions utilizing specific nutrients in transplant recipients have been 5 weeks to 12 months in duration (*Santos et al. 2000*; *Cupisti et al. 2004*; *Celik et al. 2008*). It is unlikely that lack of adequate intervention time is the result of the mixed results in the present investigation; subjects in the current study with elevated lipid levels did experience reductions in plasma lipid levels, and it may well be that the individuals without elevated plasma lipids would not experience reductions in cholesterol and TG even with longer intervention periods. Interventions in other transplant groups of shorter durations, such as 2 weeks, have shown significant changes in plasma lipid levels (*Nelson et al. 1988*), and there are also interventions lasting up to 12 months that did not show an effect on plasma lipids (*Santos et al. 2000*). Most dietary pattern interventions require longer intervention periods to allow adoption of new dietary habits; in comparison, the menu plan in the present study was a set meal plan with prescribed food types and quantities. A longer dietary phase may have increased the likelihood of reduced dietary compliance over time. Therefore, differences between intervention efficacies in transplant populations may have to do with patient selection and type of intervention as opposed to duration.

FA composition of plasma lipids did not reflect changes in FA intake

despite consumption of EPA/DHA through the dietary supplements. However, most of the plasma lipid FA showed trends towards increase in EPA and DHA, though most did not reach statistical significance. It could be that dietary EPA/DHA are preferentially stored in other tissue lipids instead of incorporated into plasma lipids for transportation, or that higher dosages are required for significant change in plasma lipid FA compositions. TPL FA composition and concentrations showed the greatest response to dietary therapy and showed trends toward increasing levels of EPA/DHA following diet intervention in the subjects. Phospholipid FA composition is believed to be a better marker of EPA/DHA status compared to other plasma lipids such as plasma TG (*Fekete et al. 2009*). Badalamenti et al (1995) found that treatment of 3.6 g/day EPA/DHA in liver transplant patients reduced plasma FA 20:4n6 from 6.2% to 4.8% and increased 20:5n3 from 0.4% to 4.6% and 22:6n3 from 1.8% to 3.9% (*Badalamenti et al. 1995*). The baseline percent contribution of 20:4n6, 20:5n3, and 22:6n3 are similar in the present study compared to that of Badalamenti et al (1995), but the post-dietary intervention levels do not reach the levels obtained by these investigators. Therefore it may be that higher supplemental amounts of fish oil are required to reflect significant changes in FA fractions in transplant subjects. Changes in some key FA (20:4n6, 20:5n3, and 22:6n3) from plasma lipid fractions were related to bar compliance and nutrient intake (**Table 7-7**), therefore it could be that total fish oil intake influences plasma individual FA concentrations.

Plasma TG levels and reductions in plasma TG was related to baseline lipogenesis in control subjects but not in transplant subjects. This may be because the rates of lipogenesis in the transplant subjects were so low that they would be unlikely to be reduced further. It could also be due to the fact that lipogenesis appeared to be lower in the transplant subjects despite comparable plasma TG levels for most of the subjects, and because other factors such as particle clearance or production may be more significantly

related to plasma TG levels in these subjects. EPA/DHA are believed to lower plasma TG levels in part by suppressing lipogenesis by reducing SREBP-1c expression, as well as by upregulating FA oxidation (*Davidson 2006*; *Jump et al. 2008*; *Di Nunzio et al. 2010*). Therefore, the reduction in lipogenesis after dietary intervention in Control subjects may be due to the effects of EPA/DHA consumption.

Interestingly, plasma levels of and changes in plasma TG were related to basal cholesterol synthesis and changes in LDL-c levels and cholesterol synthesis rates in transplant subjects. If intestinal cholesterol absorption is reduced in response to the dietary components such as phytosterols and fibers, the liver may be upregulating cholesterol synthesis as well as LDL-receptors. Upregulation of LDL-receptors would encourage lipoprotein clearance which would serve to reduce plasma levels of cholesterol as well as TG. Alternatively, PUFA have been suggested to reduce VLDL synthesis by downregulating microsomal TG transfer protein required for lipoprotein assembly (*Minihane 2009*), which may also reduce plasma TG levels by reducing TG incorporation and release into VLDL particles.

Increases in plasma cholesterol levels could result from an increase in synthesis, increase in absorption from dietary or biliary sources, reduced plasma clearance, or a combination of these factors. The control subjects in the present study showed a reduction in plasma cholesterol levels also without a significant change in fasting cholesterol synthesis. It is predicted in the control subjects that there may have been a reduction in cholesterol absorption owing to the dietary supplement phytosterols and fibers that contributed to this reduction in plasma cholesterol levels despite no change in cholesterol synthesis. The majority of the transplant patients in the present study showed an increase in plasma cholesterol levels after dietary intervention in the absence of an increase in cholesterol synthesis. Therefore, rise in plasma cholesterol levels may have been due to either increased

cholesterol absorption or reduction in plasma lipoprotein clearance. Phytosterols are reported to reduce intestinal cholesterol absorption and were provided at ~2 g/day in the current study, comparable to the dosage suggested by the American Heart Association (*Antonopoulos 2002*).

Therefore it is possible that lipoprotein clearance may have been impaired by the diet intervention. The majority of patients experiencing increases in plasma cholesterol levels were the ITx patients, who had also previously been maintained on statin therapy despite having plasma lipid levels at or below current recommendations. Statins lower plasma cholesterol levels by reducing hepatic cholesterol synthesis which causes a compensatory upregulation of LDL-receptors, resulting in increased lipoprotein uptake by the liver and clearance from the circulation (*Reihnér et al. 1990*; *Raggatt et al. 2002*). Tacrolimus immunosuppression is suggested to impair activity of LPL, which is a key enzyme in lipoprotein modification for removal from the circulation (*Tory et al. 2008*; *Tory et al. 2009*). Impairment of LPL can lead to reduced removal of lipids from the circulation, contributing to elevated plasma lipid concentrations. One possibility is that the upregulation of LDL-receptors by statin therapy partially off-sets the deleterious effects of Tacrolimus on LPL, thereby helping to maintain plasma lipid clearance from the blood. The patients in the current study were requested to abstain from statin therapy for 1 week prior to the baseline testing day, and for the last 2 weeks of the dietary intervention. Due to reduction in intestinal cholesterol absorption, phytosterols may also induce an increase in hepatic cholesterol synthesis and upregulation of hepatic LDL-receptors (*Ruiu et al. 2009*; *Calpe-Berdiel et al. 2009*; *AbuMweis et al. 2009*). It may be that the potential upregulation of LDL-receptors by phytosterol intake is not enough to offset the effects of Tacrolimus on plasma lipid clearance, and that a stronger effect inducible by statins is required in these patients. It is important to note that these transplant patients had normal

lipid levels in absence of statin therapy, as well as after the diet intervention when plasma cholesterol levels were increased.

Response to dietary therapy in the transplant patients was related to baseline cholesterol synthesis rate, suggesting that those with higher cholesterol synthesis had lower reduction and actually greater increase in plasma cholesterol levels in response to diet therapy. In addition, the degree of change in plasma cholesterol levels was negatively correlated with percent change in whole-body and hepatic synthesis, suggesting that an increase in cholesterol synthesis is associated with a greater decrease (or, on the contrary, a lesser increase) in plasma cholesterol levels. This finding may be a reflection of greater response to phytosterol-induced reduction of cholesterol absorption. Percent change in cholesterol synthesis was also negatively correlated with baseline cholesterol synthesis in the transplant patients, suggesting that the higher the baseline cholesterol synthesis the lower the change in cholesterol synthesis with diet intervention. Taken together, these results indicate that individuals with high basal cholesterol synthesis experienced greater increases in plasma cholesterol levels and lesser change in cholesterol synthesis in response to dietary therapy compared to those with lower rates of cholesterol synthesis. It may be that those with lower rates of synthesis naturally have higher rates of intestinal cholesterol absorption, as suggested in the literature (*Miettinen et al. 1989*; *Miettinen et al. 2000*; *Santosa et al. 2007b*). If this is true, than it could be expected that those individuals with naturally higher absorptive capacities would respond better to the current dietary intervention emphasizing phytosterols and fibers, which would reduce cholesterol absorption and cause a compensatory increase in cholesterol synthesis while still achieving reduction in plasma cholesterol levels. Conversely, it may be that the individuals with higher cholesterol synthesis have lower cholesterol absorption and therefore phytosterols would have a relatively weaker influence on blocking intestinal

cholesterol absorption, as suggested by other interventions utilizing phytosterols (*Gylling et al. 2002*; *Rideout et al. 2010*). For example, Rideout et al (2010) showed that individuals with lower basal cholesterol synthesis responded better to phytosterol therapy than individuals with higher basal cholesterol synthesis, and a previous investigation had also shown no different in 24h fasting FC-FSR after a period of intervention using phytosterol milks (*Rideout et al. 2009*; *Rideout et al. 2010*). Unfortunately markers of cholesterol absorption were not measured in the present study, but would be of interest to include in future studies in transplant patients. This relationship between basal cholesterol synthesis and change in plasma cholesterol levels after diet intervention were not significant in the control subjects, but this may be due to the smaller range in plasma cholesterol reduction (+4% to -13%), and perhaps would be significant if a larger group of subjects were studied.

