Effects of Low Fat Versus High Fat Cheese on Glucose Homeostasis and Hepatic Lipid Metabolism in Prediabetic and Type 2 Diabetic Rats

by

Anik Ririe Ziezold Hanning

A thesis submitted in partial fulfillment of the requirements for the degree of

Master of Science

in
Nutrition and Metabolism

Department of Agricultural, Food and Nutritional Science
University of Alberta

Abstract

Background: Type 2 diabetes (T2D) is a disease characterized by insulin resistance and pancreatic beta cell failure. Lifestyle interventions, including dietary interventions, are the first line of treatment for this disease. At present, Canada's Food Guide recommends choosing low fat cheese over high fat cheese, however, current literature suggests a more complex relationship exists between cheese consumption and risk of T2D. Several fatty acids abundant in cheese have been shown independently to have beneficial effects on glucose homeostasis. Therefore, it is of particular interest whether low fat cheese and high fat cheese affect diabetes outcomes differently.

Objectives: (1) To determine the impacts of cheese feeding on *in vivo* responses to glucose and insulin in prediabetic and T2D rats. (2) To explore the mechanisms by which cheese affects metabolism in prediabetic rats using an untargeted metabolomic analysis. (3) To use the results of the *in vivo* studies and metabolomics assays to direct additional investigations of the effects of cheese feeding in prediabetic rats.

Methods: Two cohorts of rats, one prediabetic model (8-week old Sprague Dawley rats), and one T2D model (retired male Sprague Dawley breeder rats, 5-6 months old), were used. For each cohort, N = 64 animals were randomized to receive high fat diet (HFD) or low fat diet (LFD) for four weeks. In the T2D model, HFD rats underwent streptozotocin (STZ) administration at week 5 to induce a T2D phenotype. At the start of week 6, all HFD-fed animals (prediabetic and T2D models) were randomized to either continue on HFD or begin one of two cheese diets: HFD + high fat cheese (HFD+HFCh), or HFD + low fat cheese (HFD+LFCh) diet. HFD and cheese diets were isocaloric and matched for macronutrient composition. After 7-8 weeks of feeding, rats underwent either an oral glucose tolerance test (OGTT) or insulin tolerance test (ITT), and were

euthanized the following week for tissue collection. In the prediabetic cohort, serum was sent for metabolomic analysis, and both the serum and liver lipidome were analyzed. Further, histology was conducted in a sub-sample of prediabetic rats' livers.

Results: Food intake and body weight were similar between groups in both cohorts. There was no effect of diet on HOMA-IR, glucose-insulin AUC index, or fasted insulin and glucose in either cohort. In the T2D cohort OGTT blood glucose was significantly higher in the HFD+HFCh group and HFD groups, relative to LFD, while HFD+LFCh was not. In the prediabetic cohort, both HFD+LFCh and HFD+HFCh groups demonstrated improved insulin sensitivity relative to HFD during an ITT, while OGTT blood glucose was unaffected by diet. Metabolomic analyses revealed alterations in several phosphatidylcholine metabolites in serum of cheese-fed, prediabetic rats, while overall serum lipids remained unaffected by diet. Hepatic triglyceride accumulation was increased in prediabetic HFD+LFCh, with cholesterol ester accumulation also increased, but not reaching significance. A similar trend was observed in HFD+HFCh. Liver histology revealed markedly increased oil red-O staining in the livers of prediabetic animals fed cheese.

Conclusion: T2D rats that consumed HFD+LFCh had improved glucose tolerance that was not explained by body weight or insulin tolerance changes. Prediabetic rats that consumed either HFD+LFCh or HFD+HFCh demonstrated improved insulin sensitivity during an ITT. These rats also demonstrated increased liver triglyceride and cholesterol ester accumulation. This may have been due to altered phosphatidylcholine metabolism. These data suggest that either HFCh or LFCh may improve insulin sensitivity in a prediabetic model, while only LFCh improves glucose tolerance in a model of T2D.

Preface

This thesis is an original work by Anik R. Z. Hanning. The research project, of which this thesis is a part, received research ethics approval from the University of Alberta Animal Care and Use Committee, “Effect of naturally-occurring trans fats on insulin-secreting cells”, AUP232, 2017.

Some of the research conducted for this thesis was completed in collaboration with other members of the Chan lab. *In vivo* lab work completed in the cohort of prediabetic rats was begun by Xiaofeng Wang, Alexandra England, and Kristina MacNaughton. Analysis of serum metabolomic samples was done at the University of Alberta Metabolomics Innovation Centre by Erin Zhang. Analysis of serum and liver lipidome samples was performed by Audric Moses in the Lipidomics Core Facility at the University of Alberta. Finally, liver histology data was gathered by Taylor Huber and Zohre Hashemi. All *in vivo* procedures were completed alongside Xiaofeng Wang and Nicole Coursen. Study diets were designed by Catherine Field and Susan Goruk.

Acknowledgments

The completion of a thesis is rarely a solo affair, and therefore I would like to express my eternal gratitude to all of those who have helped me in the completion of this document. First and foremost, I must thank my supervisor, Dr. Cathy Chan. I frequently tell people how much I lucked out getting you as a supervisor. Your guidance, sense of humour, and super-human time management skills have been tremendously helpful, and inspirational. I can only hope to someday be as efficient and organized as you are. I would like to thank my committee members, Dr. Spencer Proctor and Dr. Gina Rayat, who always managed to know which questions I did not want them to ask (and then ask them). You poked holes in my arguments, took apart my every statement, and showed me how to put them back together to turn this thesis into a cohesive and logical document.

To the members of the Chan lab, past and present, thank you for taking in someone who could not even pipette properly and showing me the ropes of lab bench work. Special thanks to Zohre Hashemi, Xiaofeng Wang, Hui Huang, and Carina Yang who tolerated endless, and often repetitive questions, and were always happy and willing to go above and beyond the call of duty to help me. Animal work would never have been as enjoyable or simple without Nicole Coursen, and our animal procedure karaoke parties. To Fatheema Subhan, thank you for all your assistance with the statistics I had to learn. To Carolina Archundia Herrera, for your excellent PowerPoint presentation critiques, and Alexandra England, for learning lab procedures alongside me. Thank you to our summer students Mortaza Hassanabad and Taylor Huber, for showing that the best way to learn is to teach. I would like to acknowledge the financial support of Dairy Farmers of Canada for the personal and project funding.

