Gangliosides in Plasma and Chylomicrons of Control Participants and Individuals with Diabetes Fed both a High- and Low-Fat Diet

by

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Abstract

Aim: To investigate whether the plasma and chylomicron ganglioside profiles of participants with type 2 diabetes differ from those of matched control participants at 0, 2, 4, 6 and 8 hours postprandial when individuals are fed both a high- and low-fat diet for three days each. **Background**: Type 2 diabetes is a disease that impacts a growing portion of the population increasing the risk of cardiovascular disease, and leading to early mortality. Previous studies have reported that gangliosides, bioactive lipids, specifically GM3, can lead to the insulin receptor dissociating from the caveolae-1 complex in the cell membrane in adipocytes. Mice that have GM3 synthase knocked out have increased insulin sensitivity. **Methods**: In the present study, plasma samples from matched individuals with type 2 diabetes and control participants were fed both a high- and low-fat diet for three days each in a randomized cross-over controlled study. Plasma samples from 0, 2, 4, 6, and 8 hours postprandial, were collected, extracted and analyzed. Chylomicron samples from 0 and 4 hours were also analyzed. Gangliosides were extracted from plasma/chylomicron samples using a Folch technique and analyzed using a triple quad mass spectrometer. **Result**: The overall trend in both the plasma and chylomicron data indicated that there was no significant difference between ganglioside content in the plasma of individuals who were fed both a high- and low-fat diet in either the control participants or participants with diabetes. However, the majority of the plasma data showed a significant difference between ganglioside content over collection timepoints. **Conclusion**: The results indicate that there is no

significant difference in ganglioside profiles of non-diabetic participants and participants with diabetes, matched for age, gender and body mass index, or that a high- vs low-fat diet influences plasma/chylomicron ganglioside profiles.

Preface

Plasma and chylomicron samples were obtained from a previous study conducted by Dr Michaelann Wilke. Dr Goh isolated the lipoproteins from the fresh blood samples and assisted with the statistical analysis. Dr Glen Shoemaker operated the LC mass spectrometer. The initial study was approved by the Faculty of Agriculture, Forestry and Home Economics and Human Research Ethics board at the University of Alberta. Analysis of blood samples for the purpose of the present study was also approved by the Health Research Ethics Board Biomedical Panel at the University of Alberta.

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I dedicate this thesis to my family (Mom, Dad, Julie, Allison, Grandma Honey and Grandpa Jim).

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Abbreviations

M=Mono

D=Di

T=Tri

GG=Gangliosides

TG=Triglyceride

BMI=Body Mass Index

UDP=uridine diphosphate

TLC= Thin layer chromatography

TNF alpha = Tumor necrosis factor alpha

LCMS= Liquid chromatography-mass spectrometry

HBAlc = Hemoglobin A1c

HOMA=Homeostatsis model assessment

FFAs= Free fatty acids

ALT=Alanine transaminase

LDL= Low-density lipoprotein

HDL= High-density lipoprotein

TC= Total cholesterol

VLDL= Very low-density lipoprotein

NHANES= National Health and Nutrition Examination Survey

ApoB= Apolipoprotein B

ApoE= Apolipoprotein E

Hr= hour

D_2O= Deuterium oxide

SE= Standard error

V= Volume

Chapter 1: Introduction

1.1 Gangliosides

1.1.1 Ganglioside Composition/ Nomenclature

Gangliosides are bioactive lipids composed of a fatty acid chain, carbohydrate moieties, and sialic acid (1). Gangliosides are found ubiquitously in cell membranes and are involved in growth, differentiation and cell signaling. Gangliosides are abundant in the nervous system and brain (2). Since gangliosides are amphiphillic molecules, the molecule is orientated so that the fatty acid portion is inserted in the membrane while the charged groups project outwards (3).

A nomenclature system has been developed to classify the numerous ganglioside species. In general, ganglioside nomenclature is derived from the number of sialic acids and sugar moieties present in the molecule. The 'G' in the name indicates ganglioside. The mono (M), di (D) or tri (T) in the ganglioside name indicates the number of sialic acids in the ganglioside, and the number in the nomenclature (1, 2, or 3) is indicative of the number of sugar moieties (2). The number of sugar moieties is determined by subtracting the number in the ganglioside name from 5. For example, in GM1, there are four sugar moieties on the molecule (4). The subscript 0, a, b or c that is found after the letters indicates the pathway used in synthesis (5). Gangliosides are a class of abundant amphiphillic diverse lipids found in the human body.

1.1.2 Ganglioside Biosynthesis

Ganglioside formation starts in the endoplasmic reticulum where the ceramide tail of the ganglioside is formed. Ceramide is then transferred to the Golgi apparatus where ceramide is modified through addition of sugar moieties. Glycosyltransferase transfers the glucose molecule, which is bound to UDP, to the ceramide, thereby forming glucosylceramide. Glucosylceramide is used in the synthesis of the majority of gangliosides. The process of creating glucosylceramide occurs on the luminal side of the Golgi apparatus. Lactosylceramide is then formed through the addition of UDP-galactose to glucosylceramide via galactosyltransferase I (4). Lactosylceramide is sialated through the use of Sial-T1, Sial-T2 and Sial-T3, which results in the production of GM3, GD3 and GT3, respectively. GM3, GD3 and GT3 can, therefore, be converted to more complex gangliosides in the 0, a, b and c series (6). The luminal side of the Golgi apparatus is where more complex ganglioside species are synthesized. There are four different series of gangliosides (0, a, b, and c); the gangliosides in these respective series contain 0, 1, 2, or 3 sialic acids (4). The gangliosides are synthesized through addition of galactose, N-acetylgalactosiamine and sialic acid by N-acetyl-galactosaminyltransferase, galactosyl transferase and sialyl-transferase, respectively (7). The rate of complex ganglioside synthesis is dependent on expression of glycosyltransferases. After gangliosides are synthesized, gangliosides are transported to the cell membrane (8). See Figure 1 for ganglioside synthesis.

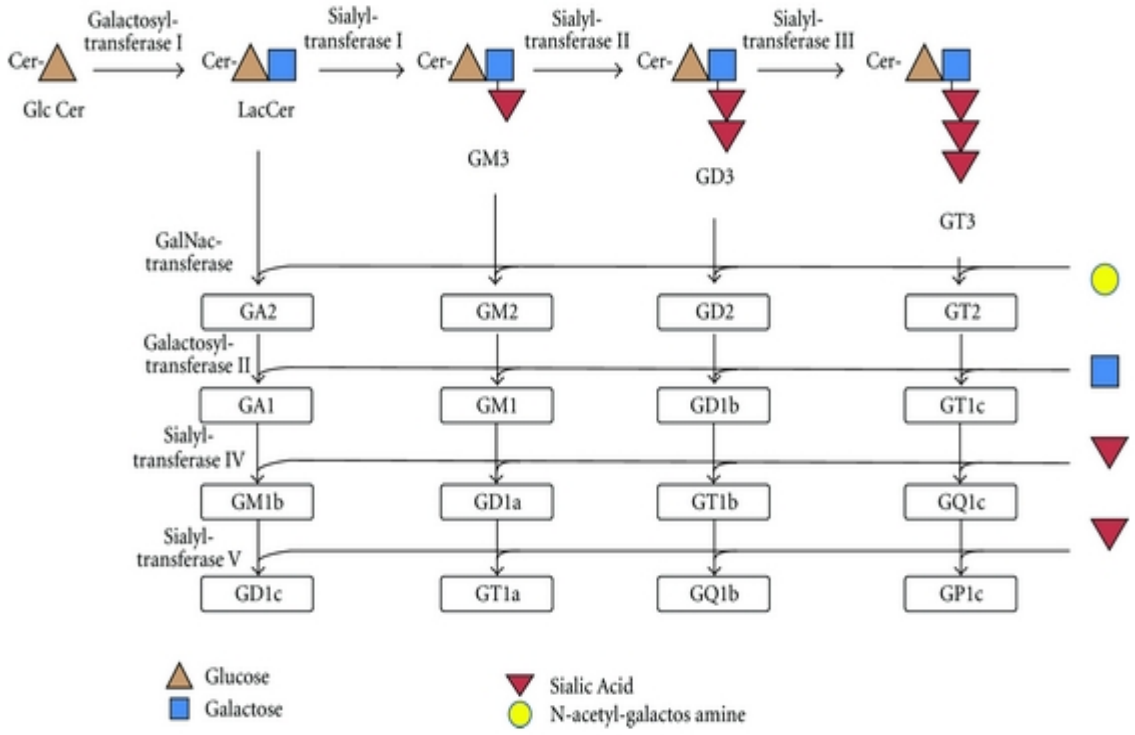

Figure 1: General scheme for ganglioside synthesis. Network of ganglioside synthesis; steps are also reversible. “G” denotes “ganglioside;” “A” denotes “asialo” or lacking sialic acid; “M” denotes “monosialo,” “D” denotes “disialo;” numbers denote carbohydrate sequence. Adapted from Malisan and Testi (5). Cer: ceramide; GlcCer: glucosylceramide; LacCer: lactosylceramide; GalNac: N-acetylgalactosamine. (Taken from Miklavcic et al.,(9)).

Galactosyltransferase, sialyltransferase I and sialyltransferase II all bind substrates with high affinity and specificity (6). Gangliosides found in the “0” series and in the “c” series are found in small amounts in humans. It is also important to note that regulation of production of different gangliosides is not well understood (4). Palmitate has also been shown to be a precursor for ceramide synthesis. Increasing the supply of palmitate causes an increase in the quantity of sphingolipid in the tissue (10). Ganglioside metabolism is a complex process that leads to the production of a variety of different gangliosides.

1.1.3 Ganglioside Degradation

Ganglioside degradation starts with the removal of the ganglioside residues. The process of degradation occurs through the endocytosis-endosome-lysosome pathway. Gangliosides with multiple sialic acid moieties are broken down into gangliosides that contain only one sialic acid. Galactose moieties are then removed from the monosialated ganglioside by B-galactosidase, which is a non-specific enzyme. The N-acetyl-galactosamine is removed by B-N-acetylhexosaminidase. Laceramide is degraded by both B-glucosidase and B-galactosidase to a ceramide. The ceramide is then split into a long chain base and a fatty acid. This process is accomplished through ceramidase (7). See Figures 2 and 3.

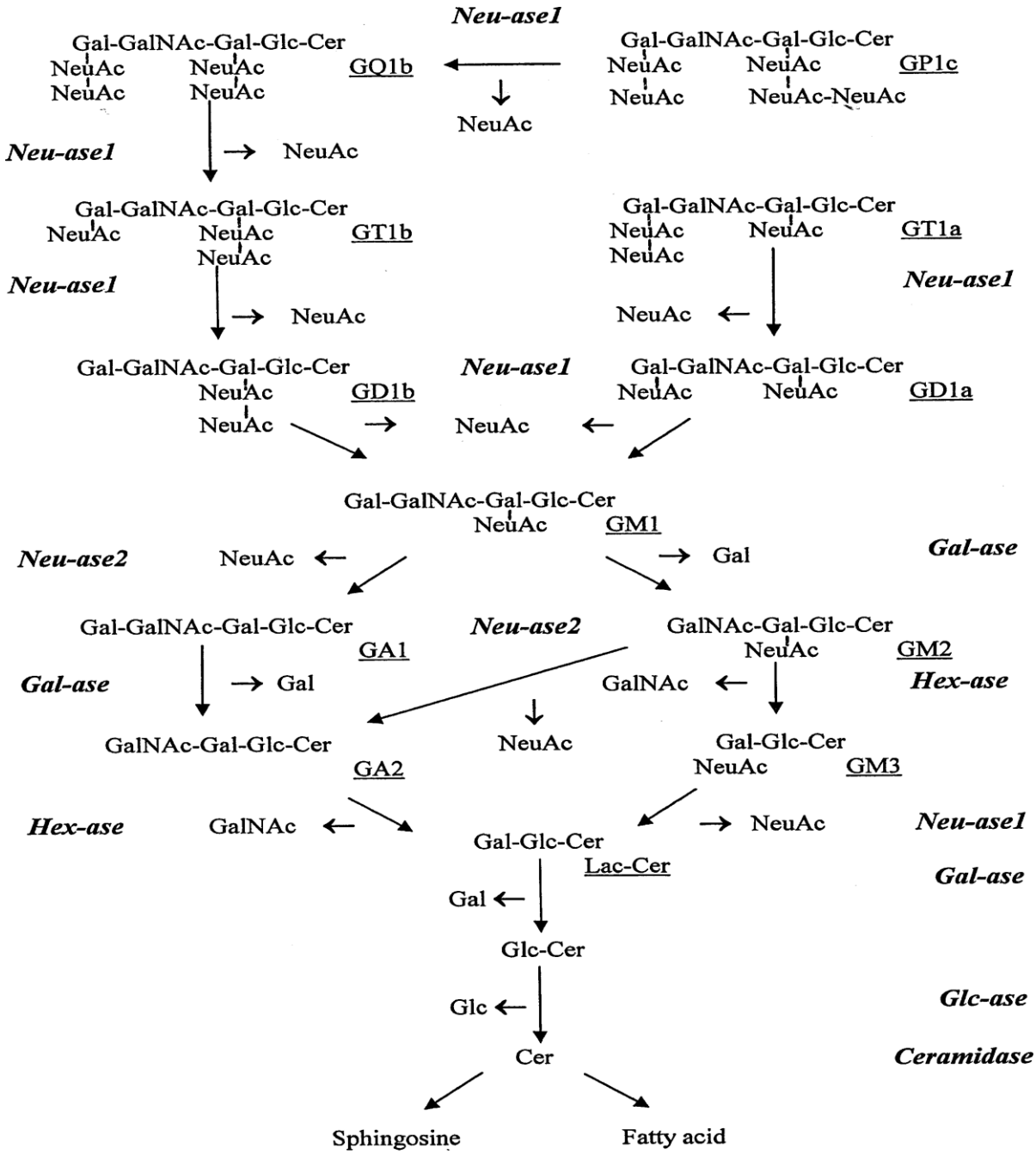

Figure 2. Scheme of the degradation pathway of gangliosides. Neu-ase 1 (neuraminidase or sialidase 1) and Neu-ase 2 (neuraminidaseor sialidase 2) are two different enzymes; Gal-ase, **β**-galactosidase; Hex-ase, **β**-hexosaminidase; Glc-ase, **β**-glucosidase. (Abstracted from Tettamanti et al. (7)).