A limitation of this study is that more transplant patients with elevated lipid levels were not included. However, an advantage of the present study was that almost all of the subjects were on the same immunosuppressive medication, allowing for comparisons between individuals with different metabolic characteristics. On the other hand, this fact also limits generalization to patients taking other types of immunosuppressive medications. In this study a reasonably high level of compliance was achieved for the overall diet and the supplement components, and there were no drop-outs during the course of the intervention. Part of the high rate of compliance was likely due to the nature of the supplement bars provided, which were small and incorporated all functional nutrients targeted in the study. This allowed for the use of a supplement that was portable and easy to incorporate into daily dietary patterns. In addition, the supplement bars were generally well-received. The menu log book served as a 4-week dietary food record providing for detailed

analysis of dietary consumption and deviations from the menu plan. It could be suggested that the base menu plan may be a healthier diet than what the control and transplant subjects would normally consume, and that this dietary change could be responsible for the changes in plasma lipid levels noted in the control subjects and some of the transplant subjects. This is unlikely given that reductions in plasma cholesterol and TG levels in healthy subjects is difficult to achieve, and expected to be even more difficult to achieve in individuals with altered metabolism. In addition, the 50% reduction in plasma TG levels in some of the control subjects would be highly unlikely to occur due to the base menu plan alone and is likely only achievable by dietary supplementation with EPA/DHA.

In conclusion, this is the first study to investigate a dietary intervention in islet transplant recipients, and the first to investigate use of fish oil and phytosterols in liver transplant patients for reducing plasma lipid levels. In summary, this study shows that a dietary intervention may be effective in hyperlipidemic transplant patients but further studies with larger sample sizes are required. Dietary components may elicit surprising responses in plasma lipid levels in transplant patients that may be related to underlying regulation of cholesterol synthesis. The potential exists for tailoring of dietary protocols to transplant patients depending on individual rates of basal cholesterol synthesis, however this concept requires further investigation.

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8. Conclusions and Future Directions

In the present investigation, it is apparent that normal plasma cholesterol or triglyceride concentrations may not reflect normal cholesterol and fat metabolism. As such, determination of lipid metabolic abnormalities specific to individual disease states is important. Determination of metabolic aberrations can then serve to guide treatment in such disease states, and in some cases may lead to development of markers or indicators of disease status or severity. In addition, aberrations in lipid metabolism may interfere with treatment modalities such as dietary therapy; therefore, identification of potential mechanisms can allow for targeting of appropriate therapies and may help to suggest which types of subjects respond to specific therapies.

8.1. Hypothesis #1: De novo lipogenesis will be higher and cholesterol synthesis lower in brittle Type 1 diabetes.

8.1.1. Conclusions

This study was undertaken to quantitatively determine FA and cholesterol synthesis in Type 1 diabetes. There had been no previous studies on DNL in individuals with Type 1 diabetes. In previous studies cholesterol synthesis had been proposed to be lower in people with Type 1 diabetes compared to non-diabetic control subjects, while cholesterol absorption was proposed to be higher (*Gylling et al. 2004*).

Contrary to the hypothesis, lipogenesis and cholesterol synthesis were not different in individuals with brittle Type 1 diabetes compared to healthy control subjects. In matched subjects, hepatic DNFA and cholesterol synthesis tended to be lower in the diabetes group compared to control, whereas whole-body cholesterol synthesis showed mixed results. Postprandial hepatic DNFA was also not different between groups, but tended to be lower in the

diabetes group at all timepoints. Neither fasting or postprandial DNFA was correlated with plasma insulin levels in the diabetes subjects. Fasting plasma lipid levels were comparable between diabetes and healthy subjects. Postprandial plasma lipid levels were not different between groups at any timepoint measured, however plasma TG levels tended to be higher in the diabetes group at 6 and 8 hours compared to control subjects.

8.1.2. Significance of Findings and Future Directions

Postprandial measurements of plasma TG suggested that there may be delayed lipoprotein clearance after meal consumption in Type 1 diabetes. Future studies aimed at answering this question could measure activity of LPL during postprandial testing to determine if this enzyme is impaired in diabetes contributing to reduced particle modification and clearance. In addition, levels of ApoB48 indicating chylomicron particle number could also be measured at postprandial timepoints to investigate intestinal lipoprotein production. If cholesterol absorption indeed prevails over cholesterol synthesis, suggested by Gylling (2004), cholesterol could be isotopically-labelled and fed to participants in tandem with deuterium-labelled water in order to quantitatively determine if cholesterol absorption is elevated and cholesterol synthesis lower in Type 1 diabetes. However, the cost of isotopically-labelled cholesterol compounds may impede this type of investigation. An alternative investigation could test the effect of dietary phytosterols on plasma lipid levels in individuals with Type 1 diabetes, in addition to measurement of deuterium incorporation into newly synthesized cholesterol. By this method, it could potentially be determined if there is a relationship between response to phytosterol therapy and cholesterol synthesis in diabetes. If absorption is higher and synthesis lower in Type 1 diabetes, then phytosterol or ezetimibe therapy targeting intestinal cholesterol absorption may be more appropriate for management of plasma cholesterol.

In this particular study, insulin injections were not controlled due to the brittle and unpredictable nature of diabetes in these subjects. However, it was found that plasma insulin levels were not correlated with lipogenesis or cholesterol synthesis at fasting or postprandial timepoints. Future studies could quantify lipogenesis and cholesterol synthesis in people with diabetes with greater glycemic control, as well as under timed or controlled insulin dosing, to determine if insulin excess or deficiency has an impact on rate of FA or cholesterol synthesis. Alternatively, people with Type 1 diabetes controlled by multiple daily injections (similar to the subjects investigated in this study) could be compared to individuals utilizing insulin pumps designed to more closely mimic endogenous insulin secretion, in order to determine if insulin fluctuations or bolus influences lipid synthesis.

It has been suggested that hepatic lipogenesis may be lower in Type 1 diabetes in animal models, however this suggestion is in contrast to the observation that a significant proportion of individuals with diabetes have detectable fatty liver. A possible way to reconcile this question is to perform liver biopsies on T1D individuals with and without fatty liver confirmed by imaging methods. Liver samples could then be examined for hepatic lipid content, as well as deuterium incorporation into newly synthesized FA. However, the invasiveness of this type of investigation is an obvious limitation.

8.2. Hypothesis #2: Cholesterol synthesis will be lower and de novo lipogenesis will be higher in patients with liver failure.

8.2.1. Conclusions

This study was undertaken to investigate and quantify FA and cholesterol synthesis in patients with liver failure. Previously there have been no direct evaluations of lipogenesis and cholesterol synthesis in patients with hepatic failure. It is proposed that lipogenesis is elevated while cholesterol

synthesis is reduced, effects which may be mediated through HCV.

Plasma cholesterol levels were found to be lower in the liver failure group, while plasma TG levels were marginally higher. Fasting plasma insulin was significantly higher in the liver failure group. Analysis of VLDL FA composition showed lower essential PUFA content in the liver failure group compared to control subjects. Whole-body and hepatic lipogenesis were higher, while whole-body and hepatic cholesterol synthesis were lower in the liver failure group compared to controls. Hepatic lipogenesis was positively correlated with plasma TG levels in the liver failure group. When patients with and without HCV were compared, hepatic lipogenesis trended towards higher in patients with HCV. Cholesterol synthesis did not appear to be different between patients based on HCV status, however the small sample size limits conclusive comparisons. Therefore, despite low plasma lipid levels, lipogenesis is elevated in liver failure patients, which may contribute to development and progression of steatosis. Low plasma cholesterol levels may actually be indicative of reduced hepatic function leading to low cholesterol synthesis.

8.2.2. Significance of Findings and Future Directions

In the present study it was found that hepatic lipogenesis was positively related to plasma TG levels. It would be of interest to perform a study investigating whether degree of steatosis was related to either measured lipogenic rate or plasma TG level in patients with liver failure, particularly in patients with different primary causes of hepatic dysfunction. In this way, it could be determined if and how plasma TG levels could be used as markers for individuals who may have elevated fatty acid synthesis as well as steatosis.

It would be of interest to particularly focus on individuals with HCV. This is because HCV appears to influence both TG and cholesterol

metabolism; HCV may promote lipogenesis and TG synthesis thereby encouraging steatosis and contributing to hepatic damage, and HCV may also interfere with cholesterol synthesis for viral replication means, diverting cholesterol synthesis intermediates towards viral-related pathways and away from sterol synthesis. It would be interesting to use deuterium to measure lipogenesis and cholesterol synthesis in a larger group of patients with and without HCV to determine if lipogenesis and cholesterol synthesis are related to steatosis and HCV viral load. Potentially the deuterium method could be used as a marker for steatosis or HCV progression and severity in HCV-infected individuals.