Finally, thank you to my friends and family for your patience and support, and to Glenn for being my self-appointed thesis-writing taskmaster.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

15:0	Pentadecanoic acid
17:0	Heptadecanoic acid
AKT	Protein kinase B
ANOVA	Analysis of variance
AUC	Area under the curve
BG	Blood Glucose
BMI	Body Mass Index
CAD	Canadian dollar
CDA	Canadian Diabetes Association
cDNA	Complementary deoxyribonucleic acid
CHD	Coronary heart disease
CHO	Carbohydrate
CLA	Cis-9, trans-11 conjugated linoleic acid
CVD	Cardiovascular disease
ddH_2O	Distilled water
DI-MS	Direct ionization-mass spectroscopy
DNA	Deoxyribonucleic acid
GC	Gas-liquid chromatography
GLUT4	Glucose transporter type 4

HbA_{1C}	Glycated hemoglobin (Hemoglobin A_{1C})
HDL	High density lipoprotein
HF	High fat
HFD	High fat diet (control)
HFD+HFCh	High fat cheese dziet (experimental)
HFD+LFCh	Low fat cheese diet (experimental)
HFD/STZ	High fat diet / streptozotocin
HIV/AIDS	Human immunodeficiency virus infection and acquired immune deficiency syndrome
HOMA-IR	Homeostatic model assessment – insulin resistance
IDF	International Diabetes Federation
IGT	Impaired glucose tolerance
IP	Intraperitoneal
IRS-1	Insulin receptor substrate
ITT	Insulin tolerance test
LDL	Low density lipoprotein
LF	Low fat
LFD	Low fat diet (control)
MF	Milk fat
MetS	Metabolic syndrome
NMR	Nuclear magnetic resonance spectroscopy

OGTT	Oral glucose tolerance test
PDK1	Phosphoinositide-dependent kinase-1
P13K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PIP2	Phosphatidylinositol 4,5-bisphosphate
PIP3	Phosphatidylinositol (3,4,5)-trisphosphate
PKB	Protein Kinase B
PLS-DA	Partial least squares discriminant analysis
qPCR	Quantitative real-time polymerase chain reaction
RPM	Rotations per minute
SD	Sprague Dawley
STZ	Streptozotocin
t-16:1n-7	Trans-palmitoleate
T1D	Type 1 diabetes mellitus
T2D	Type 2 diabetes mellitus
TBST	Tris-buffered saline with 0.1% Tween 20
USD	United States Dollar
VA	Trans-vaccenic acid
VLDL	Very low density lipoprotein
W/V	Weight per unit volume
W/W	Weight per unit weight

Chapter 1: Literature Review

1.1 Thesis Introduction

This thesis addresses the hypothesis that diets rich in cheese improve the maintenance of normoglycemia in rats with prediabetes or type 2 diabetes (T2D). Moreover, diets that have been supplemented with low fat (<20% milk fat) cheese and high fat (≥20% milk fat) cheese will be compared, to examine whether cheese fat content can affect the endpoints we explore. Finally, should cheese consumption prove to be beneficial, the mechanisms through which this process may occur will be further examined.

1.2 Introduction to Diabetes

The regulation of blood glucose levels is essential for maintaining proper functioning of tissues that rely almost entirely on glucose for fuel, such as the brain and red blood cells. Under normal conditions, plasma blood glucose levels are maintained within a tight range. Glucose metabolism must be dynamic, and respond rapidly to continuously changing inputs of glucose. Moreover, minimum blood glucose levels must be maintained to ensure proper functioning of these tissues[1].

Diabetes mellitus is the result of an inability to secrete or produce insulin, leading to chronic hyperglycemia. It is classified into two main forms, type 1 diabetes (T1D) and T2D. T1D arises from the autoimmune-mediated destruction of pancreatic β-cells, which results in absolute insulin deficiency[2]. T1D accounts for 3-5% of all diabetes cases

globally[3]. T2D is a disease characterized by the combination of insulin resistance, wherein normal or elevated insulin levels produce an attenuated biological response, and β-cell failure. This insulin resistance manifests as decreased sensitivity to insulin-mediated glucose disposal. T2D accounts for roughly 95% of diabetes cases globally[3]. When insulin resistance first manifests, the body will hypersecrete insulin to compensate for its reduced ability to act. This state is known as hyperinsulinemia, and is often the point at which symptoms of impaired glucose metabolism begin to manifest. Prediabetes is a state of impaired glucose tolerance and impaired fasting glucose, albeit less pronounced than in patients with T2D. Eventually, the pancreas is unable to meet increased insulin demands, and progresses to overt T2D.

1.3 Causes of Type 2 Diabetes

Environmental factors, including diet and lifestyle, appear to have a large impact on T2D susceptibility. T2D occurs most frequently in middle-aged and older adults, although T2D is increasingly prevalent in the young[3]. Modifiable risk factors of T2D include obesity, over- or poor nutrition, and physical inactivity[3]. Obesity is a particularly important risk factor, as excess body weight can lead to insulin resistance. Insulin resistance leads to increased insulin requirements, and increased demand on the pancreas to produce insulin[4]. Eventually this increased demand cannot be met, leading to the development of T2D. Obese adults have a 2- to 4-fold risk of developing T2D, and 75.6% of Canadian adults with diabetes are classified as either overweight or obese[5]. Diet is the most important factor contributing to body weight, therefore it is not surprising

that diets high in calories and glycemic load, and low in fibre may predispose individuals to T2D[6]. A hereditary component is also present. In instances when one parent has T2D, the lifetime risk of offspring developing T2D is ~40%, and significantly increases to ~70% if both parents have T2D[7]. While genes may predispose an individual to diabetes, environmental and/or behavioural factors are necessary for the activation of that genetic susceptibility. In one study, 91% of cases of T2D were attributable to lifestyle factors. It was reported that nonsmokers who maintained a BMI <25, consumed diets high in fibre and polyunsaturated fat and low in saturated fat and glycemic load, exercised regularly, and consumed moderate alcohol had a 90% lower risk of T2D compared to those who did not[6]. Therefore, lifestyle factors, more specifically diet, are the focus of this thesis. One major focus of this thesis will be to investigate whether cheese feeding can prevent or improve the diabetic phenotype in insulin resistant and T2D rats.