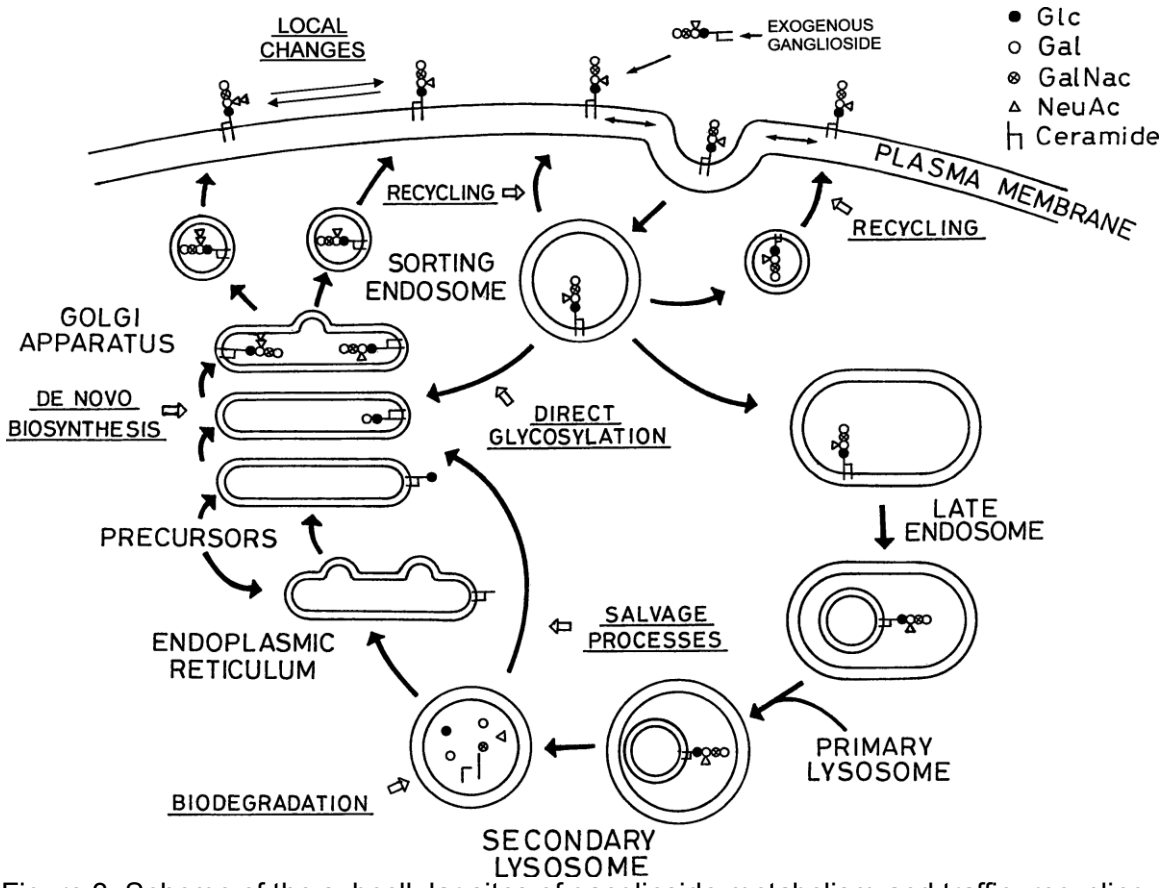

Figure 3. Scheme of the subcellular sites of ganglioside metabolism and traffic: recycling to the plasma membrane; de novo biosynthesis in the endoplasmic reticulum and Golgi apparatus; direct glycosylations at the Golgi apparatus level; degradation in the late endosomes/lysosomes; salvage processes at the endoplasmic reticulum/Golgi apparatus level; local changes (glycosylayions/deglycosylations) at the plasma membrane level. (Taken from Tettamanti et al.,(7)).

In addition to being degraded, gangliosides can be altered during insertion in the plasma membrane. It has been shown that the number of sialic acids in a ganglioside can be modified by sialidase enzymes in the plasma membrane. Sialidase enzymes can lead to production of monosialated gangliosides or the production of Lac-ceramide (7). Neu3, a plasma membrane associated with sialidase, can cause desialylation of gangliosides in the cell membrane. B-hexoaminidase, B-glucosidase and B-galactosidase are also active on the cell

surface, and play a role in modulating the gangliosides in the cell membrane that are involved in cell signaling, as well as other processes. It has also been shown that Sial-T2 can add a sialic acid moiety to GM3 in the cell membrane (6).

Gangliosides have been shown to be recycled during the degradation process. For example, Lac-ceramide may be formed during the degradation process and released for use in synthesis of new gangliosides. It was found that when mice were injected with GM1, which had a radioactive sphingosine, radioactive GM2 and GM3 were produced in the liver, thereby indicating that the sphingosine of the GM1 molecule is recycled. Additional experiments have been conducted with other components of gangliosides and support the idea of recycling (7). Gangliosides can be recycled or degraded through a variety of complex pathways and cellular processes.

1.1.4 Dietary Intake of Gangliosides

Gangliosides are found to be most abundant in foods from animal origin (11). Gangliosides are especially abundant in the central nervous system (CNS), spleen, liver, kidney and gut of animals. A study conducted in Edmonton, AB, which investigated the most abundant sources of gangliosides using 7-day food records of 19 healthy individuals (4 males and 15 females), determined that there were seven predominant ganglioside-containing foods that were consumed. The most abundant were: egg yolk (1.73 µg/g), regular-fat cooked ground beef (1.70 µg/g), regular-fat ground raw beef (1.57 µg/g), regular canned tuna (0.83 µg/g), regular-fat yoghurt (0.73µg/g), regular-fat cheddar cheese (0.72 µg/g), and 1%

milk (0.51µg/g). Seventy percent of individuals consumed less than 100µg/4187kJ, which is approximately 100µg/1001 kcal. No individuals consumed more than 600µg/2000kcal (12). The quantity of ganglioside consumption is dependent on the amount of food consumed that is of animal origin.

1.1.5 Dietary Ganglioside Uptake/ Modification of Intestinal Mucosa

Experiments where gangliosides were provided to cells have shown that diffusion and endocytosis are two of the processes used to uptake gangliosides. This process has been found to be dependent on the concentration of gangliosides present, the type of cell, the temperature and the length of time the cell is incubated (13). Once transported into the plasma membrane, the ganglioside can be recycled and transported to the Golgi apparatus for modification, degradation, or transfer from one side of the cell membrane to the other (14).

When dietary gangliosides are provided to animals, changes in the intestinal mucosa, plasma and brain ganglioside composition are evident. When rats were provided with gangliosides that contained greater than 80% GD3 for 2 weeks, there was an increase in total gangliosides in plasma, intestine and brain when compared to controls, with the highest amount of gangliosides being found in the intestine and the lowest in the brain. Specifically, in the intestine, there was an increase in the quantity of GD3 and a decrease in the level of GM3. Rats provided with dietary gangliosides also had decreased ratios of cholesterol to

gangliosides in the intestinal mucosa and brain, when compared to control participants. GM3 was found to be generally localized on the apical side of the membrane while GD3 was localized on the basolateral side. As a result, it is possible that a change in ganglioside composition has the potential to change the function of different organs by altering membrane composition function (2). Dietary gangliosides have also been shown to reduce degradation of the occludin tight junction (15). In addition, dietary gangliosides reduce the amount of cholesterol, caveolin protein expression, as well as pro-inflammatory mediators in the cell membrane. It has been shown in rats that a diet high in polyunsaturated fatty acids does not modify the quantity of gangliosides in the intestine (2). Dietary gangliosides are both taken up by the enterocytes and modify membrane composition, thereby possibly altering function.

1.1.6 Ganglioside Function/ Relation to Diseases in the Body

The function of gangliosides in the body is largely unknown. Knockout studies have shown that when one group of gangliosides is knocked out, there is only a mild impact on phenotype. Conversely, knocking out all of the gangliosides has severe neurological effects and fatal results (16). A variety of studies have demonstrated that gangliosides play a role in maintaining nerve tissues, and are involved in regulating cellular events. Gangliosides are also needed for proper cell adhesion in the embryonic stage (17). Gangliosides play a role in the differentiation of cells, as the composition of GD3:GM3 changes between differentiated and undifferentiated cells (18). Gangliosides are involved in neural

tube formation, and mylenation of nervous tissues. Studies have shown that gangliosides are also involved in cell signaling and adhesion (19). Gangliosides may also be involved in the maturation of the retina, and may play a role in the development of the photoreceptor membrane and, therefore, visual function (73).

Many of the functions of gangliosides have been discovered through the study of different disease states. There are a number of genetically determined ganglioside-related diseases. Tay-Sachs is one of these diseases. Tay-Sachs is a disease that results from an inborn error in lysomal function, which causes a lack of B-N- acetylhexoaminidase. As a result of this deficiency, there is an accumulation of GM2 in the neurons. In infants, this neurological disease can result in blindness, regression of developmental skills, seizures and death (20). Gangliosides are complex molecules involved in numerous processes in the body. Malfunction of ganglioside production or function can have a profound pathological impact.

1.2 Insulin Resistance and Type 2 Diabetes

Insulin resistance is defined as “the decreased ability of cells or tissues to respond to physiological levels of insulin” (21). In a state of insulin resistance, individuals experience hyperinsulinemia. When functioning normally, insulin reduces output of hepatic glucose, increases glucose uptake by muscles, and decreases fatty acid release from adipocytes via lipolysis (22). Insulin helps to reduce the output of glucose in the liver by activating enzymes used in the conversion of glucose to fatty acids, such as in fatty acid synthase (23).

Therefore, in a state of insulin resistance, both glucose metabolism and fatty acid metabolism are impaired, contributing to higher glucose and fatty acid levels in the blood (22). Insulin resistance is a condition that is present in many individuals along with other symptoms such as high blood pressure, dyslipidemia and cardiovascular disease. Insulin resistance is present in individuals with type 2 diabetes; however, not all individuals with insulin resistance develop type 2 diabetes. As long as the body can continue to secrete a higher amount of insulin to maintain relatively normal glucose levels, the individual will avoid development of diabetes (24).

The number of adults and children with type 2 diabetes is increasing. According to the Public Health Agency of Canada, 2.4 million or 6.4% of Canadians are living with diabetes (25). Ninety percent of the individuals diagnosed with diabetes in Canada have type 2 diabetes(26). Beta cell dysfunction is a defining factor in the onset of type 2 diabetes. Prior to being diagnosed with type 2 diabetes, many individuals experience insulin resistance. It is the combination of both insulin resistance and decreased insulin production that contributes to the high blood glucose and fatty acids levels in the blood (22).

The development of type 2 diabetes results from both lifestyle and genetic factors. Obesity and low levels of physical activity have been shown to increase the chance of developing type 2 diabetes (27). Diets high in sugar, soft drinks, fries, and snack foods, and low in fruits and vegetables are also associated with the development of type 2 diabetes since nutrient-poor diets are associated with

an increased risk of obesity (28). Although lifestyle factors greatly influence the development of type 2 diabetes, genetic factors also play a role. It has been found that individuals with a family history of type 2 diabetes have a 2.4 fold increased chance of developing this disease with approximately 15-25% of individuals developing hyperglycemia or diabetes if a first degree relative has type 2 diabetes (22). For example the rs12255372 located in the *TCF7L2* gene is associated with susceptibility to the development of type 2 diabetes and may vary between different ethnicities. *TCF7L2* encodes for "an endocrine transcription factor that controls the production of proglucagon gene". This gene then encodes for glucagon, and glucagon-like peptide 1, which promotes insulin secretion and inhibits glucagon secretion (29).

Diagnosis of type 2 diabetes occurs when an individual has a fasting glucose level greater than or equal to 7.0 mmol/L, a random glucose level over 11.0 mmol/L, a hemoglobin A1C over 6.5% or a glucose tolerance test with a result of 11.1 mmol/L or greater two hours after a sweet beverage is consumed (30). In addition to hyperglycemia, individuals with type 2 diabetes may experience symptoms such as increased thirst, increased/ frequent urination, weight loss or gain, extreme fatigue, impaired/ blurred vision, reoccurring infection, slow healing, numbness in extremities, and sexual impairment (56). Individuals with type 2 diabetes also can experience numerous complications with the disease including damage to multiple organs in the body such as the kidneys, eyes and the nervous system, as well as cardiovascular disease (24).

Type 2 diabetes is a multifaceted disease that impacts the health of many people in Canada.