8.3. Hypothesis #3: Fatty acid and cholesterol synthesis will be elevated in post-transplant patients.

8.3.1. Conclusions

This study was undertaken to investigate and quantify FA and cholesterol synthesis in patients after transplantation under immunosuppressive medication. Hyperlipidemia after transplant is common and is attributed to immunosuppressive drug use, however the mechanisms responsible have not been elucidated. Previously there have been no direct evaluations of lipogenesis and cholesterol synthesis in post-transplant humans. It is proposed that lipogenesis and cholesterol synthesis are elevated post-transplant and may be mechanisms contributing to post-transplant hyperlipidemia.

Contrary to the hypothesis, fasting whole-body and hepatic lipogenesis was significantly lower in islet transplant patients compared to control and liver transplant subjects. When subjects were divided based on BMI, in case differences in adiposity and related factors were concealing differences between groups, it was determined that whole-body and hepatic lipogenesis was significantly lower in the normal BMI transplant group

compared to control subjects. Lipogenesis was similar between higher BMI transplant and control subjects despite markedly higher plasma lipid levels in the higher BMI transplant subjects compared to controls. In contrast, fasting whole-body and hepatic synthesis of free or esterified cholesterol was not significantly different between groups, even when subjects were divided based on BMI. Therefore it is unlikely that lipogenesis and cholesterol synthesis are mechanisms by which immunosuppressive medication induces hyperlipidemia.

8.3.2. Significance of Findings and Future Directions

This study focused primarily on Tacrolimus-based immunosuppression, and only one transplant subject was taking Sirolimus. Therefore it would be of interest to recruit a larger group of subjects to determine if major immunosuppressive medications including Tacrolimus, Sirolimus, and Cyclosporine have similar effects on inhibiting lipogenesis. It would also be of interest to measure plasma LPL activity to gauge lipoprotein particle clearance efficiency, as well as ApoB48 to determine chylomicron particle number, in order to determine if elevations in plasma lipid levels may instead result from impaired lipoprotein clearance or elevated lipoprotein synthesis and secretion. Further, studies of lipoprotein turnover could be implemented which would yield valuable information regarding postprandial lipid metabolism kinetics.

It is difficult to determine whether the degree of hyperinsulinemia and insulin resistance exacerbates aberrations in plasma lipid metabolism and contributes to elevated plasma levels, particularly plasma TG levels, in post-transplant patients. Therefore, it would be of interest to perform a larger study examining distinct groups of post-transplant subjects that differ in insulin sensitivity, including subjects with normal fasting insulin, elevated fasting insulin but normal plasma glucose levels, and insulin resistance.

The very low rate of lipogenesis in the normal weight transplant subjects may indicate a greater importance of dietary fat intake and composition compared to healthy subjects, particularly for intake of unsaturated FA. The products of de novo lipogenesis are primarily saturated FA; the principal FA synthesized is palmitic acid. By comparison, there is relatively low rates of synthesis of oleic acid (18:1) even in the control subjects; therefore in the transplant patients dietary intake of monounsaturated and polyunsaturated fat may be particularly important due to the even lower rates of de novo synthesis compared to the control subjects.

8.4. Hypothesis #4: A dietary intervention incorporating a variety of nutrients will reduce plasma lipid levels, de novo lipogenesis, and cholesterol synthesis in post-transplant patients.

8.4.1. Conclusions

The diet intervention reduced plasma cholesterol levels in the control subjects, and reduced plasma TG levels in control subjects with baseline plasma TG >1.0 mmol/L. In contrast, the diet intervention increased plasma cholesterol levels in the ITx group and had mixed results in the LTx group with some subjects exhibiting decreases, no change, or increases in plasma cholesterol and TG. Since the majority of ITx and LTx subjects had normal baseline plasma lipid levels, it may be that inducing a reduction further is extremely difficult and unlikely to occur in this population except by pharmacologic intervention. The diet intervention was effective in reducing plasma cholesterol and TG in two LTx subjects with elevated baseline plasma lipid levels, indicating that this type of diet intervention may be successful for hyperlipidemic post-transplant patients.

The diet intervention reduced fasting whole-body and hepatic lipogenesis in the control subjects but not in the transplant groups. This finding again may be due to the already very low rates of lipogenesis noted in

the transplant patients. The diet intervention did not significantly change fasting cholesterol synthesis in any of the groups. Interestingly in the transplant subjects response to dietary therapy was related to baseline cholesterol synthesis rate and with change in cholesterol synthesis after the diet intervention. These findings suggest that those with higher cholesterol synthesis had lower reduction and actually greater increase in plasma cholesterol levels in response to diet therapy, and an increase in cholesterol synthesis was associated with a greater decrease (or, on the contrary, a lesser increase) in plasma cholesterol levels. Therefore the potential exists for measurement of basal cholesterol synthesis to allow for targeting treatment, however further studies are required.

8.4.2. Significance of Findings and Future Directions

Potential further research could include studying a larger group of transplant subjects with adequate sample numbers for normolipidemic and hyperlipidemic subjects to examine the dietary intervention on plasma lipids and further investigate the relationship between basal cholesterol synthesis and response to dietary therapy. If the relationship holds strong across a variety of subjects and plasma cholesterol levels, the potential exists for the use of deuterium for assessment of cholesterol synthesis in transplant patients with the purpose of predicting who might respond to certain dietary or pharmacological therapies. In addition, it would be prudent to conduct a study investigating the effects of this diet intervention in addition to statin or ezetimibe therapy to confirm the relationship between cholesterol synthesis and response to treatment. Specifically, it should be realized that some individuals will require pharmacological treatment in addition to dietary therapy in order to manage plasma lipid levels. In addition, it would be of benefit to perform a larger study in transplant patients that measures cholesterol absorption at baseline and also after dietary intervention. Measurement of cholesterol absorption could be performed ideally using

isotopically-labelled cholesterol, however the costs associated with this type of investigation limit its use. Alternatively, markers of absorption as used by Miettinen and Gylling may be used (*Nissinen et al. 2008*), however use of dietary phytosterols in the intervention may have to be considered (*Sarkkinen et al. 1998*).

It would be of interest to perform a postprandial study investigating the acute effects of the supplement bar consumption on plasma lipid levels as well as cholesterol and FA synthesis. The supplement bar utilized in this study has wide-spread application to many other individuals with or at risk for hyperlipidemia. Therefore it would be interesting to perform a dose-response study in a large number of subjects varying in plasma lipid levels and other characteristics in order to determine if a minimum intake is required to see benefits in plasma lipid levels, and if so, what this minimum intake is for various types of subjects. A potential criticism of the present study is that all of the subjects were placed on a standardized diet. This was done in order to increase generalizability across the subjects. In addition, it is unlikely that a significant effect would have been observed if subjects had been consuming a typical "Western" diet as well as different diets. However, if the intent is to potentially promote the supplement bar as a dietary aid in managing plasma lipid levels either in transplant patients or in the general population, investigation of the effects of the supplement bar in free-living subjects with self-selected diets should be performed. If a large group of subjects would be observed potential dietary patterns could be identified that would be more likely to produce beneficial effects on plasma lipid levels.

8.5. General Comments and Limitations

Due to the difficult nature of recruiting and testing patients from clinical populations, there are inherent limitations in study design and patient characteristics that may affect observations and conclusions from the

data presented herein. A few of these limitations will be discussed.

The subjects in the Control vs. T1D analyses were well-matched for gender as this was one of the objectives for this analysis. However, there were differences in numbers of males and females recruited for the liver failure, and islet and liver transplant groups. There may be differences between males and females mediated by estrogen that may influence cardiovascular risk and particularly regulation of lipid metabolism. For example, FA beta-oxidation mediated by PPARα may be greater in pre-menopausal females compared to males, and PPARα may be increased by estrogen (*Yoon 2009*; *Foryst-Ludwig et al. 2010*). Increased FA oxidation normally causes reduction in FA synthesis; therefore mismatch of females, particularly pre-menopausal females, between groups may influence measured rates of DNL between groups. Estrogen appears to affect DNL, however the evidence is conflicting. Estrogen has been associated with reduced expression of key lipogenic enzymes such as ACC and FAS in adipose tissue (*Lundholm et al. 2008*; *Foryst-Ludwig et al. 2010*). For example, ovariectomized rats showed greater hepatic gene expression of SREBP-1c, ChREBP and ACC compared to sham rats, and these effects were normalized with estrogen replacement (*Pighon et al. 2010*). Conversely, estrogen treatment in Fischer rats increased activity of FAS and ACC in the liver but reduced activity of these enzymes in the mammary gland of these animals (*Abraham et al. 1980*). There are very few investigations of differences in DNL in humans between men and women. Faix et al (1993) found that DNL in women in the luteal phase was comparable to men, but was higher in the follicular phase (*Faix et al. 1993*). The higher rates of DNL during the follicular phase was only partially related to estradiol levels ($r^2 = 0.39$), and progresterone levels were not related to the lower observed rates of DNL in the luteal phase (*Faix et al. 1993*). In contrast, Tran et al (2010) studied women in the follicular phase and found that DNL was significantly lower than in men (*Tran et al. 2010*). Sex-related differences

in DNL may be due to differences in insulin sensitivity, body weight, and food intake or by an influence of estrogen on LPL or PPARα activity (*Macotela et al. 2009*; *Foryst-Ludwig et al. 2010*). Alternatively, there is the possibility that estrogen exerts tissue-specific effects, such as suppressing adipocyte lipogenesis while stimulating hepatic lipogenesis. Estrogen and related sex hormones do not appear to influence cholesterol synthesis, as evidenced by similar rates of cholesterol synthesis in the follicular and luteal phases of the menstrual cycle (*Faix et al. 1993*). However, administration of physiological doses of estrogen in female rats increased hepatic expression of HMGCoA-r (*Parini et al. 2000*). Unfortunately the limited sample size of the present analyses precludes conclusions about sex-related differences in lipid synthesis.