In Canada, T2D is diagnosed using one of four measures: Hemoglobin A_{1c} (HbA_{1c}) > 6.5%, fasting blood glucose ≥7.0 mmol/L, or blood glucose ≥11.1 mmol/L 2 hours after a 75 g oral glucose tolerance test (OGTT), or random fasting glucose >11.1 mmol/L[8,9]. HbA_{1c} is a measure of average blood glucose over 3 months, expressed as the percentage of hemoglobin that is glycated[10]. A 75 g OGTT is a test of glucose metabolism where the subject ingests 75g of glucose orally, and their blood glucose is monitored following the food bolus. Prediabetes is the term assigned to those with impaired glucose tolerance (IGT), impaired fasting glucose (IFG), or moderately elevated HbA_{1c}. Prediabetes is diagnosed by HbA_{1c} 6.0-6.4%, fasting blood glucose 6.1-6.9 mmol/L (which indicates IFG), or 2-hour prandial glucose following a 75g OGTT that is 7.8-11.0

mmol/L (which indicates IGT)[9]. Figure 1.1 illustrates the diagnosis of T2D and/or prediabetes in Canada.

Figure 1.1: Screening and Diagnosing of Diabetes in Canada Flow Chart

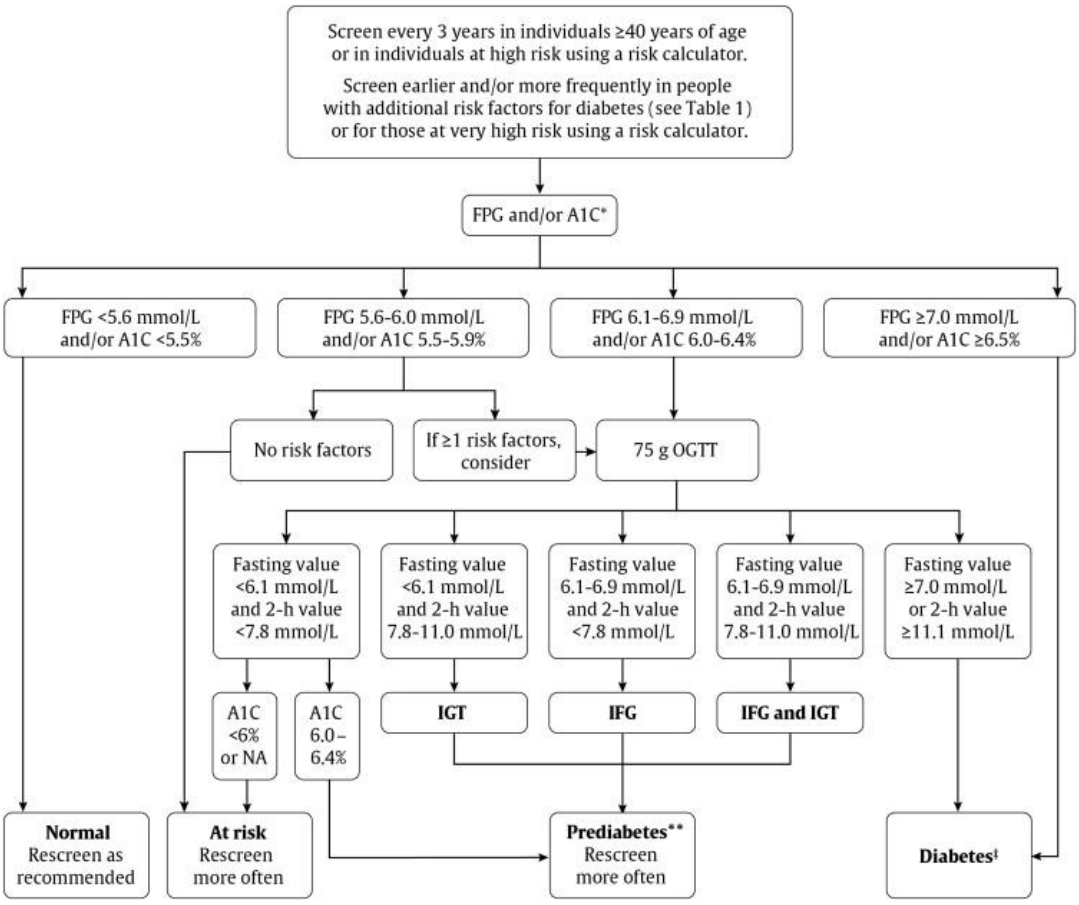


Figure created by [11]

1.4 Diabetes Prevalence and Economic Burden

As of 2008-2009, an estimated 2.4 million, or 6.8% of Canadians were living with diagnosed diabetes, with an additional 450,000 estimated to be living with undiagnosed diabetes[12]. Diabetes is on the rise, with a 70% increase in rates of diabetes diagnosis since 1998/1999. It is projected that in 2018/2019, 3.7 million Canadians will be living with

diagnosed diabetes[12]. In Canada, diabetes incidence is 3.6 and 5.3 times higher among First Nations men and women, respectively, than Canadian men and women[13]. Diabetes prevalence is reported to be 17.2%, 10.3%, and 7.3% among First Nations people living on-reserve, off-reserve, and among Métis, respectively[12]. At least 1 in 10 First Nations adults claim they "never or hardly ever" consume milk or milk products[14]. Worldwide, an estimated 415 million people live with diabetes, with a projected prevalence of 642 million cases by 2040[15].

A 2009 report from the Canadian Diabetes Association (CDA) found that diabetes accounted for approximately 3.5% of public healthcare spending in Canada[16]. The total economic burden of diabetes in Canada was predicted to be $12.2 billion CAD in 2010, rising to $16.9 billion CAD by 2020. Interventions aimed at diabetes prevention are of enormous benefit, not only due to increases in life expectancy and health outcomes, but from an economic standpoint as well. Although there is large variation from country to country, the vast majority of health economics studies attest to the cost-effectiveness of diabetes prevention[17].