1.3 Gangliosides and Insulin Resistance

Studies have shown a connection between gangliosides, specifically GM3, and insulin resistance. The insulin receptor, which is involved in insulin signaling, binds caveolae-1 and GM3 independently with no association between GM3 and the caveolin-1 complex. However, when GM3 accumulates in the lipid raft, it causes dissociation of the caveolin-1 complex from the insulin receptor. TNF alpha, which is involved in inflammation, has been shown to increase GM3 synthesis in adipocytes (21)(31). This dissociation results in the impaired functioning of the insulin receptor. This change is reversible, as it has also been shown that through the addition of D-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (D-PDMP), an inhibitor of ganglioside biosynthesis, insulin receptor function can be restored (32). Further, phosphorylation of the insulin receptor, and tyrosine phosphorylation of (insulin receptor substrate) IRS-1 are reduced in adipocytes when incubated with GM3 (33). Mutant mice lacking GM3 synthase have increased insulin sensitivity due to more rapid insulin receptor phosphorylation in the skeletal muscle. It was also found that the mice did not become insulin resistant, despite being fed a high-fat diet used to induce insulin resistance (34). Collectively this suggests that gangliosides play a role in insulin resistance at a mechanistic level. However, there are no known mechanistic human studies.

Despite the recurring theme of gangliosides being associated with insulin resistance, there is controversy in the literature as to which gangliosides are most involved. Transgenic mice that carried the human NEU3 gene encoding for sialidase 3, an enzyme in the membrane that removes sialic acid from glycolipids, developed insulin resistant diabetes and exhibited hyperglycemia and hyperinsulinemia by 18-22 weeks of age. The transgenic gene was expressed in multiple tissues with the main ones being the muscle, pancreas and heart. The diabetic mice also had less stimulation of tyrosine phosphorylation and insulin receptor phosphorylation, and lower glycogen synthase activitiy, which is also indicative of impaired insulin signalling. Increased levels of GM1 and GM2, which are poor substrates for sialadase, were found in various tissues of the transgenic mice along with a decrease in GM3 levels and higher levels of lactoceramide. It was found that GM2 and GM1 inhibited insulin receptor phosphorylation, while GM3 and GD1a did not impact insulin receptor phosphorylation. Therefore, the authors concluded that overexpression of NEU3 may lead to insulin resistance; however, the gangliosides they believe to be involved differ from those believed to be responsible for insulin resistance in previous studies (35). Further supporting evidence for the involvement of gangliosides with insulin resistance, other than GM3, comes from a study that reported that obese diabetic mice had increased levels of GM2, GM1 and GD1a in adipose tissue when compared to adipose tissues in control mice, whereas no change in the quantity of GM3 was observed (36).

Pharmaceutical agents that inhibit enzymes in the ganglioside synthesis pathway have been developed to provide insight into the role of gangliosides in insulin resistance. A study conducted in diet-induced obese diabetic mice and Zucker diabetic rats used the pharmaceutical agent (1R,2R)-nonanoic acid[2-(2',3'-dihydro-benzo [1,4] dioxin-6'-yl)-2-hydroxy-1-pyrrolidin-1-ylmethyl-ethyl]-amide-L-tartaric acid salt (Genz-123346) to inhibit glucosylceramide synthase, which is involved in the initial step of converting ceramide to glycosylceramide. It was found that the obese diabetic mice that were given Genz-123346 had lower glucose and hemoglobin A1c levels, as well as improved glucose tolerance when compared to the placebo treated diabetic rats. The treatment also helped to prevent the loss of beta cells in rats (57). Similar results were found in another study where a pharmaceutical compound N-(5'-adamantane-1'-yl-methoxy)-pentyl-1-deoxynojirimycin (AMP-DNM), which inhibits glycosylceramide synthase, was provided to rats and mice models of type 2 diabetes and obesity - namely, C57B1/6J mice fed a diet to induce glucose intolerance, ob/ob mice, and ZDF rats. AMP-DNM was found to lower circulating levels of glucose, improve glucose tolerance, reduce hemoglobin A1C, and improve insulin sensitivity in the liver, as there was increased phosphorylation of the insulin receptor and mTOR(10).

In addition to animal and cell culture experimental studies, the few human studies that have been conducted reveal an association between higher fasting serum in individuals with type 2 diabetes, individuals with hyperlipidemia, and

individuals with both type 2 diabetes and hyperlipidemia ganglioside concentrations compared to healthy controls. It was found that ganglioside serum levels in both hyperlipidemic and in hyperglycemic individuals were higher than in control participants (1.6 and 1.4 fold higher, respectively). It was also found that individuals with type 2 diabetes who had extreme visceral fat had elevated serum GM3 levels when compared to people with diabetes with normal amounts of visceral fat (37). In addition, a two-fold increase of ceramide in the muscle of obese participants has been noted (38).

Cell culture, animal and human experimental evidence supports the idea of insulin resistance being a micromembrane disorder (32). Gangliosides have been shown to mechanistically alter function of the insulin receptor, as GM3 has been shown to cause dissociation of the caveolin-1 complex from the insulin receptor (21)(31). It has also been shown that the inhibition of enzymes in the ganglioside metabolic pathway leads to improved glucose profiles and improved insulin sensitivity (57)(10). Further evidence from human studies shows that individuals with diabetes and elevated levels of visceral fat have altered ganglioside profiles when compared to control participants.

1.4 Ganglioside Analysis

The most common method for ganglioside analysis cited in the literature is thin layer chromatography (TLC). Lipids are extracted from a tissue sample and purified of salts and impurities; the lipid is then dissolved in chloroform and methanol and spotted 1.5 cm from the bottom of the TLC plate. The plate is

placed in a tank and immersed in approximately 1cm of the chromatographic solvent system used in the separation process. The lipid is then carried by the chromatographic solvent system, separating on the TLC plate. The plate is then dried and can be stained by Ehrlich reagent to detect gangliosides.

Although TLC allows for the separation and detection of gangliosides, the limitations are that TLC does not allow for determination by mass or for detection of different molecular species and is very time consuming. This technique, despite its drawbacks, is affordable and accessible. Currently, the best technique for ganglioside analysis is high-pressure liquid chromatography and analysis by mass spectrometry (LCMS). This combination allows for very rapid precise characterization, classification and quantification of the different ganglioside species. Barriers to LCMS include high capital costs and professional training requirements (58). Methodology with regards to ganglioside analysis by mass spectrometry is presented in the Methods section of Chapter 3. TLC and LCMS are two viable techniques for ganglioside separation and detection.

1.5 Other Lipids

1.5.1 Structure and Function

Lipids are a large class of molecules divided into numerous classifications. Lipids are defined by solubility in non-polar solvents. Some of these classes include non-esterified fatty acids, glycerolipids (mono, di and triacylglycerols), glycerophospholipids (phosphatidylethanolamine, phosphatidylcholine, phosphotidylserine), sphingolipid (sphingosine, ceramide), and steroids. Each

class of lipids differs in both structure and function of the molecule. The majority of lipids in the diet and mammalian tissue are found in the esterified form as mono, di or triacylglyceride (triglycerides). Triglycerides are composed of a glycerol backbone and three fatty acid molecules (39). Triglycerides act as a form of energy storage for humans and are often found surrounded by amphiphilic phospholipids (40).

According to data collected by NHANES, dietary lipids comprise between 25.1 (5^{th} percentile) - 42.9% (95^{th} percentile) of the diet with the mean energy intake at 33.3% (41). Fat can be obtained through dietary consumption, or produced through de novo lipogenesis in the liver. One of the main products of de novo lipogenesis is palmitate. Palmitate is produced in response to a high glucose load in the blood and is rapidly converted to oleic acid (42).

Dietary fat is associated with functional and metabolic changes in the body. Consumption of fat has been shown to have a satiating impact by altering the hormones released from the gut (43). Dietary fat alters membrane structure by altering biosynthesis of phospholipids. This alteration in membrane function due to structural changes in the lipid can result in changes in regulation and function of a cell by altering hormone binding, gene regulation, and many other cellular processes (44). Dietary fat also plays a role in altering insulin binding to receptors. A diet with a high polyunsaturated to saturated ratio increases insulin binding resulting in increased uptake of glucose. (45). Lipids are complex, diverse molecules involved in the regulation of many cellular processes.

1.5.2 Fat digestion and absorption

Dietary lipid digestion starts in the mouth with lingual lipase (46). The process of digestion is continued in the stomach by gastric lipase (47). Both lingual lipase and gastric lipase specifically digest the sn-3 position of the triglyceride. The dietary fat is transported to the small intestine where it combines with pancreatic juice and bile, which contain pancreatic lipase, colipase and bile salts necessary to break down and emulsify the triglyceride into fatty acids and monoglycerides. Digestion of triglycerides by pancreatic lipase occurs primarily in the upper part of the jejunum. Pancreatic lipase is responsible for the majority of lipid breakdown, acting mainly on fatty acids found in the sn-1 and 3 positions, and resulting in the production of 2-monoacylglycerol and free fatty acids (48). Bile acids help to emulsify the fat, increasing the amount of surface area available for digestion (46). Absorption of fatty acids and monoglycerides occurs with the aid of bile acid through the formation of a micelle, which transports the lipids through the aqueous medium (46)(48).

Once fat components have entered the enterocyte, triglycerides are re-formed, allowing for packaging and secretion of chylomicrons into the lymphatic system. Medium chain triglycerides (8-12 carbons) can be released directly into the bloodstream (46). Chylomicrons are mainly composed of triglycerides, cholesterol ester, cholesterol and phospholipids. Phospholipids make up most of the membrane along with protein and cholesterol esters. Chylomicrons are composed of approximately 95% triglycerides and contain apoB-48, as the distinguishing proteins (49).

Normally chylomicron levels peak at 4 hours postprandially in the blood stream (50). Triglycerides in chylomicrons are broken down by lipoprotein lipase. Lipoprotein lipase is a rate-limiting enzyme responsible for clearance of chylomicrons from the circulatory system. When fatty acids are released from the chylomicron, the fatty acids are taken up primarily by adipose tissue or muscle for storage or oxidation. The type of dietary fat influences clearance rate of chylomicrons from the blood, as polyunsaturated fat is cleared faster than saturated fat (46). The size and composition of the meal, quantity of fibre and fat, all influence the triglyceride and postprandial response. A diet high in fat increases the triglyceride response relative to a low-fat diet (51). A diet high in monounsaturated fat decreases the production of apoB-48 relative to a diet high in saturated fat (74). Simple sugar has also been shown to influence the lipid postprandial response. When simple sugar (sucrose or fructose) was added to a fatty meal, it increased lipid postprandial response. However, soluble dietary fibre has been shown to reduce the number of chylomicrons produced (53). The process of digestion and the absorption of fat is a complex process involving multiple steps and factors.

1.5.3 Chylomicron Secretion in Individuals with Type 2 Diabetes

Individuals with type 2 diabetes often have dyslipidemia. Dyslipidemia is thought to be due to the fact that these individuals experience insulin resistance. The impaired response to insulin results in higher non-esterified fatty acid levels

both in a fasting state and at 2 hours when compared to control participants. It has also been shown that MTP, a protein critical for assembly of chylomicrons, is upregulated in patients with insulin resistance causing an increase in the number of chylomicrons produced and a decrease in chylomicron size (54). Overproduction of chylomicrons may be a main contributing factor to the state of dyslipidemia in individuals with type 2 diabetes (55). For individuals with type 2 diabetes, the clearance rate of chylomicrons is slower than in control participants: this may be due to the deficiency of apoE protein (54). Low grade inflammation that is present in type 2 diabetes may also contribute to dyslipidemia. TNF-alpha is associated with increased chylomicron production in the intestine. When the intestine is in a state of insulin resistance, it upregulates de novo lipogenesis causing increased production of fat, which may also be a factor in dyslipidemia in individuals with type 2 diabetes (55). Individuals with type 2 diabetes often have altered lipid profiles and overproduction of chylomicrons.

Chapter 2: Rationale, Hypothesis and Objectives.

2.1 Rationale

The development of leading edge technology for ganglioside analysis by LC triple quad mass spectrometry has enabled fast and sensitive analysis of ganglioside molecular species. Animal research has also shown that dietary ganglioside causes modification in intestinal gangliosides (2). Recent publications note the role of gangliosides in insulin resistance. Insulin resistance in adipocytes is associated with an increased level of GM3 (31). It was also found that individuals with type 2 diabetes who had extreme (> 200 cm^2) amounts visceral fat exhibited elevated GM3 levels in fasting blood samples when compared to people with diabetes with normal amounts of visceral fat (37). Limited research has been conducted on the composition of gangliosides in fasting plasma and no known research has been conducted on the ganglioside content of chylomicrons or the ganglioside content in postprandial plasma in either control participants or individuals with type 2 diabetes. Gangliosides are involved in many different processes in the body and it is possible that changes in both the ganglioside content in the plasma and chylomicron fractions contribute to the pathology of diabetes. There are no studies in the literature on the role of high- or low-fat diets on chylomicron or plasma ganglioside composition.

The present research was completed to determine whether or not the intestine may play a role in ganglioside secretion, as well as to determine

whether or not ganglioside composition in the plasma or chylomicrons may be indicative of type 2 diabetes. Understanding if the fat content of a diet influences ganglioside secretion may eventually influence recommendations given to individuals with diabetes, and may also provide further insight into the role of fat in the diet, and the development of insulin resistance.

2.2 Hypothesis

Working Hypothesis:

Gangliosides are secreted from enterocytes after a meal and supplied to peripheral tissues as part of the chylomicron particle.

Specific Hypothesis:

1. Ganglioside content in plasma reflects secretion of fat in chylomicrons.

2. Changes in specific gangliosides in the plasma occur as a result of gangliosides secreted from the gut.

3. Gangliosides secreted from enterocytes are different in participants with type 2 diabetes compared to non-diabetic participants at fasting versus fed states after consuming high- and low-fat diets for three days each.

2.3 Objectives

Hypotheses one and three will be tested by determining:

1. If there is a relationship between the quantity of total gangliosides and TG secreted in plasma after participants were fed high- versus low-fat diets in a randomized cross over design.