The washout period for lipid-lowering medications, both statins and fibrates, in the present study was 1 week. This length of time was determined based on the half-lives of these medications, with the longest half-life being estimated at 30 hours for Atorvastatin (*Bellosta et al. 2004*), and 20 hours for fenofibrate (*Najib 2002*). A week is anticipated to be long enough for the effects of statin on cholesterol synthesis to subside, although very few studies have investigated this. In one investigation, 24h after statin cessation in healthy volunteers cholesterol secretion was still similar to statin treatment levels, which were 80% of the control pre-treatment values (*Mitchell et al. 1991*). However, another investigation using mevalonic acid levels as a marker of cholesterol synthesis found that mevalonic acid levels were not significantly different from baseline (untreated) levels by 1 week after cessation of statin therapy in patients with familial hypercholesterolemia and further investigation found rebounding of mevalonic acid levels by 3 days after cessation of statin (*Pappu et al. 2003*). Similarly, an investigation of simvastatin in healthy volunteers, serum lathosterol (a marker of cholesterol synthesis) was reduced by statin treatment and rebounded to pre-treatment

levels within 7 days after cessation of statin (*De Cuyper et al. 1993*). Finally, levels of mevalonic acid and lathosterol increased after statin therapy in healthy volunteers, and were actually higher after 8 days washout in an investigation by Pfohl et al (1998) (*Pfohl et al. 1998*).

While the effects on cholesterol synthesis induced by statin medication are likely removed after a week's time, the LDL-c levels may not have returned to true baseline after 1 week of statin cessation. In investigations by Pappu et al (2003) and Pfhol et al (1998), LDL-c levels were higher after 1 week of statin cessation but did not yet reach original baseline levels. In other investigations, LDL-c has been found to increase 18% after 1 week washout of statin, but remain 15% lower than pre-treatment values (*Chen et al. 2009*). Therefore a washout period of greater than 1 week may be required for LDL-c levels to return to normal after statin therapy. This may be due to the longer half-life of LDL particles of 1-3 days, as compared to chylomicrons and VLDL which have particle half-lives of ~15 min and 2-3 hours, respectively (*Nilsson-Ehle et al. 1980*; *Frayn 2003*). The longer circulating half-life of LDL particles has particular implications for the T1D and ITx groups, in whom the majority of subjects were taking statin medication. Analysis of LDL-c levels in patient histories at previous timepoints when patients were on statin medication revealed significantly higher LDL-c levels at time of testing (i.e. after a week washout of statin medication) for both T1D and ITx groups (data not shown), indicating that there was washout of statin effects on LDL-c levels; however it is not possible to determine if plasma LDL-c levels reached true baseline levels after this 1-week washout and in fact could have increased further if a longer washout period was used. Underscoring this, the finding of higher LDL-c levels after the diet intervention in the ITx group may be therefore due to the relatively longer washout period during the diet intervention (final 2 weeks) as compared to the initial testing period (1 week), and not due to a deleterious

effect of the diet intervention.

The half-life of fenofibrate is reported to be 20 hours (*Najib 2002*), however there is a paucity of reports detailing time required for washout of fibrate effects on aspects of lipid metabolism. Fenofibrate is a PPARα agonist which induces FA beta-oxidation; as stated previously, upregulation of beta-oxidation acts to concomitantly reduce FA synthesis (*Chapman 2003*). Fenofibrate was recently shown to increase DNL in mice (*Oosterveer et al. 2009*), however fenofibrate has previously been shown to reduce DNL in individuals with T2D (*Forcheron et al. 2002*). Therefore, it is possible that fenofibrate may independently reduce DNL by virtue of PPARα activation, and it is unclear how long this effect may last after cessation of fibrate therapy. This has implications for the LTx group in which two of the patients were on fibrate medication. It was concluded that DNL was lower in the transplant groups compared to the control group. It cannot be excluded that inadequate washout of fenofibrate did not affect these results, however the fact that the DNL rates noted in the two individuals taking fenofibrate were not significantly different (i.e. would not be considered outliers) within the transplant group suggests that lasting effects of fenofibrate are unlikely to influence the overall interpretation of these findings.

8.6. References for Conclusions and Future Directions

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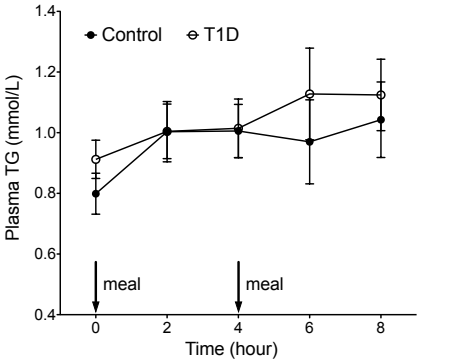
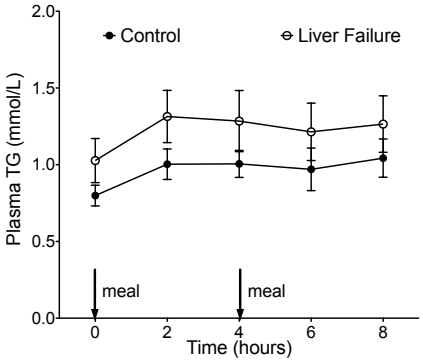
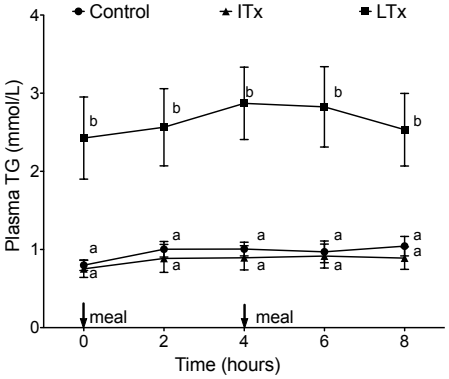
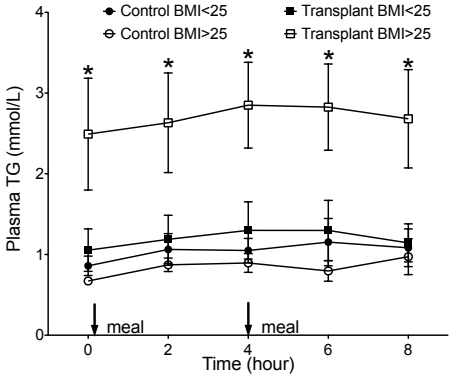
9. Appendix A - Postprandial plasma lipids, glucose, insulin, and DNFA

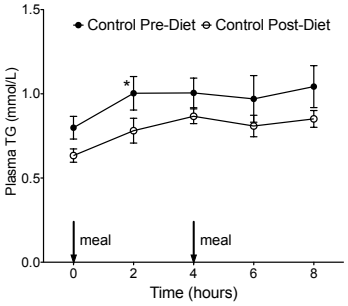
Figure A-I: Postprandial plasma triglyceride