1.5 Diabetes Health Consequences

The long-term health complications associated with poorly controlled diabetes are mainly due to hyperglycemia. They include microvascular complications such as retinopathy, nephropathy, and sensory neuropathy, and macrovascular complications such as cardiovascular disease. The CDA reports that ~80% of Canadians with diabetes die from a heart attack, and that 42% of new kidney dialysis patients in 2004 had

diabetes[16]. Those living with diabetes have a 3-fold greater risk of being hospitalized with cardiovascular disease when compared to the national average[12]. Diabetes is the leading cause of blindness in Canada, in addition to causing 7 out of every 10 non-traumatic limb amputations[16]. In 2009-2010 it was reported that 36.5% of Canadian adults with diabetes were living with two or more serious chronic conditions, such as hypertension, heart disease, mood disorder, chronic obstructive pulmonary disease, or arthritis. Moreover, diabetes exacerbates many infectious diseases, including tuberculosis, HIV/AIDS, and malaria[3]. Finally, diabetes also takes a toll on mental wellbeing, with 25% of people with diabetes suffering from depression[16].

It is well-established that blood glucose control is linked with risk of diabetes complications. The United Kingdom Prospective Diabetes Study reported that each 1% decrease in HbA_{1c} corresponded to a 21% decrease in diabetes-related death, 14% decrease in risk of myocardial infarction, and a 37% decrease in microvascular complications[18]. However, insulin is also a major regulator of lipid metabolism[19] and dyslipidemia in T2D is well-established[20] and an independent risk factor for cardiovascular disease[21]. Therefore, understanding the factors that regulate blood glucose and lipid control, as well as developing effective strategies to improve glycemia and lipidemia are important areas of research. Diet is an important component of any treatment or prevention plan, and can be effective with or without the aid of pharmaceutical agents.

1.6 Mechanisms of Glucose Homeostasis

Homeostasis, in biology, is the maintenance of physiological processes, ensuring that all biological levels remain within a set range or stable equilibrium. Alterations in this balance often result in a diseased state, as can be seen in the irregular glucose metabolism that characterizes diabetes mellitus.

Following the ingestion of carbohydrate (CHO), plasma blood glucose will rise. A rise in plasma glucose triggers insulin release from the pancreatic β-cells in the islets of Langerhans. Insulin is circulated by the blood, travelling towards its target tissues, where it binds to the insulin receptor and initiates insulin dependent glucose transport. In the body, insulin upregulates glycogen and lipid synthesis as well as the esterification of fatty acids, and downregulates glycogenolysis, gluconeogenesis, lipolysis, and proteolysis[19]. Glucose is taken up into the cells and either used for energy through glycolysis, or stored as glycogen. These three processes result in a decrease in plasma blood glucose. When glucose drops below normal physiological levels, glucagon, produced in the alpha cells of the pancreatic islets, is released into the bloodstream. At its target tissues, glucagon promotes glycogenolysis, glycolysis, and gluconeogenesis[19]. These three processes result in the conversion of glycogen, glycerol (from triacylglycerol), or amino acids (from protein) into glucose. This glucose is released into the bloodstream, raising plasma glucose levels to their normal physiological levels. In this way, insulin and glucagon work together to maintain blood glucose levels. This relationship is illustrated in Figure 1.2.

Figure 1.2: Mechanisms of Glucose Homeostasis

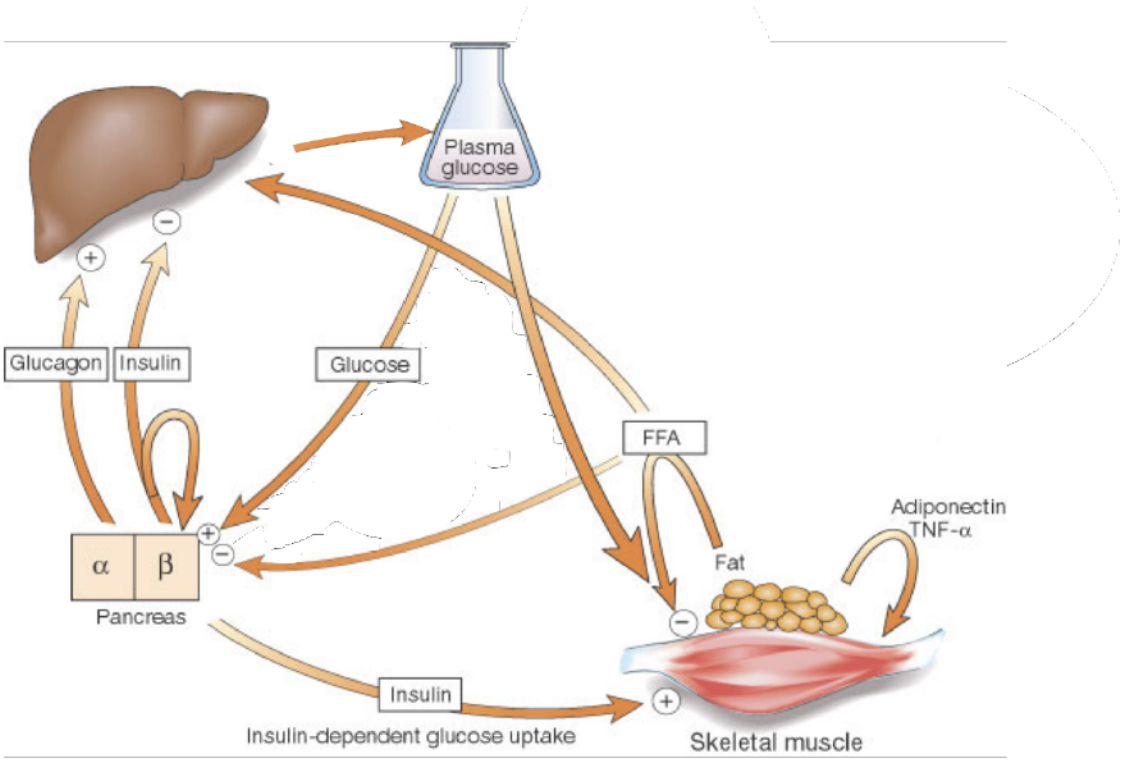


Figure adapted from[19]. When plasma glucose is elevated, insulin is secreted from the pancreatic β-cells. Insulin decreases hepatic glucose release, while increasing glucose uptake, and storage in fat and muscle cells. Fat cells release free fatty acids (FFA), which cause reduced muscle glucose uptake, as well as insulin secretion, and glucose production in the liver. In addition, fat cells secrete the adipokines leptin and TNF, which play a role in the regulation of food intake, energy expenditure, and insulin sensitivity.