2. If there is a difference in relative percentage and total abundance of GD3, GM3 and GD1 in the plasma of individuals with diabetes and that of control participants at fasting, 2, 4, 6, and 8 hour timepoints postprandial.

3. If the relative percentage of GD3, GD1 and GM3 in a chylomicron differ in the chylomicron fraction of control participants and participants with type 2 diabetes at 0 versus 4 hours.

4. If there is a difference in the relative percent of saturated, monounsaturated and polyunsaturated gangliosides present in the chylomicron fractions of control

participants and participants with diabetes fed both a high-fat diet and low-fat diet.

Hypotheses two and three will be tested by determining:

1. If there is a difference in the relative percentage of saturated, monounsaturated and polyunsaturated gangliosides, and major ganglioside species present in the plasma of control participants and participants with diabetes in fasting and postprandial (2 hr, 4 hr, 6 hr, 8 hr) samples.

2. If there is a difference in the relative percentage of specific gangliosides in the chylomicron fractions at 0 versus 4 hour timepoints in control participants versus participants with diabetes.

3. If there is a difference in the relative percentage of saturated, monounsaturated and polyunsaturated gangliosides and major ganglioside species present in the chylomicron fraction of control participants and participants with diabetes in fasting and 4 hour postprandial samples.

4. If there is a difference in the relative percentage of major ganglioside species in the plasma of participants with diabetes and control participants at fasting, 2, 4, 6 and 8 hours after being fed both a high-fat diet and low-fat diet.

Chapter 3: Fasting and Postprandial Plasma Ganglioside Levels in Relation to High- and Low-fat Diets in Control Participants and Individuals with Diabetes.

3.1 Introduction

Gangliosides are bioactive lipids composed of a fatty acid chain, carbohydrate moieties, and sialic acids (1). These bioactive lipids are found ubiquitously in cell membranes and are involved in growth, differentiation and cell signaling. Gangliosides are commonly found in the nervous system and are abundant in the brain (2). Recent studies have shown a connection between gangliosides, specifically GM3, and insulin resistance. The insulin receptor, which is involved in insulin signaling, binds caveolae-1 and GM3 independently with no physical association between GM3 and the caveolin-1 complex. However, when GM3 accumulates in the lipid raft, it causes dissociation of the caveolin-1 complex from the insulin receptor. This dissociation results in impaired insulin receptor function (21)(31). Further evidence supports the role of GM3 in insulin resistance. A study in humans investigated the association between fasted serum ganglioside concentrations and the following groups compared to healthy controls: individuals with type 2 diabetes, individuals with hyperlipidemia, and people with both type 2 diabetes and hyperlipidemia. It was found that serum levels of GM3 were 1.6 fold higher in individuals with hyperlipidemia and 1.4 fold higher in hyperglycemic individuals compared to control participants. It was also found that individuals with type 2 diabetes who had extreme visceral fat had elevated GM3 levels when compared to people with diabetes with normal amounts of visceral fat (37). However there is controversy as to which

gangliosides are involved in the development of insulin resistance. GM2 and GM1 were found to inhibit insulin receptor phosphorylation in insulin resistant diabetic mice, while GM3 and GD1a did not impact the insulin receptor phosphorylation (35). Limited research has been conducted on the composition of gangliosides in fasting plasma of individuals with type 2 diabetes versus control participants in a fasting state, and no studies have investigated postprandial plasma ganglioside levels in humans. The objective of the present study was to determine ganglioside (GM3, GD3, and GD1) profiles and composition at fasting, 2, 4, 6, and 8 hour postprandial timepoints in matched pairs, each consisting of an individual with diabetes and a control subject fed both a high- and low-fat diet for three days each in a crossover design.

3.2 Participants and Methods

Samples for this study were collected for a prior study examining the impact of carbohydrate and fat intake on fasting and postprandial triglyceride levels and rate of hepatic de novo lipogenesis (59). Participants were recruited from the outpatient Metabolic Clinic at the University of Alberta Hospital, from the diabetic registry, and from a list of respondents compiled from an earlier study. Ten control individuals and eleven individuals with type 2 diabetes were recruited. All individuals provided informed consent. Individuals completed a case history and provided information on food aversions and preferences. The initial study was approved by the Faculty of Agriculture, Forestry and Home Economics and Human Research Ethics board at the University of Alberta. The analysis of

blood for the purpose of the present study was also approved by the Health Research Ethics Board Biomedical Panel at the University of Alberta.

Baseline information was used to screen participants. Information collected included: BMI, waist circumference, a 12 hour fasting blood sample, which allowed for determination of total cholesterol, LDL and HDL cholesterol levels, as well as triglyceride, glucose, free fatty acid, insulin and hemoglobin A1c levels. Individuals were excluded if triglyceride levels were found to be >4.0 mmol/L or if the individual was on lipid lowering drugs. Individuals taking other forms of medication were asked to record medication use and to continue to use medications as instructed. Medications and laboratory results were reviewed by an endocrinologist (59).

3.2.1 Characteristics of participants

Individuals with diabetes and control participants were matched based on gender, age and BMI. Baseline data were obtained a minimum of one month before beginning the study (Table 1) (59).

Table 1: Fasting baseline characteristics of participants who completed the study protocol.

	Control Group	Individual with Diabetes
Gender (M/F)	3/4	3/4
Age (yr)	51.4 ± 9.2	50.0 ± 8.8
BMI (kg/m^2)	33.5 ± 8.3	33.2 ± 7.5
Weight (kg)	93.4 ± 24.1	91.9 ± 15.4
Waist (cm)	105.6 ± 15.8	105.6 ± 13.5
Glucose (mmol/L)	5.0 ± 0.4*	6.2 ± 1.1
HBAlc (%)	5.3 ± 0.4*	5.9 ± 0.5
Insulin (U/ml)	9.9 ± 7.7	13.6 ± 7.9
HOMA	2.22 ± 1.7	3.85 ± 2.4
FFAs (mmol/L)	0.88 ± 0.4	0.66 ± 0.3
Triglycerides (mmol/L)	1.35 ± 0.4	1.96 ± 0.8
TC (mmol/L)	5.41 ± 0.8	4.76 ± 0.6
HDL (mmol/L)	1.34 ± 0.2	1.21 ± 0.2
LDL (mmol/L)	3.46 ± 0.7 *	2.67 ± 0.6

Mean ± Standard Deviation * indicates significant difference ($p \leq 0.05$) (Modified table taken from Wilke et al.(59)).

3.2.2 Study design

All participants were fed both a high- and low-fat diet for three days each. Between diets there was a washout period of one month. Information regarding participants' background diets was obtained. Participants were asked to record all food, drinks and medications for the seven days prior to consuming the test diets. Individuals picked up packaged meals from the Human Nutrition Research Unit at the University of Alberta. Instructions indicated that participants were to

consume only the diet provided with the exception of calorie free fluids, and omit all alcohol and vigorous exercise. Participants were not informed as to which diet they had received. Containers were to be returned unwashed in order to determine the level of compliance of the individual.

Individual daily energy needs of the participants were estimated based on the Harris Benedict equation, with the inclusion of an activity factor. The low-fat or high-carbohydrate diet was high in complex carbohydrates – with limited simple sugars - and consisted of less than 25% fat. The high-fat diet provided was low in carbohydrates and consisted of approximately 35% fat. The same menu was used for the high- and low-fat diets with the same amount of calories being provided. To achieve a diet of 35% energy from fat for the high-fat diet, canola oil, which is mainly monounsaturated fatty acids, was added. To increase the amount of polyunsaturated fatty acids in the low-fat diet, flax seed and safflower oil were added. At each meal (breakfast, lunch and dinner) a third of the participant's daily energy was provided (Table 2). Similar macronutrient composition and energy was provided at all three meals. The same meals were consumed for all three days of the study. Each diet was blind and randomized. Each participant completed both dietary treatments (high- and low-fat diets) and, therefore, acted as his/her own control. Providing the diets to participants allowed for control of food intake and also served as a measure of compliance (59).

Table 2: Menu items consumed in high- and low-fat diets.

Breakfast	Lunch	Dinner
Orange juice *Blueberry oatbran muffins *Scrambled egg *Bread	*Whole wheat rotini *Tomato base pasta sauce (vegetables, beef, mozzarella) *Peas Apple	*Turkey sandwich Raspberry newtons Melon pieces

Meals were identical on all 3 days of diet intervention for both high and low-fat diets. To meet specific fatty acid amounts and composition, safflower oil, flax oil and 50% less fat margarine were added to low fat items; canola oil and canola margarine were added to high-fat items as indicated by *. (Taken from Wilke et al.(59)).

3.2.3 Analytical methods

Both the composition of the background diet (7 days prior to study) and the study diet were analyzed using Food Processor II nutrient analysis computer software (VP9.6.2, Esha Research 2004). Study diets were prepared based on calculated recipes and the fat from the diet was extracted and analyzed. The fatty acid profile of oils and fat were analyzed by Gas Liquid Chromotography (61). [See Appendix for Diet composition (Table 21) and Fatty acid composition (Table 20)].

Meals were picked up on Monday between 7:00 and 8:00 am and completed 7-day food records were handed in. On Monday and Tuesday, individuals consumed the prepared meals. On Wednesday at 7:30 am blood collection started and individuals were asked to return food containers from the

previous day. Blood samples from participants were collected through the insertion of an intravenous catheter into the forearm of each participant. The first fasting blood was collected into a tube containing disodium EDTA. The test breakfast along with D_2O was consumed within 15 minutes of being served. Participants then fasted for 12 hours. At 2, 4, 6, and 8 hours after the test breakfast was consumed, blood was collected. Immediately after the blood samples were collected, lunch was consumed and dinner was provided and was consumed a minimum of 12 hours before the last blood draw at 9:00 am the next day. During the three-day diets, alcohol was not consumed and participants did not participate in intense exercise for 24 hours before blood collection. Blood was collected into tubes containing lithium heparin for analysis of plasma lipids, glucose and insulin, which were analyzed by the University of Alberta Hospital Laboratory. Other blood parameters were analyzed from blood collected into sodium heparin tubes (59).

Blood samples were immediately centrifuged at 1000 x g for 10 minutes at 4 °C in order to separate out plasma. The samples were stored in the -80 °C freezer until used. A Folch was performed on 250 µl of plasma to extract lipids (gangliosides) for analysis (60). A solution of 1.25 ml of 2:1 choloform: methanol was added to 250µl of plasma with 28ul of 0.025% $CaCl_2$, which was used for extraction. The top layer of the Folch extract was obtained and dried down using nitrogen gas. The ganglioside content of plasma was analyzed using an Agilent 6430 Triple-Quad LC/MS system (Santa Clara, CA). Ganglioside samples were prepared in 50/50 water and methonal prior to injection into the mass

spectrometer. Ganglioside MS measurements were obtained using an Agilent 6430 Triple-Quad LC/MS system (Santa Clara, CA) operating in negative ion mode. Deprotonated gas-phase ions of the various ganglioside species were obtained using electrospray ionization, with the electrospray needle held at -4500 V. The MS operated in multiple reaction monitoring mode (MRM). A library of theoretical precursor ions was generated for over 600 gangliosides of specific ceramide and carbohydrate composition. The first quadruple mass filter was set to scan for these specific precursor ions, allowing each to sequentially pass into the hexapole collision cell where ions were fragmented using collision induced dissociation (CID). Gangliosides readily lose a sialic acid moiety when subjected to CID; thus, the second quadrupole mass filter was set to only allow this characteristic fragment ion (*m/z* 290) to reach the detector. The CID and ion source voltages for each ganglioside class (ie GM, GD) were optimized using the Agilent Optimizer software. Data acquisition and analysis were carried out using the Agilent Mass Hunter software package. The quantity of gangliosides was calculated relative to an external standard. Any area response under 50 was determined to be non-distinguishable and was not used for quantitative purposes.

3.2.4 Statistical method

Statistical analysis was computed using SAS (Version 9.3, SAS Institute Inc., USA). Two-factor repeated measures of analysis of variance was used to

determine statistical significance between collection timepoints, diet type and groups (individuals with diabetes and control participants), and if there were interactions between the different variables. If an overall statistical difference was detected by the two-factor repeated measure analysis of variance, then a Duncan's multiple analysis of variance was used to determine specifically what values were statistically different. Statistical difference was determined if the $p < 0.05$. A student T- test was used to determine statistical significance between high- and low-fat diets and control versus individuals with type 2 diabetes.

3.3 Results

Participants were included in the results only if they completed both the high- and low-fat arms of the study and complied with study guidelines. One recruitment obstacle was the exclusion of individuals with type 2 diabetes who were on lipid lowering drugs. Four participants withdrew before starting the study, two participants after completing only one diet, and one due to illness (59).

3.3.1 Dietary intake

All food provided was consumed by the majority of the participants. If the full meal was not consumed, the food was weighed and caloric content was determined. The uneaten food was then subtracted from the food provided to the subject in the second dietary arm. A questionnaire was used to determine whether or not individuals were blinded. Only 4 out of the 14 participants were able to correctly identify whether the diet was low or high fat. Caloric intake and

macronutrient distribution was estimated to determine usual diet from 7-day food records. Average caloric intake estimated prior to the consumption of the high- and low-fat diets was 2269 and 2197 kcal respectively for individuals with diabetes, and 2269 and 1725 respectively for control participants. The average protein macronutrient percentage distribution for the diet consumed in the seven days prior to the high- and low-fat diets for individuals with diabetes was 17% for both groups, and 17% and 18% respectively for the control participants. The average percentage of the macronutrient intake that was carbohydrate consumed seven days prior to intervention was 48% for both the high- and low-fat diets for individuals with type 2 diabetes, and 48% and 49% respectively for high- and low-fat diets for the control group. Average fat intake was found to be 35% and 36% prior to intervention of high- and low-fat diets, respectively, for individuals with diabetes, and 35% and 34%, respectively, for control participants (59).