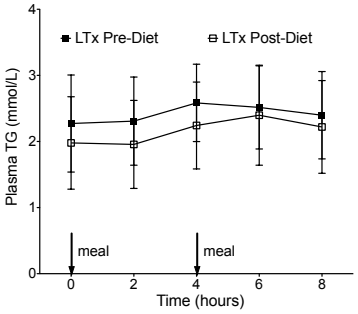


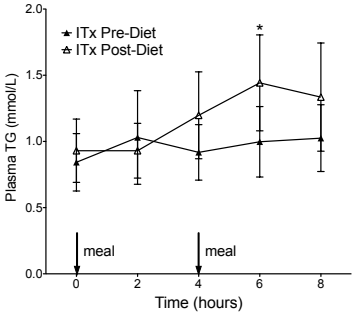












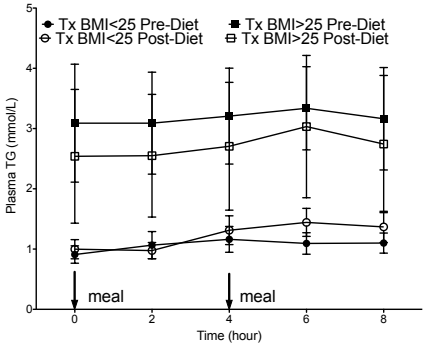


Figure A-II: Postprandial plasma total cholesterol

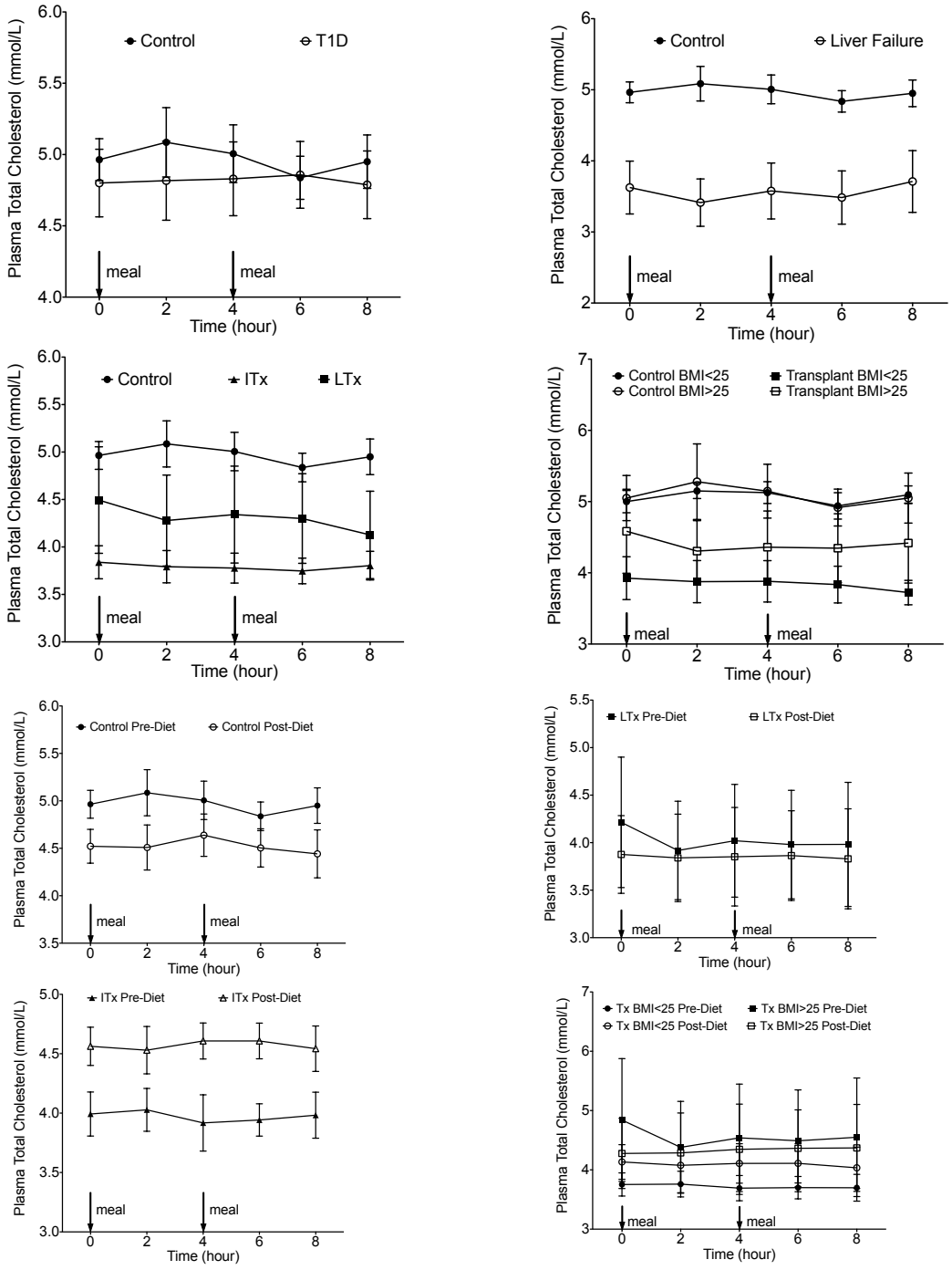