1.7 Molecular Mechanisms of Insulin Action

As described above, blood glucose rises following ingestion of food. Elevated blood glucose triggers a release of insulin from the β-cells in the islets of Langerhans. The insulin is taken up in the blood and delivered to insulin receptors in skeletal and cardiac muscle, as well as adipose tissue. At its target tissues, insulin initiates the insulin signal transduction pathway. First, insulin activates the insulin receptor's tyrosine kinase

domain and recruits insulin receptor substrate (IRS-1), which binds to phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)[22]. PI3K causes phosphatidylinositol 4,5-bisphosphate (PIP2), a membrane lipid, to be converted into phosphatidylinositol (3,4,5)-trisphosphate (PIP3)[22]. PIP3 is recognized by protein kinase B (PKB), also known as AKT, as well as phosphoinositide-dependent kinase-1 (PDK1). The result is the phosphorylation and activation of PKB. In its active form, PKB phosphorylates TBC1D4, thereby inhibiting its GTPase-activating domain [22]. This causes proteins in the next cascade to remain active, which triggers the translocation of GLUT4[22]. GLUT4 is the insulin-regulated glucose transporter found mainly in adipose and muscle cells. Under normal conditions GLUT4 remains inside the cells in intracellular vesicles. When activated, GLUT4 allows for the facilitated diffusion of glucose into the muscle or fat cells [23]. Once inside the cell, glucose is quickly phosphorylated by glucokinase in the liver, or hexokinase in all other tissues. The resulting glucose-6-phosphate either enters glycolysis, or is polymerized into glycogen.

Figure 1.3 Molecular Mechanisms of Insulin

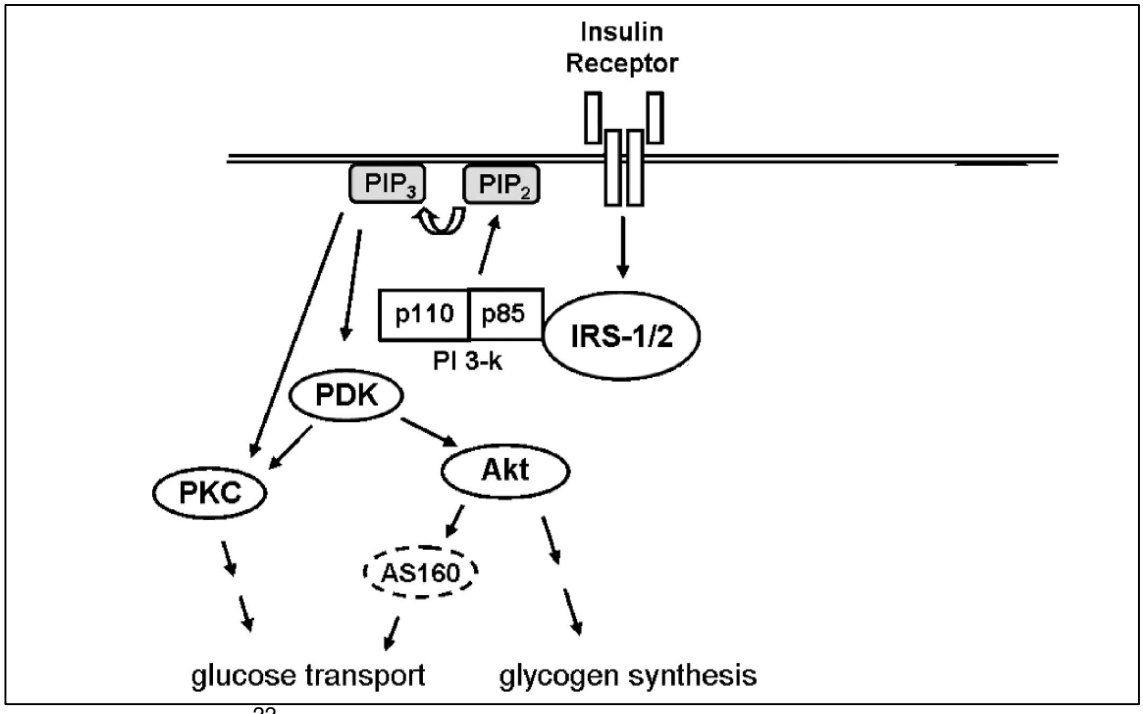


Figure adapted from [22]. Insulin signaling pathway in skeletal muscle.

1.8 Lipid Metabolism Overview

Following the consumption of a meal, fat reaches the small intestine and in the enterocytes is packaged into triglycerides along with cholesterol, phospholipids, and apolipoproteins into chylomicrons for transport to the blood vessels[1]. In the blood, chylomicrons are quickly hydrolyzed into smaller chylomicron remnant particles. These remnant particles are removed from circulation by the liver. In the liver, nutrients are packaged into other lipoprotein particles such as high density lipoproteins (HDL), low density lipoproteins (LDL), and very low density lipoproteins (VLDL)[1]. Chylomicrons, VLDL, and LDL are responsible for the transport of lipids to peripheral tissues. In these peripheral tissues, the lipid constituents of these particles are used for energy, storage,

and cell membrane structural integrity[1]. Simultaneously, HDL particles serve to transport cholesterol from peripheral tissues back to the liver to be excreted[1].