3.3.2 Fasting and Postprandial Glucose, Insulin and Triglyceride Measures.

There was no difference between the insulin or glucose response related to either diet for individuals with type 2 diabetes or for control participants. However, there was a significant increase in glucose 2 hours after consumption in individuals with diabetes consuming a low-fat diet when compared to either the high- ($P<0.001$) or low-fat control group ($P<0.01$). (Figure not shown (59)). There was also higher insulin concentration at 2 hours in the group of individuals with diabetes consuming the low-fat diet, compared to the control group that

consumed either diet (P<0.05) (Figure not shown (59)). A significant difference was found between triglyceride concentrations at different collection timepoints. Triglycerides were found to be highest in the 4 hour postprandial sample (Table 5). There was no significant difference between the high- and low-fat diets nor between individuals with diabetes and control participants (Table 3, Table 4).

Table 3: Plasma triglyceride levels (mmol/l) in participants fed high- and low-fat diets.

Diet	TG
High-fat	1.92 ± 0.10
Low-fat	2.05 ± 0.11

Sample size: 84 in each group. No significant difference was found between triglycerides in the high- and low-fat diet. P value: 0.19. Mean± standard error. Significance was determined to be P value < 0.05.

Table 4: Plasma triglyceride levels (mmol/l) in individuals with diabetes and control participants.

Participants	TG	Baseline Fasting TG
Individuals with diabetes	2.33 ± 0.13	2.05 ± 0.29
Control	1.64 ± 0.07	1.46 ± 0.14

Sample size: 84 in each group. No significant difference was found between the overall triglyceride levels of individuals with diabetes and control participants. P value: 0.16. No significant difference was found between the baseline fasting triglyceride values for individuals with diabetes and control participant. P value: 0.08. Significance was determined to be a P value < 0.05. Mean± standard error

Table 5: Plasma triglyceride levels (mmol/l) over collection timepoints.

Timepoints	TG
Fasting Baseline	1.75 ± 0.17 [c]
2 hr	2.25 ± 0.16 [ab]
4 hr	2.42 ± 0.21 [a]
6 hr	2.08 ± 0.21 [b]
8 hr	1.60 ± 0.17 [c]
Fasting 24 hr	1.83 ± 0.17 [c]

Sample size: 28 at each timepoint. A significant difference was found between the collection timepoints. P value: <0.0001. Timepoints with the same letter (a,b or c) are not significantly different. Timepoints with different letters (a, b or c) are significantly different. TG= triglyceride. Significance was determined to be a P value < 0.05. Both the baseline and fasting 24 hr samples were fasted for 12 hrs. Mean ± standard error.

3.3.3 Total and Relative Percentage of Fasted and Postprandial Gangliosides in Plasma of Individuals with Type 2 Diabetes and Control Participants.

No significant difference was found in the total quantity of plasma gangliosides (GM3, GD3 and GD1) in the plasma of individuals with type 2 diabetes and control participants, nor between high- and low-fat diets (Table 6). A significant difference in the quantity of total gangliosides was found between the collection timepoints. At the 6 hour timepoint, ganglioside in the plasma was lower than both the 8 hour and fasting 24 hour timepoint ($P<0.05$) (Table 7). The highest total ganglioside level found was at the fasting 24 hour timepoint and the lowest at the 6 hour timepoint. No significant interactions were found between groups and collection timepoints, collection times and diets, or diets and groups.

A similar trend was found in the total plasma ganglioside/ triglyceride levels. No significant differences were found between high- and low-fat diets nor between individuals with diabetes and control participants; however, there was a significant difference between the collection timepoints. The fasting 24 hour

sample and 8 hour timepoint were found to be significantly different from all other timepoints (P<0.0001) (Table 7). The highest ratio of gangliosides to triglyerides was observed at the 8 hour timepoint and the lowest triglyceride to ganglioside ratio was observed at both the 2 hour and 4 hour collection timepoints. No significant interactions were found between groups and collection timepoints, collection times and diets, or diets and groups.

Table 6: Total plasma ganglioside (GG) levels in high- and low-fat diet groups and individuals with diabetes and control participants.

	Group		Diet	
Total GG (ng/ml)	Type 2 Diabetes	Control	High Fat	Low Fat
	661± 0.03	745 ± 0.04	669 ± 0.03	735 ± 0.04

Sample size: 78-79 for individuals with diabetes and control participants. P value: 0.09. Sample size: 75-82 for the high- and low-fat diets. P value: 0.18. No significant interactions were found involving control participants or individuals with diabetes or different timepoints of collection. GG= gangliosides. Significance was determined to be P value < 0.05. Mean ± standard error.

Table 7: Total plasma ganglioside (GG) levels in fasting and postprandial samples.

Timepoints	Total GG (ng/ml)	Total GG (ng/ml) /TG Ratio (mmol/l)
Fasting Baseline	664 ± 0.05^{ab}	0.43 ± 0.05^{b}
2 hr	669 ± 0.07^{ab}	0.32 ± 0.04^{b}
4 hr	707 ± 0.04^{ab}	0.32 ± 0.04^{b}
6 hr	593 ± 0.04^{b}	0.37 ± 0.05^{b}
8 hr	774 ± 0.07^{a}	0.62 ± 0.09^{a}
Fasting 24 hr	815 ± 0.08^{a}	0.59 ± 0.10^{a}

Sample size for total gangliosides: 25-27 participants. P value: < 0.05. Sample size for the ganglioside to triglyceride ratio: 26–28. P value < 0.0001. No significant interactions were found involving control participants or individuals with diabetes or the high- or low-fat diets. Timepoints with the same letter (a,b or c) are not significantly different. Timepoints with different letters (a, b or c) are significantly different. TG= Triglyceride. GG = ganglioside. Significance was determined to be P value < 0.05. Both the baseline and fasting 24 hr samples were fasted for 12 hrs. Mean ± standard error.

3.3.4 Relative percentage of GM3, GD3 and GD1 in the plasma.

No significant difference was found between individuals with type 2 diabetes and control participants nor between high- and low-fat diets in relative percent of GM3, GD3 and GD1. GM3 was found to be the most abundant ganglioside in the plasma followed by GD3 and GD1. A significant difference was found between the collection timepoints and levels of GM3, GD3 and GD1. The highest relative percent of GM3 was found at the 6 hour collection timepoint, while the lowest was observed at fasting baseline (P value: 0.0007) (Table 8). A significant difference was found between the relative percentage of GD3 in fasting baseline and postprandial samples (P value: 0.002). The fasting baseline sample was found to be significantly larger than the 2, 6, and 8 hour timepoints, while the 6 hour timepoint was statistically different from the 4 hour and fasting baseline, as well as the fasting 24 hour timepoint. The highest relative percent of GD3 was found to be highest at fasting baseline and lowest at the 6 hour collection timepoint (Table 8). There was also a significant difference between collection sequence timepoints, as the 4 hour timepoint for GD1 relative percent was significantly larger than the value recorded at the 2 and 6 hour timepoints. A significant difference exists between the 6 hour timepoint and both the 4 hour and 8 hour timepoints for the relative percent of GD1. The lowest relative percent of GD1 was at the 6 hour collection timepoint and the highest at the 4 hour collection timepoint (Table 8). No significant interactions between groups and

collection timepoints, collection times and diets, or diets and groups for the relative percent of GD1, GD3 or GM3 were observed.

Table 8: Relative percentage of plasma GM3, GD3 and GD1 in fasting and postprandial sample.

Timepoints	GM3 %	GD3 %	GD1 %
Fasting Baseline	96.3 ± 0.67^{c}	3.30 ± 0.67^{a}	0.40 ± 0.13^{bac}
2 hr	98.7 ± 0.28^{a}	0.98 ± 0.19^{cd}	0.32 ± 0.12^{bc}
4 hr	96.9 ± 0.42^{bc}	2.40 ± 0.32^{ab}	0.70 ± 0.16^{a}
6 hr	99.1 ± 0.20^{a}	0.82 ± 0.17^{d}	0.12 ± 0.12^{c}
8 hr	97.8 ± 0.41^{ab}	1.74 ± 0.33^{bdc}	0.49 ± 0.13^{ba}
Fasting 24 hr	97.3 ± 0.56^{bc}	2.23 ± 0.49^{bac}	0.43 ± 0.14^{bac}

Sample size: 25-27 participants for the relative percent of GM3. P value: 0.0007, Sample size: 25-27 people for the relative percent of GD3. P value: 0.002. Sample size: 25-27 for the relative percent of GD1. P value: 0.01. No significant interactions were found between control participants or individuals with diabetes or the high- or low-fat diet. Timepoints with the same letter (a,b or c) are not significantly different. Timepoints with different letters (a, b or c) are significantly different. Significance was determined to be P value < 0.05. Both the baseline and fasting 24 hr samples were fasted for 12 hrs. Mean ± standard error.

3.3.5 Total quantity of GM3, GD3 and GD1 in the plasma.

No significant difference was found between individuals with type 2 diabetes and control participants, nor between high- and low-fat diets when determining the total quantity of GM3, GD3 and GD1 in the plasma (Table 9). There was no significant difference found between total quantity of GM3; however, there was a trend towards significance (P value: 0.06) with the fasting 24 hour timepoint being the highest and the 6 hour timepoint being the lowest (Table 10). There was a statistically significant difference ($P<0.05$) between the timepoints for the total quantity of GD3, as the 6 hour timepoint was significantly different from fasting baseline and the fasting 24 hour collection timepoint. The fasting 24 hour value was significantly different from the 6 hour and 2 hour

timepoints. The fasting 24 hour collection timepoint had the highest quantity of gangliosides, while the 6 hour timepoint had the lowest quantity (Table 10). There was also a statistically significant difference between collection timepoints for the total quantity of GD1 (P value: 0.0005). The 4 hour collection timepoint was significantly higher than all other timepoints (Table 10).

Table 9: Total quantity of plasma GM3, GD3 and GD1 (ng/ml) levels in high- and low-fat diet groups.

Diet	Total GM3 (ng/ml)	Total GD3 (ng/ml)	Total GD1 (ng/ml)
High-fat	651 ± 0.03	18.1 ± 0.003	5.80 ± 0.001
Low-fat	713 ± 0.04	16.0 ± 0.002	4.02 ± 0.001

Sample size GM3: 76-82. P value: 0.19. Sample size GD3: 80-83. P value: 0.44. Sample size: 81-82. P value: 0.48. Sample size GD1: 81-83. P value: 0.41. Significance was determined to be P value < 0.05. Mean ± standard error.

Table 10: Total quantity (ng/ml) of plasma GM3, GD3 and GD1 in fasting and postprandial samples.

Timepoints	GM3 (ng/ml)	GD3 (ng/ml)	GD1 (ng/ml)
Fasting Baseline	639 ± 0.05	22.1 ± 0.004 [a b]	5.00 ± 0.002 [b]
2 hr	657 ± 0.07	10.0 ± 0.002 [b c]	3.08 ± 0.002 [b]
4 hr	680 ± 0.04	20.4 ± 0.004 [a b c]	10.4 ± 0.003 [a]
6 hr	586 ± 0.04	8.21 ± 0.002 [c]	1.11 ± 0.001 [b]
8 hr	752 ± 0.07	17.9 ± 0.004 [a b c]	5.00 ± 0.001 [b]
Fasting 24 hr	786 ± 0.07	23.9 ± 0.008 [a]	4.62 ± 0.001 [b]

No significant difference was found involving individuals with diabetes and control participants or between the high- and low-fat diet. Sample size for GM3 (ng/ml): 25-27. P value 0.06. Sample size for GD3 (ng/ml): 26-28. P value: < 0.05. Sample size for GD1 (ng/ml): 26-28. P value: 0.0005. Timepoints with the same letter (a, b or c) are not significantly different. Timepoints with different letters (a, b or c) are significantly different. Significance was determined to be P value < 0.05. Both the baseline and fasting 24 hr samples were fasted for 12 hrs. Mean ± standard error.

3.3.6 Percentage of Monounsaturated, Polyunsaturated and Saturated GM3, GD3 and GD1 Gangliosides.

All percentages for gangliosides were calculated as a proportion of total gangliosides. A significant difference was found between the collection

sequences for the percent of monounsaturated (GM3, GD3 and GD1), saturated (GM3, GD3) and polyunstaturated (GD1, GD3 and GM3) gangliosides (Table 11,13,14). The percent of GD1 saturated ganglioside was found not to be a contributing factor and, therefore, the data was not included in the table (Table 14). The percentage of monounsaturated GM3 gangliosides was found to be over half of the total gangliosides present in the plasma. Compared to both the respective polyunsaturated and saturated levels of GM3, GD3 and GD1, it is apparent that monounsaturated gangliosides are the most abundant in the plasma making up between approximately 76.5-81% of the total gangliosides measured when GM3, GD3 and GD1 are included (Table 11). GM3 contains the highest percentage of gangliosides in monounsaturated, polyunsaturated and saturated gangliosides, followed by GD3 and GD1, respectively (Table 11,13,14). At 2 hr postprandial there was a significant difference in the GD1 monounsaturated ganglioside content between individuals with diabetes versus control participants)(Figure 4). The relative percentage of GD3 polyunsaturated ganglioside was higher in the group of individuals with diabetes when compared to the control participants (Table 12).

Table 11: Percentage of GD1, GD3 and GM3 gangliosides that are monounsaturated.