Figure A-III: Postprandial plasma glucose

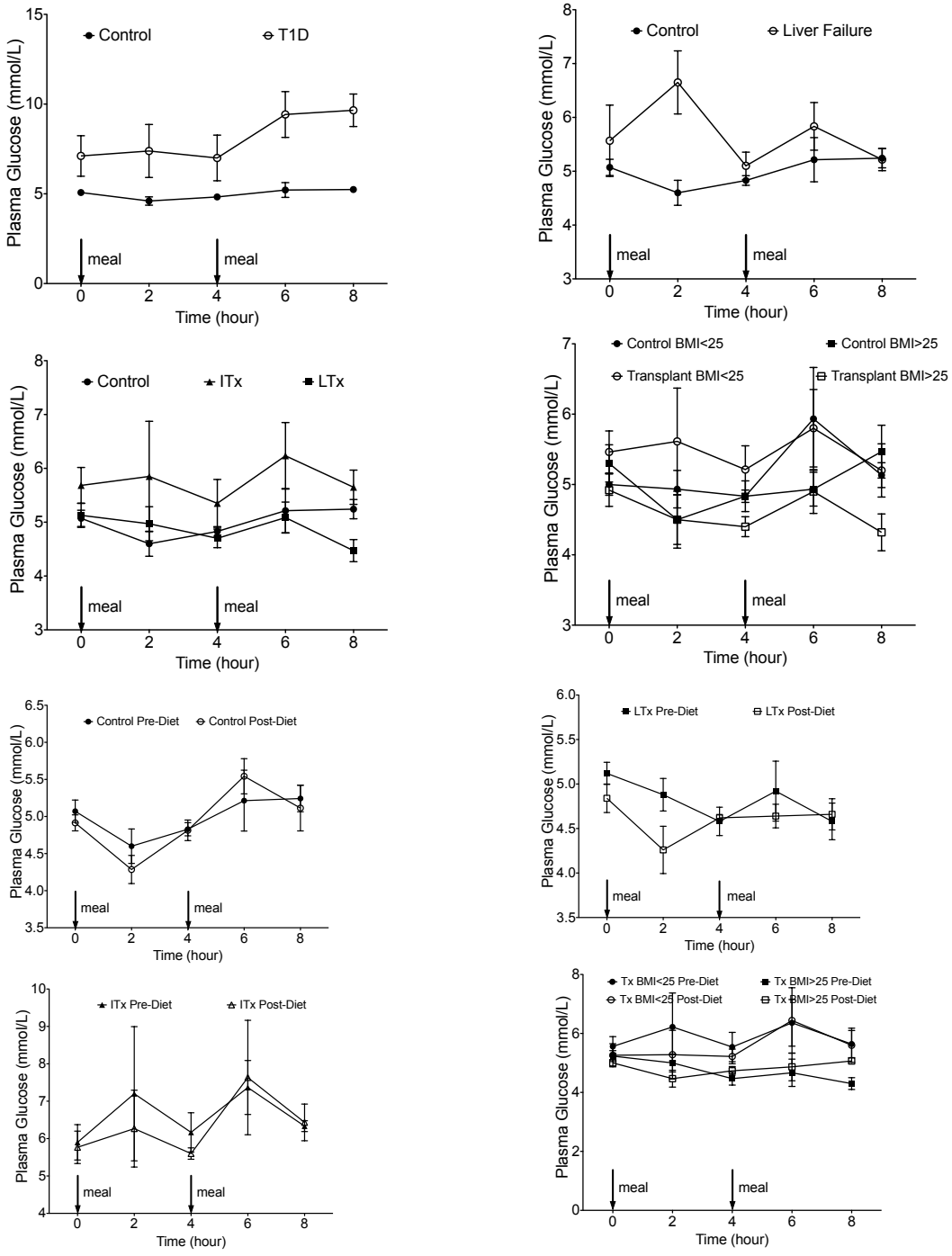


Figure A-IV: Postprandial plasma insulin

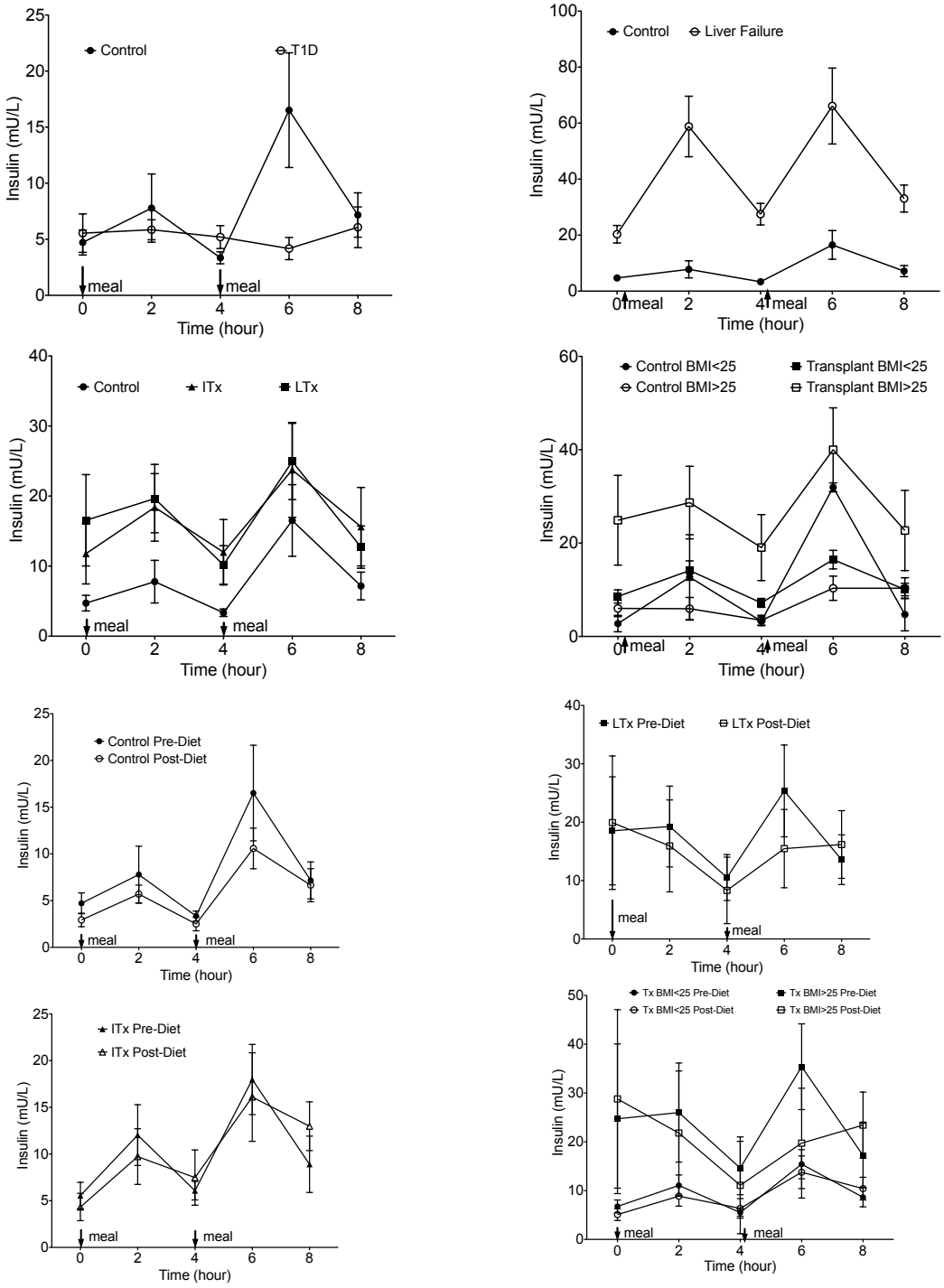
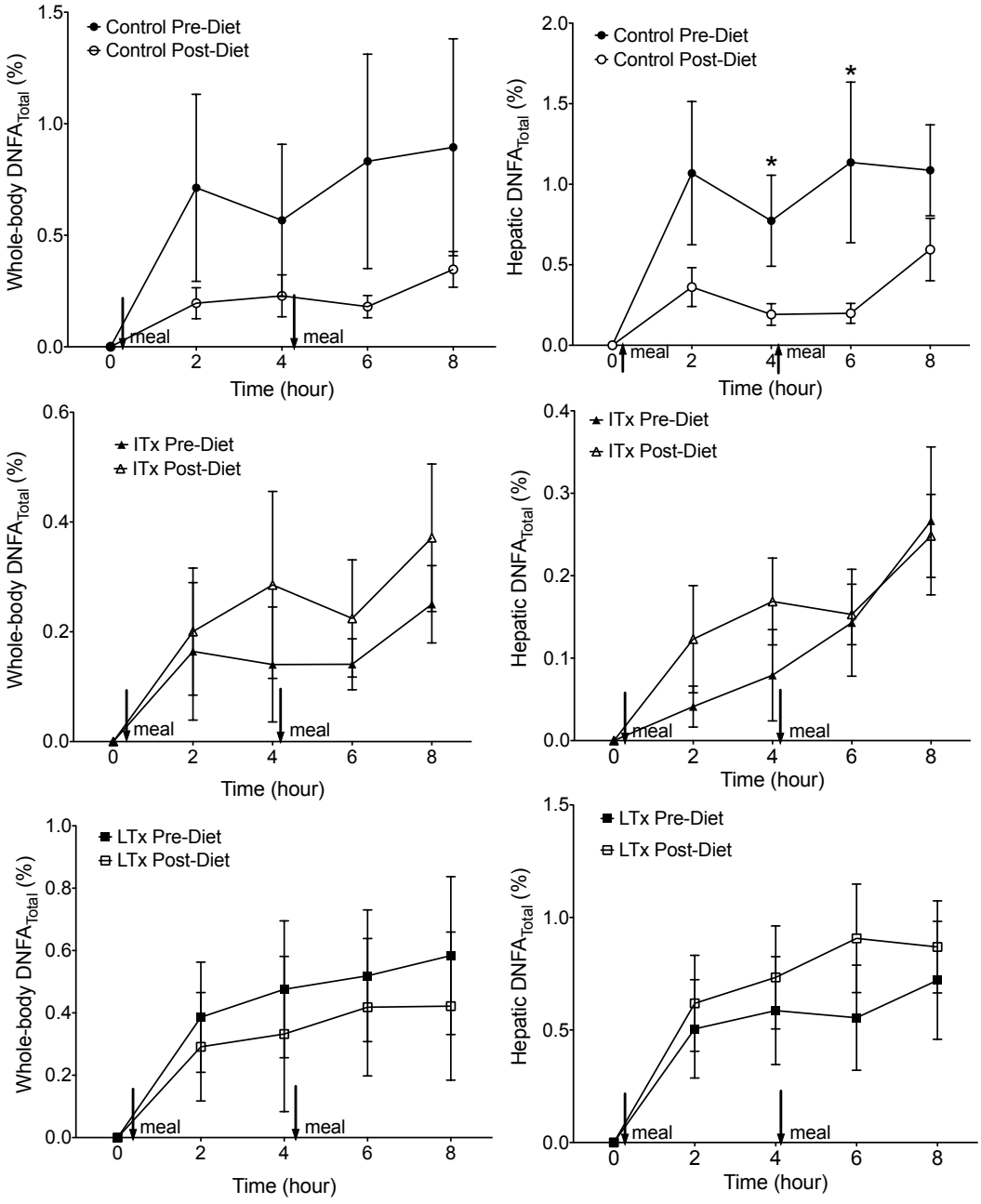