1.9 Cheese Consumption and Cardiovascular Disease

As a food containing large amounts of saturated fat, cheese is often considered to have a negative impact on blood lipid profiles, and overall cardiovascular health. However, recent systematic reviews have concluded that overall, cheese consumption leads to either neutral, or modest positive benefits on overall cardiovascular health and markers of disease[24,25]. Cheese intake has been shown to have no association with CVD[26,27], or coronary artery disease[26,28,29]. Moreover, cheese consumption has been found to be associated with a reduced risk of stroke[26,28–30], and hypertension[31–37]. Next, it has been shown that substituting cheese for meat or carbohydrate meals had no impact on LDL cholesterol, apoB, HDL:LDL ratio, HDL:total cholesterol ratio, or post-prandial TG[38]. One study reported that using cheese in the place of butter resulted in reductions in both LDL and HDL cholesterol, while fasting TG were unaffected[39]. In line with these findings, another study reported that fasting TG were not affected when subjects consumed 40 g of dairy fat per day, from either cheese or butter[40]. These studies suggest that overall cheese consumption may have a slight positive impact on cardiovascular health.

1.10 Hepatic Lipid Accumulation and T2D

Counter-regulatory hormones such as glucagon regulate hepatic glucose release in the fasted state, while insulin promotes the conversion of glucose into lipid in the fed state. Like insulin action in muscle, in the liver insulin activates the insulin receptor kinase, resulting in the phosphorylation of IRS-1 and IRS-2. This leads to the activation of PI3K and AKT-2. AKT-2 promotes glycogen synthesis and inhibits gluconeogenesis[41] (Figure 1.4).

Hyperinsulinemia is a compensatory mechanism that results from increased insulin resistance in peripheral tissues. However, while hyperinsulinemia is necessary for the maintenance of normoglycemia when there is insulin resistance, other pathways may not be as affected by insulin resistance, resulting in dysfunction. In the liver, insulin resistant subjects typically demonstrate selective hepatic insulin resistance alongside impaired glucose homeostasis, yet also demonstrate enhanced insulin-induced hepatic *de novo* lipogenesis[42]. In mice with T2D, insulin fails to suppress gluconeogenesis, while still activating lipogenesis, resulting in a state of hyperglycemia and hypertriglyceridemia[43]. Peripheral tissue insulin resistance promotes ectopic fat deposition[44,45]. Studies conducted in the lean offspring of people with T2D have indicated that intramyocellular lipid accumulation, and insulin resistance in muscle precede the development of hepatic insulin resistance and overt T2D[46]. Moreover, lipid accumulation in the liver has been associated with whole body and tissue-specific insulin resistance[47–52].

The accumulation of lipid in locations other than adipose tissue (ectopic lipid accumulation) has been hypothesized to play a role in the pathogenesis of T2D. Lipid is accumulated when energy consumed exceeds energy expended. The combination of an increasingly sedentary society and the abundance of energy-dense foods has contributed, at least in part, to widespread obesity. Emerging evidence points to the notion that the accumulation of ectopic fat may be caused by overwhelming one's lipid storage capacity in adipocytes[53]. When energy is constantly consumed in excess of expenditure, lipid accumulates, and once adipocytes reach their maximum storage capacity, lipid is accumulated in ectopic sites. This theory is supported by evidence that individuals who lose weight by undergoing liposuction do not see improvements in insulin sensitivity in the muscle, liver, or adipose tissue[54]. Liposuction is a procedure that can remove significant quantities of subcutaneous adipose tissue, however, by removing adipocytes, it decreases the overall storage capacity of adipose tissue. In contrast, modest reductions in weight caused by diet or exercise result in significant improvements in insulin sensitivity[55,56]. For this reason, diabetes can be thought of as a disease of dysfunctional lipid metabolism, in addition to dysfunctional glucose metabolism. This highlights the importance of lifestyle interventions in the management and prevention of T2D.