Timepoints	GD1 % Mono	GD3% Mono	GM3 % Mono
Fasting Baseline	3.79 ± 0.45^{b}	19.0 ± 1.91^{a}	58.3 ± 1.54^{b}
2 hr	3.86 ± 0.52^{b}	9.60 ± 1.49^{c}	65.8 ± 0.93^{a}
4 hr	4.99 ± 0.46^{a}	15.5 ± 2.04^{ab}	56.1 ± 2.47^{b}
6 hr	3.74 ± 0.49^{b}	9.82 ± 0.57^{c}	65.8 ± 0.85^{a}
8 hr	3.85 ± 0.53^{b}	11.1 ± 0.90^{c}	63.9 ± 1.47^{a}
Fasting 24 hr	3.14 ± 0.47^{b}	13.1 ± 1.07^{bc}	64.0 ± 1.26^{a}

Sample size ranged from 26-28 for all groups. GD1 % Monounsaturated. P-value: 0.02. GD3 % Monounsaturated. P value: 0.0001. GM3 % Monounsaturated. P value: <0.0001. Timepoints with the same letter (a, b or c) are not significantly different. Timepoints with different letters (a, b or c) are significantly different. Mono= Monounsaturated. Significance was determined to be P value < 0.05. Both the baseline and fasting 24 hr samples were fasted for 12 hrs. Mean ± standard error.

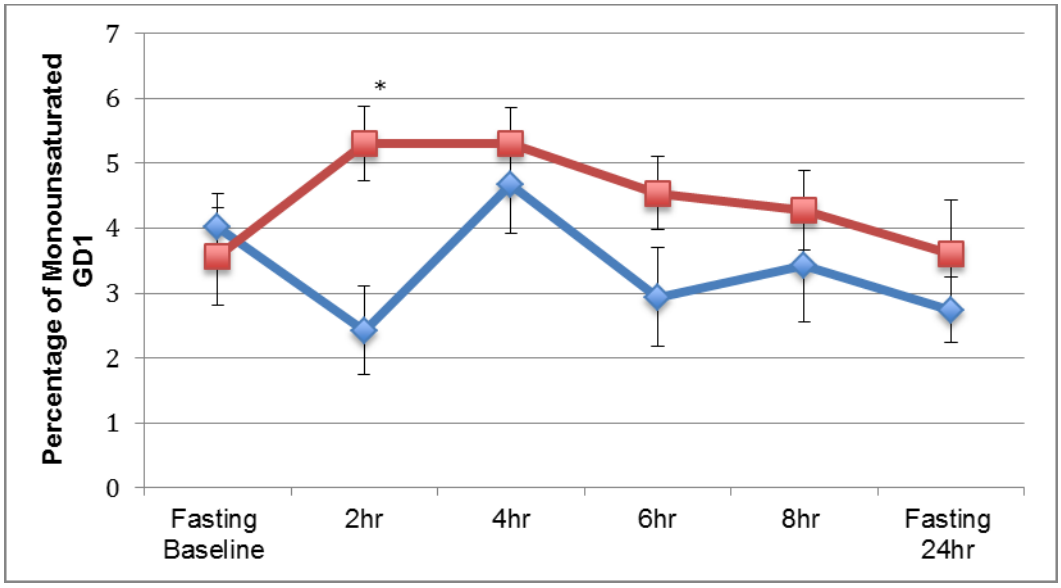

Figure 4: Percentage of GD1 gangliosides that are monounsaturated. □: Control participants . ♦: Individuals with type 2 diabetes. Sample size: 12-14. P value at 2 hr: 0.003. * indicates significant difference. Significance was determined to be P value < 0.05. Both the baseline and fasting 24 hr samples were fasted for 12 hrs. Mean ± standard error.

Table 12: Relative percentage of GD3 gangliosides that are polyunsaturated in individuals with diabetes and control participants.

Group	GD3% Polyunsaturated
Individuals with diabetes	3.81 ± 0.38^{a}
Control participants	2.78 ± 0.21^{b}

Sample size:79-80. P value: 0.01. Values with the same letter (a or b) are not significantly different. Values with different letters (a or b) are significantly different. Significance was determined to be P value < 0.05. Mean ± standard error.

Table 13: Relative percentage of GD1, GD3 and GM3 gangliosides that are polyunsaturated.

Timepoints	GD1 % Poly	GD3% Poly	GM3 % Poly
Fasting Baseline	0.29 ± 0.08^{b}	2.07 ± 0.38^{c}	13.1 ± 0.20
2 hr	0.24 ± 0.07^{b}	5.58 ± 0.96^{a}	12.5 ± 0.18
4 hr	0.57 ± 0.14^{a}	2.49 ± 0.34^{bc}	14.2 ± 1.31
6 hr	0.25 ± 0.11^{b}	3.31 ± 0.34^{bc}	14.0 ± 0.86
8 hr	0.15 ± 0.05^{b}	3.56 ± 0.52^{b}	13.5 ± 0.78
Fasting 24 hr	0.06 ± 0.04^{b}	3.01 ± 0.25^{bc}	14.6 ± 0.78

Sample size for all groups ranged from 22-28. GD1 % Polyunsaturated. P-value: 0.004. GD3 % Polyunsaturated. P value: <0.0001. GM3 % Polyunsaturated. P value: 0.60. Timepoints with the same letter (a, b or c) are not significantly different. Timepoints with different letters (a, b or c) are significantly different. Significance was determined to be P value < 0.05. Both the baseline and fasting 24 hr samples were fasted for 12 hrs. Mean ± standard error.

Table 14: Relative Percentage of GD3 and GM3 gangliosides that are saturated.

Timepoints	GD3% Saturated	GM3% Saturated
Fasting Baseline	0.61 ± 0.21^{a}	0.85 ± 0.23^{c}
2 hr	0.00 ± 0.00^{b}	2.15 ± 0.52^{a}
4 hr	0.37 ± 0.15^{ba}	1.25 ± 0.24^{abc}
6 hr	0.00 ± 0.00^{b}	1.11 ± 0.30^{bc}
8 hr	0.11 ± 0.10^{b}	2.00 ± 0.44^{ab}
Fasting 24 hr	0.42 ± 0.27^{ba}	1.23 ± 0.22^{abc}

Sample size for both groups ranged from 24-28. GD3 % Saturated. P value: 0.03. GM3 % Saturated. P value: 0.05. Timepoints with the same letter (a, b or c) are not significantly different. Timepoints with different letters (a, b or c) are significantly different. Significance was determined to be P value < 0.05. Both the baseline and fasting 24 hr samples were fasted for 12 hrs. Mean ± standard error.

Table 15: Abundant individual ganglioside from plasma, as a percentage of total gangliosides.

Timepoints	GM3 34,1 %	GM3 36,1 %	GD3 34,1 %
Fasting Baseline	37.6 ±1.75 [ab]	5.45 ± 0.46 [c]	8.70 ± 0.71 [b]
2 hr	38.8 ±1.62 [ab]	7.88 ± 0.47 [a]	7.86 ± 0.43 [b]
4 hr	34.6 ± 2.00 [b]	6.16 ± 0.39 [bc]	8.30 ± 0.56 [b]
6 hr	41.5 ± 1.53 [a]	7.55 ± 0.54 [a]	9.06 ± 0.51 [b]
8 hr	41.2 ± 1.48 [a]	7.10 ± 0.40 [ab]	8.95 ± 0.66 [b]
Fasting 24 hr	42.2 ± 1.09 [a]	5.71 ± 0.39 [c]	11.1 ± 0.60 [a]

GM3 34,1% sample size; 26-28. P value: 0.006. GM3 36,1% sample size: 26-28. P value: 0.0002. GD3 34,1 % sample size: 26-28. P value: 0.001. Timepoints with the same letter (a, b or c) are not significantly different. Timepoints with different letters (a, b or c) are significantly different. Significance was determined to be P value < 0.05. Both the baseline and fasting 24 hr samples were fasted for 12 hrs. Mean ± standard error.

No significant difference was found between individuals with type 2 diabetes and control participants nor between high- and low-fat diets for the relative percents of GM3 34,1, GM3 36,1 and GD3 34,1. However a significant difference was observed between the different ganglioside collection timepoints (Table 15). GM3 34,1 had the highest relative percent of ganglioside at 6, 8 and fasting 24 hour collection timepoints. A significant difference in the relative percent of GM3 34,1 is observed between the 4 hour collection timepoint and the 6, 8 and fasting 24 hour timepoints. The relative percent of GM3 36,1 was also found to vary over the timepoints. Fasting basline and fasting 24 hour collection timepoints are significantly lower than 2, 6 and 8 hour collection timepoints. The 2 and 6 hour timepoints were statistically different from the fasting 24 hour, 4 hour and fasting baseline collection timepoints. The only significantly different observation observed in the GD3 34,1 relative percent was at the fasting 24 hour timepoint, as all the other timepoints were found to be not statistically different.

Discussion

The results do not support my original hypothesis that gangliosides secreted from enterocytes are different in participants with type 2 diabetes compared to the non-diabetic participants in fasting versus fed states or after consuming high- versus low-fat diets for three days each. Between the two subject groups, it appears that overall there was no difference in the specific gangliosides secreted from the gut, as no difference was observed between the plasma ganglioside profiles of the groups. There was little variability between the plasma gangliosides profiles of an individual with type 2 diabetes versus that of the control participants when consuming either the high- or low-fat diet.

One possible explanation for the lack of variation between the two groups of participants is the use of matched controls. The two subject groups compared were matched for BMI, waist circumference, age, gender and weight. The control population exhibited HOMA-IR values that could be classified as insulin resistant; however, these participants were not diagnosed as diabetic, as glucose levels were not impacted (59). It is, therefore, possible that the lack of significance was because both groups were matched and both were insulin resistant. In a different human study, a significant difference was found between GM3 quantities in hyperglycemic individuals compared to normal participants (37). It is also important to note that the individuals with type 2 diabetes in the present study exhibited higher glucose and insulin values when consuming the low-fat diet as compared to the control group when consuming either the low- or high-fat diets.

In the present study, individuals with type 2 diabetes exhibited higher average glucose levels, which is indicative of poorer metabolic function, than participants in the control group.

One explanation for the lack of significance was that both groups likely had similar amounts of adipose tissue. Adipose tissues contain approximately 10-15 nmol of gangliosides/g with 80-90% of gangliosides being GM3 (65). It is possible that the quantity of adipose stores influence ganglioside composition and/or quantity in the plasma. A previous study reported that individuals with type 2 diabetes that had visceral fat area greater than 200 cm^2 have higher levels of GM3 than those with waist circumferences less than 200 cm^2 (37). It is, therefore, possible that because participants in the present study were matched based on BMI, no significant difference in ganglioside content was observed. Both groups were on average in their early fifties, obese, and had higher than recommended waist circumferences. Thus, it is possible that if adipose tissue contributes to plasma ganglioside levels, no significant difference was seen between the groups because both groups had similar adipose stores and, therefore, similar amounts of gangliosides were released from adipose tissue.

Triglyceride data showed that there was no significant difference between triglyceride values for the high- and low-fat diets, nor between individuals with diabetes and control participants when all timepoints and diets were averaged (Tables 3 and 4). It is possible that the lack of difference in triglyceride levels between the two groups may be because both dietary fat intakes were within the

acceptable macronutrient distribution range (20-35%) and, therefore, did not cause significant biological variability. However, triglyceride values did peak at 4 hours as would be expected (50).

There was no significant difference between total ganglioside levels in plasma between individuals with type 2 diabetes and control participants; however, both the control group and low-fat group have higher ganglioside levels. It is, therefore, possible that total ganglioside levels may reflect the metabolic status of an individual.

One of the consistent differences observed throughout the data is the variance in ganglioside levels over the different collection timepoints. One possible explanation for this difference is that there may be different rates of ganglioside absorption, release, synthesis, degradation, and recycling of gangliosides at during fasting and after a meal. Possible evidence for this is supported by data from the 4 hour collection timepoint. The relative percent of GM3 at the 4 hour timepoint decreases, while the relative percents of GD3 and GD1 increase (Table 8). This trend is supported, as the relative percent of two of the most abundant GM3 species (GM3 34,1 and 36,1) decrease at the 4 hour timepoint, while this trend is not seen in the most abundant GD3 species 36,1, where there is little variation between timepoints with the exception of the fasting 24 hour timepoint (Table 15). Consequently, it is possible that there is an increased production of GD3 and GD1 by the 4 hour timepoint. This idea that the amount of GD3 and GD1 increases at 4 hours is also supported by the

quantitative data (Table 10). The quantity of GM3 does not seem to be impacted; however the total ganglioside quantity is higher at 4 hours (Table 7).

A similar pattern was also seen when determining saturation of gangliosides. Overall, in all gangliosides species observed, monounsaturated gangliosides are the predominant species. It is possible that the abundance of monounsaturated gangliosides may have been influenced by the high proportion of monounsaturated fatty acid in the diet provided to the participants. It is also possible that there is preferential synthesis of monounsaturated gangliosides. The preferential synthesis of monounsaturated gangliosides at the 4 hour timepoint may help explain the trend in the data. Data from the 4 hour timepoint shows that there is a significant decrease observed in the relative percent of monounsaturated GM3 and an increase in the percentage of both monounsaturated GD3 and monounsaturated GD1. This data suggests that there may be favourable production or release of monounsaturated GD3 and GD1 species 4 hours after consumption. This trend, however, is not present in the polyunsaturated species. It is also possible that there is increased production of saturated GD3 gangliosides at this time; however, the trend is less pronounced.

Minor amounts of saturated GD1 were detected in the plasma and, therefore, this data was not included. However high variability was found in the levels of saturated GD3 and GM3 (Table 14).