Figure A-V: Postprandial whole-body and hepatic total DNFA



10. Appendix B - Postprandial VLDL-TG and TPL Concentration

Figure B-I: Postprandial VLDL-TG concentration

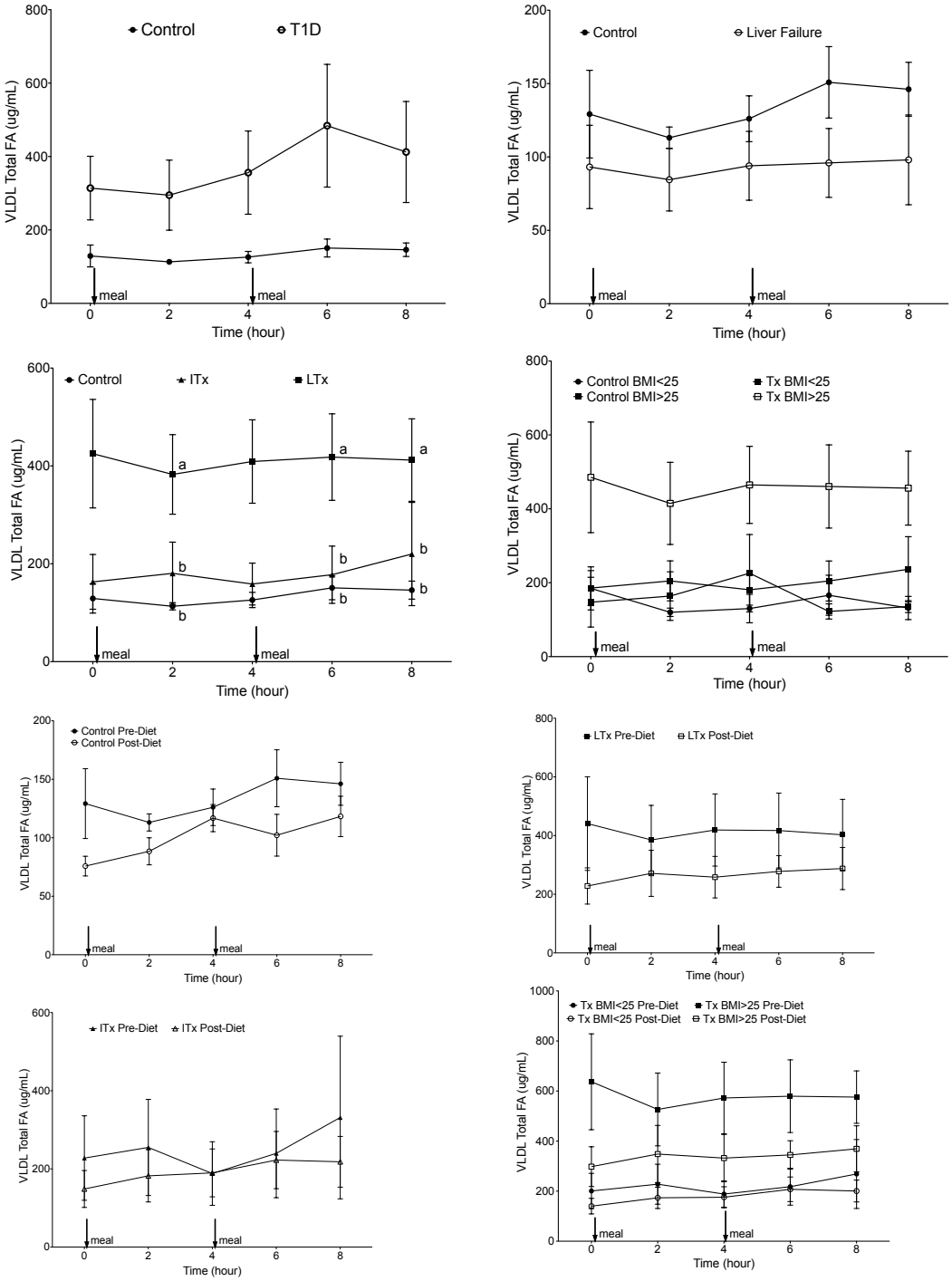
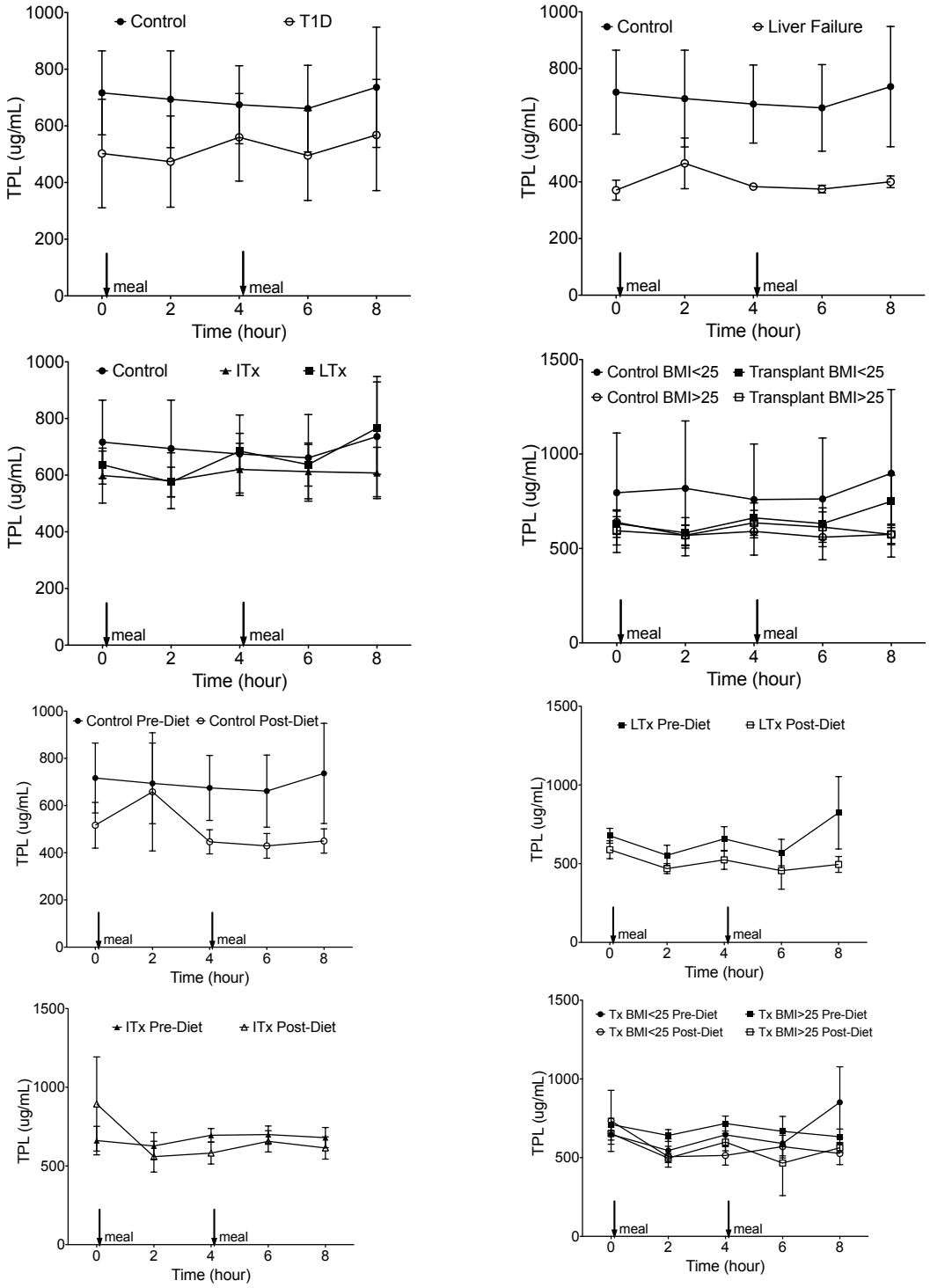
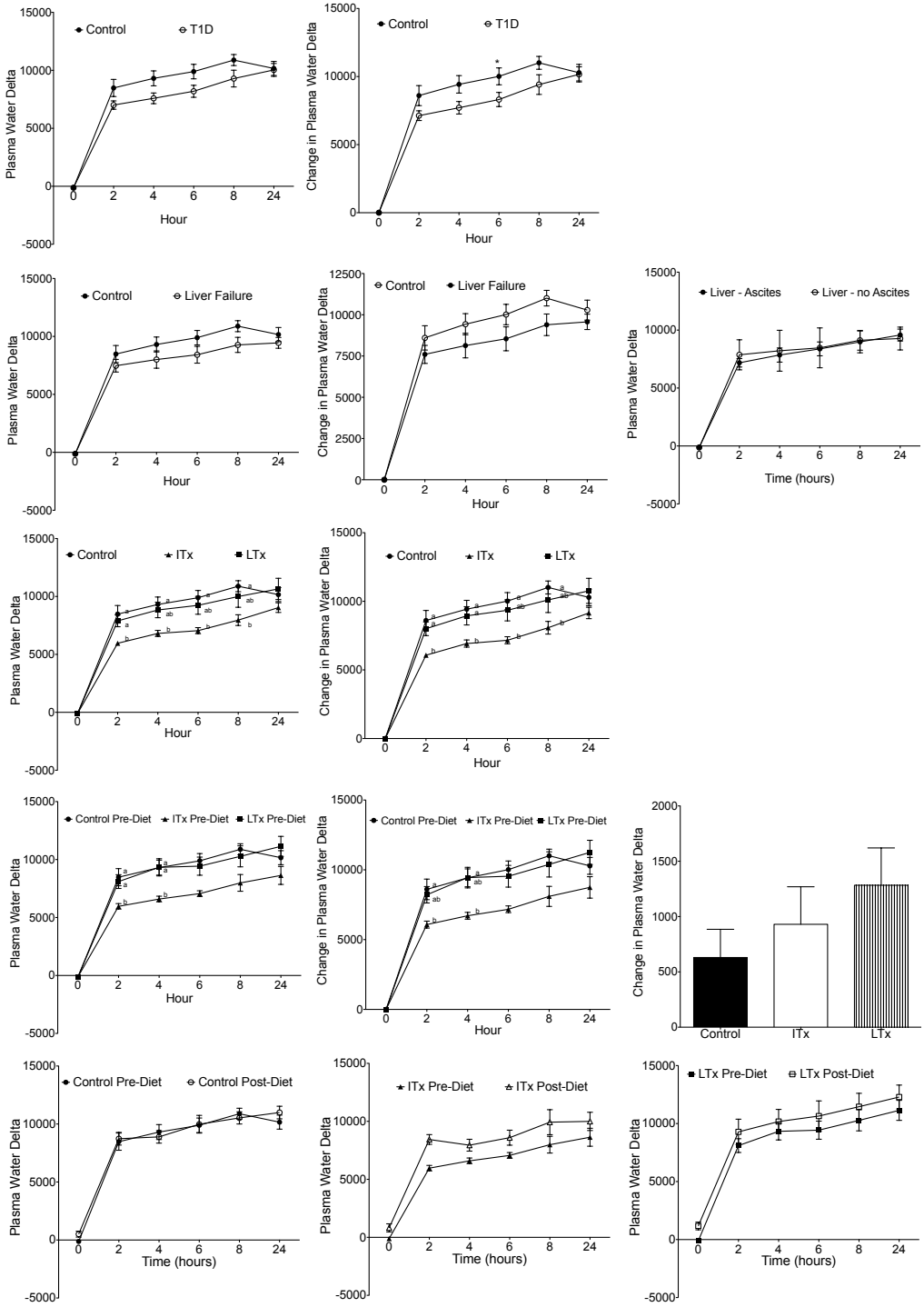