Figure 1.4 Molecular Mechanisms of Insulin Action in Liver

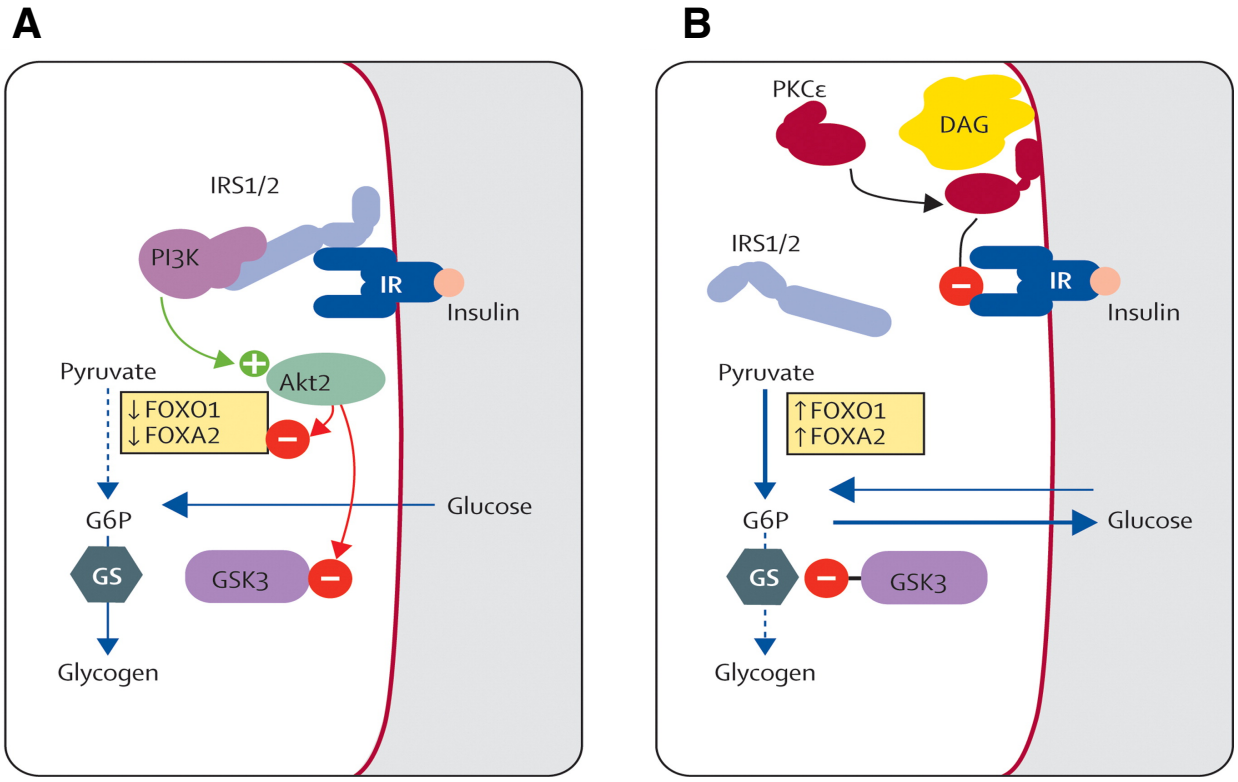


Figure adapted from[41]. Mechanisms of insulin sensitivity in (A) Insulin-sensitive liver and (B) Insulin-resistant liver. In insulin-sensitive liver, insulin binds to the insulin receptor (IR), resulting in the phosphorylation of insulin receptor substrate 1 or 2 (IRS1/2). IRS1/2 activates 1-phosphatidylinositol 3-kinase (PI3K), which activates Akt2. Akt2 inhibits the action of FOXO1 and FOXOA2, as well as GSK3, thereby promoting glycogen synthesis and inhibiting gluconeogenesis. In insulin resistant liver, the presence of diacylglycerol (DAG) and PKCε inhibit the ability of insulin to suppress hepatic gluconeogenesis and promote glycolysis.

1.11 Lifestyle Interventions

Diet and physical activity are essential in promoting health and preventing disease, and many interventions focus on one or both of these components of health. A varied, nutritious diet is essential, both as a means of preventing or reversing the progression of prediabetes to T2D, as well as for the management of T2D. Diabetes Canada and the American Diabetes Association recommend both nutrition therapy as a component of the

overall treatment plan for all types of diabetes[57,58]. It is reported that 60-70% of individuals with prediabetes will eventually progress to T2D[59,60], while nutritional therapy can assist in achieving normoglycemia in T2D, thus reducing the risk of complications.

Lifestyle interventions are often employed as a preventative measure rather than a treatment. Several studies have examined the impact of lifestyle interventions on the progression from prediabetes to T2D. One of the first interventions of its kind, the Da Qing IGT and Diabetes Study recruited individuals with IGT and randomized them to undergo one of three lifestyle interventions: (1) Diet only, (2) Exercise only, or (3) Diet and Exercise. The cumulative incidence of T2D over the 6 years of follow up was 43.8%, 41.1%, and 46.0%, respectively, compared to 67.7% in the control group[61]. Next, the Diabetes Prevention Program (DPP) was a randomized controlled clinical trial that compared the effectiveness of pharmacological intervention and lifestyle intervention. Individuals with IGT were randomized to receive either (1) standard lifestyle recommendations plus 850 mg of metformin 2x daily, (2) standard lifestyle recommendations plus placebo 2x daily, or (3) an intensive lifestyle modification program. It was reported that DM incidence was 58% lower in the lifestyle-intervention group, and 31% lower in the metformin group compared to the placebo group[62]. Further, DM incidence was 39% lower in the lifestyle-intervention group when compared to the metformin group, which suggests that lifestyle modifications can outperform pharmacological interventions in preventing the progression from IGT to T2D. However, current evidence also suggests that lifestyle interventions combined with pharmacological interventions are a viable approach, and can be successful in achieving T2D remission in

those who have been recently diagnosed. A randomized, parallel, open-label pilot trial followed Canadians who were prescribed either (1) an 8-week intensive metabolic intervention, (2) a 16-week intensive metabolic intervention, or (3) standard diabetes care. At 8-weeks, 50% of the metabolic intervention group had achieved normoglycemia, versus 3.6% in the standard care group. At 16 weeks, the standard care group remained at 3.6%, while the intensive metabolic intervention group had 70.4% of participants achieving normoglycemia[63]. Next, a recent meta-analysis examining individuals with T2D reported that overall, lifestyle interventions resulted an improvement in several risk factors associated with CVD in individuals with T2D. Lifestyle interventions led to decreased body mass index (BMI) (-0.29 kg/m^2, p=0.014), HbA_{1c} (-0.37%, p=0.001), systolic blood pressure (-0.16 mmHg, p=0.016), and diastolic blood pressure (-0.27 mmHg, p<0.001) relative to controls, although differences were not observed between groups' HDL and LDL cholesterol. Moreover, several different approaches to lifestyle interventions in those newly diagnosed with T2D are promising; more specifically, several different dietary approaches have been shown to be successful in achieving normoglycemia. In those newly diagnosed, diabetes can be reversed by intensive lifestyle interventions[64–66], low-CHO Mediterranean diets[67], or very low-calorie diets[68]. Although T2D is often thought of as a one-way road, this evidence suggests that interventions employing different dietary patterns or foods can successfully reverse its progression, if implemented soon enough.

1.12 Food Guide Recommendations in Canada

In this thesis, I examined cheese and how diets rich in cheese may affect the prevention or treatment of T2D. The health benefits of dairy products have long been contentious, in part due to their high saturated fat and sodium content. Currently, Eating Well with Canada's Food Guide recommends that individuals "select lower fat milk alternatives" and then elaborates that Canadians should "compare the Nutrition Facts table on yoghurts or cheeses to make wise choices"[69]. The Health Canada website provides guidance on cheese selection, stating that individuals should "Look for reduced fat or lower fat cheeses. Lower fat cheeses generally have less than 20% milk fat (M.F.)", as well as providing tips such as "Try a lower fat cheese such as reduced fat mozzarella, cottage or ricotta cheese in lasagnas and casseroles" and "Use a "light" cheese in place of regular cheese in sandwiches, wraps and quesadillas"[69]. In line with these recommendations, the Alberta Nutrition Guidelines for Adults places cheeses with <20% milk fat (MF) in the "Choose Most Often" category, and all other cheeses in the "Choose Sometimes" category[70]. In this thesis, low fat (LF) cheese will refer to cheese with less than 20% MF, while high fat (HF) cheese will refer to cheese with greater than or equal to 20% MF.

Emerging evidence suggests that saturated fat intake may not be associated with increased risk of cardiovascular disease. This, in turn, has led experts to question current saturated fat recommendations[71–73]. While the research surrounding saturated fat consumption and T2D is less clear, it still calls these saturated fat recommendations into question, and in turn, the wisdom of recommending low fat cheese over high fat cheese.