There is evidence in the data to support the idea that GD1 monounsaturated gangliosides and GD3 polyunsaturated gangliosides may play

different roles in individuals with diabetes and in control participants (Figure 4, Table 12). It is possible that monounsaturated GD1 may impact the severity of metabolic dysfunction, as at 2 hours postprandially, there is a significant difference in this measure between individuals with diabetes and control participants; however, no known studies in humans or animals have been done to support or refute this data. It is also possible that the GD3 polyunsaturated ganglioside may play a role in metabolic functioning, as there is a higher percentage of polyunsaturated GD3 ganglioside in individuals with diabetes when compared to the controls. The relative percent of polyunsaturated GD1 also increases at the 4 hour timepoint with reduction in the relative percent of GD3. It is possible that at this timepoint there is conversion of GD3 to GD1b or that there is increased synthesis of polyunsaturated GD1. However, there are no known studies on postprandial synthesis of polyunsaturated gangliosides. GD1a, GD1b and GD1c were not separated out due to the small quantity present in the samples.

Another trend apparent in the data is that there is a higher quantity of total gangliosides, GM3 and GD3, during fasting and at the 8 hours postprandially (Table 7, 10). This trend is reflected in relative percentage of two of the three most abundant gangliosides GM3 34,1 and GD3 34,1, as both gangliosides seem to have higher 24 hour fasting values. This trend may, in part, be explained by VLDL production/secretion and incorporation of gangliosides into lipoproteins during a fasting state, and the possible release of gangliosides from adipocytes.

It is also possible that in a fasted state there is decreased uptake of gangliosides. In a fasting state, decreased levels of insulin allow for free fatty acids to be released from adipocytes, allowing for delivery to the liver and reesterification and secretion in the form of VLDL. There is also a decreased ability of the adipose tissue to take up triglycerides during the fasting state. A similar pattern of increased release and decreased uptake may also be observed with gangliosides. However, it is important to note that there is also decreased apo B clearance and triglyceride lipolysis in a diabetic state, as insulin is a major hormone involved in uptake. Glycosylation and oxidation of lipoprotein particles may also interfere with uptake and clearance (66). It is, therefore, also possible that the state of insulin resistance in both the control participants and individuals with diabetes may be characterized by the decreased uptake of gangliosides by cells, thereby resulting in elevated ganglioside levels at the 8 hour and fasting timepoints.

GM3 and GD3 may be incorporated into apo-B lipoprotein secretion from the liver and, thus, influence plasma levels. A study performed in cells suggests that GD3 may be involved in apo-B lipoprotein secretion because when GD3 antibodies were added to the cells, there was a decrease in secretion of apoB lipoproteins in response to retinoic acid stimulation. It was also proposed that GD3 may promote secretion of triglyceride rich apoB containing lipoproteins (VLDL), while GM3 is incorporated into triglyceride poor lipoproteins (62). The idea of gangliosides being important in lipoprotein secretion is also supported by

the fact that the liver contains a relatively large number of gangliosides. Between 214-220 nmol/g of gangliosides are found in the liver with 90-91.6% being GM3 (65). A significant portion of gangliosides in humans has been found to be in the LDL component of the serum during fasting, with approximately 66% being in the LDL portion and 7% in the VLDL portion (64). However, it is unlikely that the LDL portion contributes to the differences observed, as it has been shown that there is little variability of LDL concentration in the postprandial period (71). Even though gangliosides are known to be incorporated into lipoporoteins, it appears that there is no relationship between total gangliosides and triglycerides, since a regression was done and determined not to be statistically significant.

Chapter 4: Ganglioside Levels in Chylomicron Fractions in Control Participants and Individuals with Diabetes at Fasting and 4 hours.

4.1 Introduction

Participants with and without diabetes had similar plasma ganglioside levels. It is unknown whether ganglioside content of chylomicron differs between the two groups. Chylomicron secretion in individuals with type 2 diabetes is altered compared to healthy participants, as there is an upregulation of MTP, a protein necessary for assembly of the chylomicron in a state of insulin response. As a result, there is often an increased number of small chylomicrons produced (68). Currently there is no known research on the quantity of gangliosides in chylomicrons of individuals with type 2 diabetes. However research has shown that gangliosides are present in cell membranes of enterocytes. GD3, a species of ganglioside, has been shown to localize on the basolateral side of enterocytes in rats, while GM3 localizes on the apical side (2). It is, therefore, possible that chylomicrons may act as a vehicle for the delivery of gangliosides around the body. The objective of this research is to determine if the composition of gangliosides in chylomicrons differs in individuals with diabetes and control participants in both postprandial and fasting states.

4.2/ 4.2.1 Participants and Methods /Study design

The same methods were used as in 3.2.1 and 3.2.2. Both chylomicron and plasma samples were collected from blood samples obtained from the participants.

4.2.2 Analytical Methods

Lipoprotein particles from 2 ml of plasma were separated using ultracentrifugation within 24 hours of collection. Chylomicrons and chylomicron remnants were removed in a single centrifugation (69, 70). A Folch extraction was performed on 250 µl of the chylomicron samples to extract lipids. The top layer containing gangliosides was dried using nitrogen gas. The dried down top layer was divided into two samples with two different solvent systems the first sample was prepared with 120µl 50/50 water and methanol, and the second sample with 50µl hexane, 70µl of 70% methanol, 15% water and 15% isopropyl alcohol prior to injection into the mass spectrometer. The two solvent systems were used as the solvent systems differed in polarity and, therefore, prevented clogging of the LCMS due to extraneous triglyceride from the chylomicron fraction. The total volume of each the chylomicron sample was recorded to determine percentage of total volume of chylomicron sample taken. Each chylomicron sample (250 µl) was taken from the total chylomicron sample and analyzed for ganglioside content. After extraction, the ganglioside content was quantitatively analyzed using an LC/MS system (Santa Clara, CA).

Ganglioside MS measurements were obtained using the same methods presented in section 3.2.3 of Chapter 3. However, due to low levels detected, no area cut offs were established. Instead each peak was investigated manually. Retention time and mass to charge ratio was used to determine if the peak was due to a real ganglioside. Gangliosides were quantified relative to an external standard.

4.2.3 Statistical methods

Statistical analysis was computed using SAS (Version 9.3, SAS Institute Inc., USA). Two-factor repeated measures of analysis of variance was used to determine statistical significance between collection timepoints, diet types and groups (individuals with diabetes and control participants), and to determine if there were interactions between the different variables. If an overall statistical difference was detected by the two-factor repeated measure analysis of variance, then a Duncan's multiple analysis of variance was used to determine which values were statistically different ($p < 0.05$). A student T- test was used to determine statistical significance between values for high- versus low-fat diet and control participants versus individuals with type 2 diabetes.

4.3 Results

4.3.1 Relative Percent of Gangliosides (GM3, GD3, and GD1) in the Chylomicron Fraction in Control Participants and Individuals with Diabetes at both Fasting and 4 hours Postprandial.

The most abundant ganglioside determined from the relative percent data was GM3, followed by GD3 and GD1 (Table 16). No significant difference was found between the relative percents of GD1, GD3 or GM3. The relative percent of GM3 was found to be the highest at the fasting baseline timepoint and the relative percent of GD3 was lowest at that same timepoint. The relative percent of GD1 was highest at 4 hours. No significant interactions were found between groups and collection timepoints, collection times and diets, or diets and groups.

Table 16: Relative percent of gangliosides (GM3, GD3 and GD1) in the chylomicron fraction at both fasting and 4 hrs postprandial.

Timepoints	GM3 %	GD3 %	GD1 %
Fasting Baseline	75.3 ± 3.25	18.3 ± 2.55	6.41 ± 1.08
4 hr	67.4 ± 5.15	23.2 ± 4.25	7.36 ± 1.42
Fasting 24 hr	67.3 ± 4.04	26.8 ± 4.50	5.68 ± 0.75

GD1 % sample size: 22-26. GD1%. P value: 0.53. GM3 % sample size: 21-26. GM3%. P value: >0.05. GD3% sample size: 21-25. P value: >0.05. Timepoints with the same letter (a, b or c) are not significantly different. Timepoints with different letters (a, b or c) are significantly different. No significant difference was found involving individuals with diabetes and control participants nor between the high- and low-fat diets. Mean ± standard error.

4.3.2 The Relative Percent of the Most Abundant Gangliosides in the Chylomicron Fraction in Control Participants and Individuals with Diabetes at both Fasting and 4 hours postprandial.

The most abundant ganglioside in chylomicrons is GM3 34,1, followed by GM3 36,1, and GD3 34,1 (Table 17). No significant difference was found between the relative percent of GM3 34,1, GD3 34,1, GM3 36,1 and the total number of other gangliosides. The relative abundance of gangliosides was calculated from the total ganglioside species present. The relative percent of GM3 34,1, GM3 36,1 and the total number of other gangliosides were highest at the fasting 24 hour timepoint. The relative percentage of GD3 34,1 was consistent. No significant interactions were found between groups and collection timepoints, collection times and diets, or diets and groups.

Table 17: The most abundant gangliosides from a chylomicron fraction, expressed as a percentage of total gangliosides.

Timepoints	GM3 34,1 %	GD3 34,1 %	GM3 36,1%	Total # other GG
Fasting Baseline	38.8 ± 1.87	7.50 ± 0.70	15.9 ± 1.22	14.8 ± 0.40
4 hr	38.9 ± 3.17	7.09 ± 0.73	13.8 ± 1.42	14.5 ± 0.53
Fasting 24 hr	40.9 ± 2.73	7.25 ± 0.73	17.2 ± 1.34	15.6 ± 0.41

GM3 34,1% sample size: 22-26. P value: 0.94. GD3 34,1% sample size: 22-26. P value: 0.77. GM3 36,1% sample size: 22-26. P value: 0.14. Other GG sample size: 22-26. P value: 0.18. Timepoints with the same letter (a, b or c) are not significantly different. Timepoints with different letters (a, b or c) are significantly different. No significant difference was found involving individuals with diabetes and control participants or between the high- and low-fat diets. The relative abundance of gangliosides was calculated from the total ganglioside species present. Mean ± standard error.

4.3.3 Composition of Saturated and Unsaturated Individual Gangliosides from the Chylomicron Fraction, as a Percentage of Total Gangliosides for Control Participants and in Individuals with Diabetes.

Table 18: Composition of monounsaturated individual gangliosides from the chylomicron fraction, as a percentage of total gangliosides.

Diet	GM3 Mono%	GD3 Mono %	GD1 Mono %
Fasting Baseline	72.6 ± 2.88	16.8 ± 1.87	3.26 ± 0.36
4 hr	72.5 ± 3.32	17.0 ± 2.67	2.60 ± 0.37
Fasting 24 hr	73.4 ± 3.16	14.7 ± 1.97	2.66 ± 0.35

GM3 Mono % sample size: 22-25. P value: 0.98. GD3 Mono sample size: 21-24. P value: 0.93. GD1 Mono% sample size: 22-26. P value: 0.18. Mean ± standard error.

Table 19: Composition of saturated and polyunsaturated individual gangliosides from the chylomicron fraction, as a percentage of total gangliosides.

Diet	GM3 Sat %	GD3 Sat %	GM3 Poly %
Fasting	0.93 ± 0.13	0.38 ± 0.14	2.83 ± 0.41
Baseline	0.98 ± 0.19	0.57 ± 0.26	3.60 ± 0.57
4 hr	1.68 ± 0.44	0.35 ± 0.12	3.84 ± 0.51
Fasting 24 hr			

GM3 Sat % sample size: 21-26. P value: 0.10. GD3 Sat % sample size: 21-24. P value: 0.65. GM3 Poly % sample size: 22-26. P value: 0.81. Mean ± standard error.

GM3 was found to be the most abundant monounsaturated ganglioside followed by GD3 and GD1 (Table 18). No significant difference was found

between the collection timepoints in any of the monosaturated ganglioside species (Table 18). No significant interactions were found between groups and collection timepoints, collection times and diets, or diets and groups. There was also no significant difference between the relative percent of GM3 saturated, GD3 saturated and GM3 polyunstaturated gangliosides (Table 19). The relative percent of GM3 saturated and GM3 polyunsaturated ganglioside was highest at the 24 hour fasting timepoint. The highest relative percent of GD3 saturated ganglioside was found to be at 4 hours.

4.4 Discussion

The results do not support my original hypotheses that ganglioside content in plasma reflects secretion of fat in chylomicrons or that plasma gangliosides change as a result of gangliosides secreted from the gut. There is also no evidence to support the hypothesis that gangliosides secreted from enterocytes are different in type 2 diabetes compared to the non-diabetic participants fasting versus fed states after consuming high- versus low-fat diets for three days each.

Between the two subject groups, it appears that overall there is no difference in the specific gangliosides secreted from the gut, as no difference was observed between the chylomicron ganglioside profile of the groups. The chylomicron data shown above shows little variability between the ganglioside plasma profiles of an individual with type 2 diabetes versus that of a control subject when consuming either the high- or low-fat diet.

One possible explanation for this is the similarities between the participants, as previously explained in the Chapter 3 dicussion.

Previous studies have shown that gangiosides are predominantly transported by lipoproteins in the serum of fasted individuals; however, the chylomicron fraction was not measured in those studies (64).

It appears that over the postprandial period there is a shift in the relative percent of GM3, GD3 and GD1 gangliosides. At fasting baseline there is a larger relative percent of GM3 relative to GD3 and GD1. This may be due to increased production of GM3, increased absorption of GD3 and GD1, or possible degradation of GD3 and GD1. However, at 4 hours, it appears that there is an increase in the relative percent of GD3 and GD1 and a decrease in relative percent of GM3. It is possible that the increased relative percent of GD3 observed at 4 hours is a result of intestinally produced GD3. A study done in rats showed that GD3 is predominantly found on the basolateral side of the intestinal side and, therefore, it is possible that the ganglioside is incorporated into the chylomicron prior to secretion (2). It is also possible that GD1 may be preferentially incorporated into the chylomicron fraction over GM3.