Figure B-II: Postprandial TPL concentration



11. Appendix C - Plasma Water Enrichment

Figure C-I: Plasma water enrichment and change in plasma water from baseline



12. Appendix D - Individual Data

Table D-I: DNFA and FSR of individual Control subjects

	Sex	Plasma DNL (%)	VLDL DNL (%)	FC-FSR (%)	CE-FSR (%)	V-FC-FSR (%)
Control Pre-Diet						
Ctl-1	M	0.260	0.513	20.24	1.65	7.019
Ctl-2	M	0.012	0.682	5.58	1.16	8.785
Ctl-3	F	1.156	1.289	7.56	2.05	8.621
Ctl-4	F	0.448	0.611	7.63	1.62	7.922
Ctl-5	M	0.586	0.781	9.01	2.21	10.124
Ctl-6	F	1.295	1.539	6.37	1.64	6.112
Ctl-7	F	1.420	2.043	10.52	3.12	11.503
Ctl-8	F	0.000	0.439	3.47	0.87	3.723
Ctl-9	F	2.697	2.823	8.60	2.93	9.300
Mean ± SEM		*0.875 ± 0.289*	*1.191 ± 0.272*	*8.78 ± 1.59*	*1.92 ± 0.25*	*8.123 ± 0.765*

Table D-II: DNFA and FSR of individual Type 1 diabetes subjects

	Sex	Plasma DNL (%)	VLDL DNL (%)	FC-FSR (%)	CE-FSR (%)	V-FC-FSR (%)
Type 1 diabetes						
T1D-1	F	0.712	0.634	7.09	2.16	7.69
T1D-2	F	0.989	1.350	7.63	2.01	7.56
T1D-3	M	1.029	1.618	7.03	1.74	6.78
T1D-4	M	0.263	0.284	13.98	0.65	5.43
T1D-5	F	0.769	0.307	12.15	3.62	15.23
T1D-6	M	1.367	1.569	9.17	2.58	9.65
T1D-7	M	0.000	*n/a*	2.98	0.45	*n/a*
T1D-8	F	0.430	0.502	4.76	1.78	4.01
T1D-9	F	0.552	0.714	10.10	2.37	9.26
Mean ± SEM		*0.679 ± 0.140*	*0.872 ± 0.196*	*8.32 ± 1.15*	*1.93 ± 0.32*	*8.20 ± 1.20*

Table D-III: DNFA and FSR of individual liver failure subjects

	Sex	Plasma DNL (%)	VLDL DNL (%)	FC-FSR (%)	CE-FSR (%)	V-FC-FSR (%)
Liver Failure						
Liver-1	M	3.443	1.972	1.727	0.155	2.188
Liver-2	M	2.711	3.842	0.923	0.099	0.837
Liver-3	M	2.614	4.253	2.423	0.524	1.336
Liver-4	M	2.756	2.552	1.085	0.369	1.907
Liver-5	F	0.475	1.355	2.032	0.158	2.131
Liver-6	F	0.645	0.519	1.601	0.735	1.530
Liver-7	M	1.639	1.199	0.671	0.527	0.962
Mean ± SEM		*2.040 ± 0.431*	*2.242 ± 0.526*	*1.495 ± 0.238*	*0.367 ± 0.091*	*1.556 ± 0.205*

Table D-IV: DNFA and FSR of individual islet transplant subjects

	Sex	Plasma DNL (%)	VLDL DNL (%)	FC-FSR (%)	CE-FSR (%)	V-FC-FSR (%)
Islet Transplant						
ITx-1	M	0.000	0.279	4.684	0.968	4.717
ITx-2	M	0.389	0.272	9.320	2.619	10.126
ITx-3	F	0.021	0.139	5.934	1.792	6.287
ITx-4	M	0.273	0.572	8.630	2.324	10.408
ITx-5	F	0.112	0.246	6.568	2.711	6.879
ITx-6	F	0.357	0.008	17.171	6.610	18.143
ITx-7	M	0.274	0.049	1.641	0.543	2.272
ITx-8	M	0.000	0.144	5.614	1.616	6.000
Mean ± SEM		*0.178 ± 0.058*	*0.214 ± 0.062*	*7.445 ± 1.621*	*2.398 ± 0.660*	*8.104 ± 1.716*

Table D-V: DNFA and FSR of individual liver transplant subjects

	Sex	Plasma DNL (%)	VLDL DNL (%)	FC-FSR (%)	CE-FSR (%)	V-FC-FSR (%)
Liver Transplant						
LTx-1	M	3.218	3.870	16.546	8.189	18.621
LTx-2	M	0.971	0.757	2.925	0.664	3.426
LTx-3	M	0.604	0.574	6.255	1.956	6.390
LTx-4	M	2.075	2.979	5.262	1.218	5.639
LTx-5	F	0.594	0.759	7.355	3.047	8.846
LTx-6	M	0.598	0.697	2.717	0.907	2.981
LTx-7	F	1.466	1.732	8.547	3.426	9.415
Mean ± SEM		*1.361 ± 0.374*	*1.624 ± 0.497*	*7.087 ± 1.774*	*2.772 ± 0.986*	*7.903 ± 2.010*

Table D-VI: DNFA and FSR of individual Control subjects after diet intervention and percent change from baseline

	Sex	Plasma DNL (%)	VLDL DNL (%)	FC-FSR (%)	CE-FSR (%)	V-FC-FSR (%)
Control Post-Diet						
Ctl-1	M	0.923	1.060	8.157	4.765	8.872
% change		*+255%*	*+107%*	*-60%*	*+189%*	*+26%*
Ctl-2	M	0.468	0.322	6.363	1.175	6.140
% change		*+3800%*	*-53%*	*+14%*	*+1.3%*	*-30%*
Ctl-3	F	0.413	0.975	7.199	2.687	8.797
% change		*-64%*	*-24%*	*-5%*	*+31%*	*+2%*
Ctl-4	F	n/a	0.106	9.147	1.466	7.179
% change		*n/a*	*-83%*	*+20%*	*-10%*	*-9%*
Ctl-5	M	0.309	0.218	4.972	1.521	4.018
% change		*-47%*	*-72%*	*-45%*	*-31%*	*-60%*
Ctl-6	F	0.602	0.568	5.306	1.214	7.199
% change		*-54%*	*-63%*	*-17%*	*-26%*	*+18%*
Ctl-7	F	0.444	0.134	9.414	2.991	12.632
% change		*-69%*	*-93%*	*-11%*	*-4%*	*+10%*
Ctl-8	F	0.275	0.283	7.873	1.741	9.977
% change		*n/a*	*-36%*	*+127%*	*+100%*	*+168%*
Ctl-9	F	0.279	0.292	9.915	4.486	11.419
% change		*-90%*	*-90%*	*+15%*	*+53%*	*+23%*

Table D-VII: DNFA and FSR of individual islet and liver transplant subjects after diet intervention and percent change from baseline

	Sex	Plasma DNL (%)	VLDL DNL (%)	FC-FSR (%)	CE-FSR (%)	V-FC-FSR (%)
Islet and Liver Transplant Post-Diet						
ITx-2	M	0.583	0.171	10.372	2.406	10.632
% change		*+50%*	*-37%*	*+11%*	*-8%*	*+5%*
ITx-6	F	1.527	0.651	12.239	2.524	12.156
% change		*+328%*	*+8038%*	*-29%*	*-62%*	*-33%*
ITx-7	M	0.223	0.353	6.033	0.763	6.392
% change		*-19%*	*+620%*	*+268%*	*+41%*	*+181%*
ITx-8	M	0.242	0.144	3.375	2.139	3.808
% change		*n/a*	*0%*	*-40%*	*+32%*	*-37%*
LTx-1	M	4.210	4.720	11.350	6.817	11.371
% change		*+31%*	*+22%*	*-31%*	*-17%*	*-39%*
LTx-2	M	0.286	0.605	6.068	2.005	6.938
% change		*-71%*	*-20%*	*+107%*	*+202%*	*+103%*
LTx-3	M	0.339	0.388	7.991	2.558	8.027
% change		*-44%*	*-32%*	*+28%*	*+31%*	*+26%*
LTx-5	F	0.712	1.533	8.620	3.606	11.555
% change		*+20%*	*+102%*	*+17%*	*+18%*	*+31%*
LTx-6	M	0.411	1.183	5.330	1.784	5.849
% change		*-31%*	*+70%*	*+96%*	*+97%*	*+96%*

Table D-VIII: DNFA and FSR in individual Type 1 diabetes and liver failure subjects before and after islet or liver transplant

	Sex	Plasma DNL (%)	VLDL DNL (%)	FC-FSR (%)	CE-FSR (%)	V-FC-FSR (%)
Type 1 diabetes and Liver failure before and after Islet and Liver Transplant						
T1D-3	M	1.029	1.618	7.03	1.74	6.78
ITx-4		0.273	0.572	8.630	2.324	10.408
% change		*-73%*	*-65%*	*+23%*	*+34%*	*+54%*
T1D-5	F	0.769	0.307	12.15	3.62	15.23
ITx-5		0.112	0.246	6.568	2.711	6.879
% change		*-85%*	*-20%*	*-46%*	*-25%*	*-55%*
T1D-8	F	0.430	0.502	4.76	1.78	4.01
ITx-6		0.357	0.008	17.171	6.610	18.143
% change		*-17%*	*-98%*	*+260%*	*+271%*	*+352%*
Liver-5	F	0.475	1.355	2.032	0.158	2.131
LTx-5		0.594	0.759	7.355	3.047	8.846
% change		*+25%*	*-44%*	*+262%*	*+1828%*	*+315%*