No significant difference was seen between the levels of major ganglioside species. Over all the collection timepoints, the relative percent of GM3 34,1, GD3 34,1, GM3 36,1 and total number of gangliosides remained relatively consistent (Table 17). This data suggests that the major gangliosides appear to be consistently incorporated into chylomicrons and that the minor ganglioside

species may account for the variability seen in the relative percent of GD3, GM3 and GD1.

There also appears to be little variability in the relative percent of GM3, GD3 and GD1 monounsaturated gangliosides (Table 18). Monounsaturated gangliosides are the most abundant gangliosides in the chylomicron. This may be, in part, due to preferential incorporation of monounsaturated fatty acids, as monounsaturated fat was the predominant fat provided in the diet. More variability in the relative percent of saturated and polyunsaturated gangliosides is seen across the collection timepoints. The relative percentage of both polyunsaturated and saturated GM3 are the highest at the fasting 24 hour timepoint, while GD3 saturated ganglioside is highest at 4 hours. It is, therefore, possible that the minor gangliosides produced are responsible for the changes in ganglioside composition observed in the chylomicron fractions over the collection timepoints. Gangliosides with different fatty acid saturation may play different roles in different states in the body and changes in ganglioside levels may be due to degradation, increased synthesis, and/or absorption differences.

Chapter 5: Thesis Conclusion and Future Directions

Chapter 5: Thesis Conclusion and Future Directions

5.1 Hypothesis Conclusion

The data presented in this study does not support the original working hypothesis that:

1. Ganglioside content in plasma reflects secretion of fat in chylomicrons.

2. Changes in specific gangliosides in the plasma occur as a result of gangliosides secreted from the gut.

3. Gangliosides secreted from enterocytes are different in participants with type 2 diabetes compared to non-diabetic participants at fasting versus fed states after consuming high- versus low-fat diets for three days each.

Hypotheses 1 and 2 are not supported by the findings in this thesis. The level of gangliosides detected in chylomicrons was very low compared to the ganglioside content in the plasma at all timepoints. While chylomicron data was not included herein, it is unlikely that this small quantity would be a contributing factor to the total plasma ganglioside. Investigating the plasma triglyceride to ganglioside ratio data also did not support these hypotheses. The two highest ganglioside to triglyceride ratios were noted at the 8 hour and 24 hour fasting timepoints, while the lowest ratio was noted at the 4 hour timepoint (Table 7). If plasma ganglioside content reflected the quantity of triglycerides secreted as chylomicrons, the ganglioside to triglyceride ratios would be expected to remain constant, if both chylomicron triglycerides and gangliosides were cleared at the same rate. The increased ratio at the 8 hour and fasting 24 hour collection timepoints potentially indicates that ganglioside and triglyceride clearance rates may differ. However, this increase in the ganglioside to triglyceride ratios may

also be due to the release of gangliosides during fasting. Therefore further research is needed.

There was no significant difference in the ganglioside quantity recorded in either the plasma or chylomicron fraction when high- and low-fat diets were fed, which also supports the idea that ganglioside content is independent of fat levels. It also appears that the relative percent of abundant gangliosides secreted from the gut does not alter the relative percent of specific gangliosides in plasma, as the relative percent of GM3 34,1 and GD3 34,1 is approximately the same in both plasma and chylomicrons (Table 15, Table 17). It is likely that because chylomicrons contribute so minimally to the total quantity of gangliosides in the plasma, that the chylomicron ganglioside content has little impact on specific plasma gangliosides.

There is also very limited evidence in this study to support the idea that ganglioside levels are different in control subjects and individuals with diabetes (Hypothesis 3), as the majority of plasma and chylomicron data showed no difference between the two groups. However it is possible that GD1 monounsaturated gangliosides and GD3 polyunsaturated gangliosides are metabolized differently in individuals with diabetes versus control subjects (Figure 4, Table 12). However, in order to truly conclude whether or not the hypothesis statement is true, isotope labeled gangliosides should be used to trace ganglioside content in plasma and chylomicrons.

5.2 Conclusion Chapters 3 and 4: Fasting and Postprandial Plasma and Chylomicron Ganglioside Levels in Relation to High- and Low-fat Diets in Control Participants and Individuals with Diabetes.

This study was designed to look at whether or not the plasma ganglioside profiles of GM3, GD3 and GD1 differed between individuals with diabetes and control participants fed both a high- and low-fat diet, and to look at whether or not the chylomicron fraction ganglioside profiles of GM3, GD3 and GD1 differed between individuals with diabetes and control participants fed both a high- and low- fat diet. The results indicated that, overall, there was a lack of significant difference between plasma and chylomicron profiles for these groups. The lack of significant difference could potentially be due to the similarity of the participants, as both groups were matched for BMI, waist circumference, age, gender and weight. The control population exhibited HOMA-IR values that could also be classified as insulin resistant, which may have impacted the results. Significant differences were observed over the collection timepoints in plasma. It is possible that the changes may be due to changes in ganglioside absorption, release, synthesis, degradation, and recycling. Significant differences were found between the groups regarding the relative percent of plasma GD1 monounsaturated ganglioside at the 2 hour collection sequence timepoint, and between the overall relative percents of plasma polyunsaturated GD3. Thus, it is possible that these gangliosides may impact metabolic function differently in the two groups. Overall in the chylomicron data, there was no significant difference between collection timepoints. The lack of variability in the relative percentage of

the most abundant ganglioside levels over collection timepoints in chylomicrons species also suggests that minor ganglioside species may account for more of the variability seen in the chylomicron fraction. Further research is needed to determine whether the composition of gangliosides in chylomicrons plays a physiological role in individuals with type 2 diabetes.

5.3 Limitations

This study is limited by the fact that both the control participants and individuals with diabetes were very similar in BMI and waist circumference and, consequently, this may have lead to the absence of a difference between the two groups. The sample size of the two groups was also small and, thus, limited the statistical power of the test. The study is also limited, as it is unknown as to whether or not any drugs taken by individuals in the study may alter ganglioside profiles.

5.4 Significance of the Research

This study is the first known study to examine ganglioside plasma and chylomicron profiles in individuals with type 2 diabetes and control participants at both postprandial and fasting timepoints after being fed both a high- and low-fat diet. This study is significant as there has been evidence in the literature linking gangliosides and type 2 diabetes, but relatively little research done in humans. Investigating whether or not the ganglioside profiles of these participants differ allows for a further understanding of whether or not gangliosides may play a metabolic role in type 2 diabetes and could further help understand the pathology of the disease. This research may ultimately help to contribute to a better

understanding of the role of gangliosides in insulin resistance and eventually may lead to the development of a more effective treatment of the disease.

5.5 Future Direction

Further research is needed to determine the role of gangliosides in both postprandial and fasted states. It would be beneficial to determine endogenous de novo lipogenesis of gangliosides in humans in order to understand where the majority of gangliosides are produced in a postprandial state and also to determine the roles of those gangliosides. Further research is also needed into ganglioside profile and quantity in LDL and VLDL fractions during fasted and postprandial phases in individuals with diabetes versus control participants in order to determine if the different lipoproteins may contribute to insulin resistance. It would also be beneficial to determine whether different body compositions and adipose stores influence the ganglioside content of the plasma and to determine if gender and age influence ganglioside secretion. There is evidence in rats to support the idea that ganglioside profiles may change with age (72). There is also a need for a human ganglioside feeding trials and investigation into whether dietary profile alters plasma and lipoprotein composition in individuals with diabetes and in control participants. Such research would provide a better understanding of the role of the intestine in ganglioside metabolism. Further mechanistic studies are also required into whether dietary fat (monosaturated, polyunsaturated and saturated fat) influences ganglioside composition and quantity in both the blood and in lipoprotein, and whether or not ganglioside levels impact individuals with type 2

diabetes. It is also possible that the inflammatory state of an individual impacts ganglioside metabolism in individuals with diabetes and, therefore, influences ganglioside profiles. There is also a lack of knowledge regarding how different organs in the body influence ganglioside levels in control participants and individuals with type 2 diabetes, as outlined in Figure 5. The diagram indicates that little is known about uptake, production and release of gangliosides from the liver, adipose tissue, muscle, and intestine. Further research is needed.

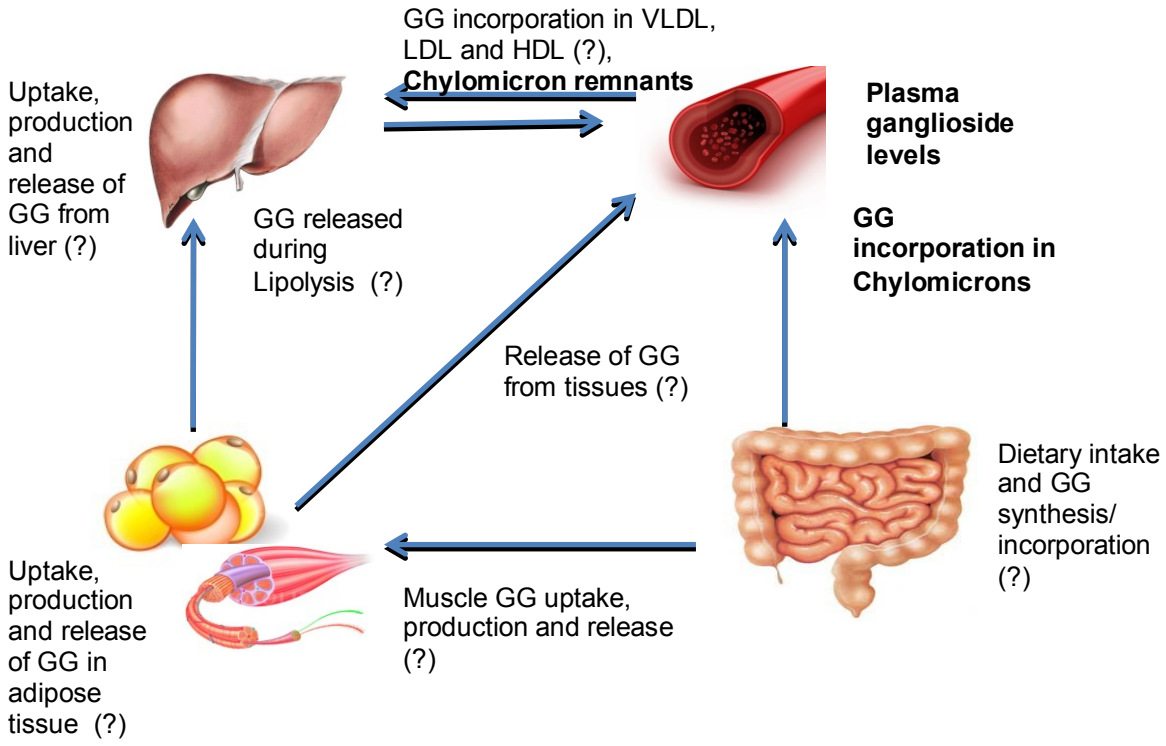

Figure 5: Unknown areas of research regarding ganglioside uptake, production and turnover in a postprandial state. Bold lettering indicates areas researched in this paper. (?) indicates unknown areas of research.

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Appendix

Table 20: Fatty acid composition of diets fed as analyzed by GLC.

Fatty Acid	**Test Meal**		**Total**	
	Breakfast		**Diet/day**	
	LF	**HF**	**LF**	**HF**
Saturated fatty acids	**6**	**8**	**4**	**6**
14:0-Myristic acid	0.4	0.6	0.3	0.5
16:0-Palmitic acid	4	5	3	4
18:0-Stearic acid	1.2	1.6	0.9	1.3
Monounsaturated fatty acids	**11**	**22**	**8**	**22**
18:1-Oleic acid	7	18	8	21
Polyunsaturated fatty acids	**10**	**8**	**11**	**9**
18:2-Linoleic acid	9	7	8	7
18:3-Linolenic acid	1.5	1.6	2.3	2.0
Other fatty acids	**1**	**1**	**1**	**1**
PUFA:SFA	**1.8**	**1.1**	**2.5**	**1.5**

After formulation, meals were prepared and analyzed for fatty acid composition. Values are mean % of energy (except PUFA:SFA) from a 2100 kcal diet (average intake for all groups). LF: lower fat diet, HF: higher fat diet. (Taken from (59)).

Table 21: Diet composition of the 2100 kcal total diet and 700 kcal test meal.

Nutrient	Test Meal		Total	
	Breakfast		Diet/day	
	LF	HF	LF	HF
Protein	**15**	**14**	**18**	**16**
Carbohydrates	**59**	**49**	**59**	**48**
Dietary Fiber (g)	**12**	**9**	**38**	**31**
Fat	**25**	**37**	**23**	**37**
Saturated Fat	5	7	5	6
Monounsaturated Fat	7	20	6	20
Polyunsaturated Fat	10	7	10	8
Trans Fatty Acid	**<1**	**<1**	**<1**	**<1**
Cholesterol (mg)	**273**	**291**	**370**	**388**
PUFA:SFA	**2.0**	**1.0**	**2.0**	**1.2**

Meals of similar fat and fibre composition were designed and adjusted to each subject's energy requirements. Values are mean % of energy (unless otherwise noted) from a 2100 kcal diet (average intake for all groups) as calculated in Food Processor after GLC of added oils and margarine fat composition was added to the database. LF: lower fat diet, HF: higher fat diet. (Taken from (59)).