1.13 Saturated Fat Intake and T2D Risk

The body of evidence surrounding saturated fatty acid intake and its impact on overall health remains inconclusive. Epidemiological evidence from large cohort studies suggests that limiting all saturated fat in the diet may aid in preventing weight gain, metabolic syndrome (MetS), T2D, and cardiovascular diseases (CVD)[8,74,75]. However, large cohort studies, wherein known risk factors and factors including fatty acid intake are controlled using multivariate analyses, report that saturated fat intake is not associated with an elevated risk of T2D[76,77]. Conversely, findings from experimental and observational studies support the notion that saturated fatty acid intake is inversely associated with insulin sensitivity[78–80]. Finally, intake of saturated fatty acids with 4–10 carbons, as well as lauric acid and myristic acid have been found to be associated with decreased T2D risk[76]. These contradictory findings suggest that it is important to consider the specific fatty acids and possibly the food matrix wherein the saturated fats are found. Further research is required to elucidate the relationship of specific fatty acids and food matrices with T2D risk.

1.14 Cheese Intake and T2D Risk: Cohort Studies

Cheese is a dairy product with a high saturated fat content. However, a closer examination of the epidemiological data surrounding cheese consumption and its impact on T2D reveals inconsistencies. Of twelve cohort studies found, three reported that cheese consumption was inversely related to risk of T2D[76,81,82], eight reported no effect[83–90] and one reported a positive association[91]. Study findings are summarized in Table 1.1.

These studies were conducted in European, Asian, American, and Australian populations; however, none of them differentiated between low fat cheese and high fat cheese.

In a large prospective nested case-cohort study within the European Prospective Investigation into Cancer and Nutrition (EPIC), researchers reported that the highest cheese consumers had a 16% decreased risk of T2D relative to lowest consumers of cheese, and that after adjusting for confounders, high consumers still had a 12% reduced risk[81]. Next, in a novel investigation Yakoob et al. investigated circulating biomarkers of dairy fat, pentadecanoic acid (15:0), heptadecanoic acid (17:0), and trans-palmitoleate (t-16:1n-7) and their association with T2D in an American population. Researchers reported that individuals with the highest 15:0, 17:0, and t-16:1n-7 had 44%, 43%, and 52% decreased risk of T2D, respectively, when compared to those with the lowest plasma concentrations[82]. This investigation is of particular interest, as it does not rely upon self-report dietary data, which can be notoriously inaccurate. However, this study examines fatty acids found in dairy, but it is not specifically about cheese. Finally, in a Swedish Cohort, Ericson et al. observed a decreased risk of T2D at higher levels of cheese consumption in women, but not in men [76].

Next, although eight separate studies reported no significant associations between cheese consumption and T2D, six had trends towards an inverse relationship between cheese consumption and T2D[83,84,86–88,90]. Interestingly, some studies indicate that there may be sex-specific differences in outcomes of cheese consumption. In an Australian population, overall highest cheese consumption was associated with a 22% lower risk of T2D relative to lowest consumers, with this trend being more pronounced in men, where

highest consumers had a 31% lower risk of T2D, although none of these findings reached significance[83]. However, these data led researchers to conclude that the positive impact of overall dairy consumption on T2D risk in men was partially driven by the effects of cheese[83]. Contrary to the findings of Grantham et al., Kirii et al. found a trend towards an inverse relationship between cheese consumption and T2D in women, but a positive relationship in men[87], although none of these findings were significant. These findings mirror those of Ericson et al. [76], who reported an inverse risk with T2D in women but not men. Next, Eussen et al.[86] nearly reached significance, reporting that high cheese consumers had a 25% decreased risk of impaired glucose relative to lowest consumers, however a p-value of 0.07 rendered this finding not significant. Interestingly, although highest cheese consumers trended towards a decrease in impaired glucose metabolism, it also trended towards a 15% increase in T2D risk, although a large p-value of 0.60 rendered this finding null[86].

Finally, although Chen et al. reported a 4% decreased risk of T2D at moderate-low levels of cheese consumption, moderate-high, and highest levels of cheese consumption were associated with 1% and 8% increased risk, respectively. Overall a 7% increase in risk was reported for every one serving increase of cheese per day[91]. In summary, the epidemiological evidence regarding cheese consumption and diabetes risk is inconsistent, and inconclusive. While some studies report positive effects of cheese on T2D risk, others report harm. The vast majority of studies are unable to reach significance in their conclusions.

Table 1.1 Cohort Studies Extraction Table

Study	N	Population	Cheese HR	P-Value
Inverse Association				
Slujis et al., 2012	16,154	European	0.88	0.01
			0.56	0.01
			0.57	0.01
Yakoob et al., 2016	3,333	American	0.48	<0.001
Ericson et al., 2015	26,930	Swedish	0.92	0.02
No Association				
Choi et al., 2005	41,254	American Males	0.88	0.69
Eussen et al., 2016	2,391	Dutch	0.75	0.07
			0.69	0.39
			0.83	0.48
Grantham et al., 2013	5,582	Australian	0.78	0.53
			0.88	0.39
Kirii et al., 2009	59,796	Japanese	1.12	0.56
Liu et al., 2006	37,183	American Females	0.8	0.45
Struck et al., 2013	5,953	Danish	0.97	CI 0.82,1.15
O'Connor et al., 2014	4,000	British	1.04	0.76
Diaz-López et al., 2016	3,454	Mediterranean	1.38	0.1
Positive Association				
Chen et al., 2014	194,458	American	1.08	0.04

1.15 Cheese Intake and T2D Risk: Systematic Reviews and Meta Analyses

Overall, the cohort study evidence regarding cheese consumption and T2D is contradictory, and inconclusive. However, when these various study results are synthesized in systematic reviews and meta analyses, a clearer picture emerges. In a systematic review and dose-response meta-analysis, it was reported that there was a 9%

decreased risk of T2D among highest compared to lowest cheese consumers, and that each 50 g per day portion of cheese was associated with an 8% decreased risk of T2D[92]. Another systematic review and dose-response analysis concluded that highest cheese consumers had an 18% decreased risk of T2D relative to their lowest consumer counterparts, and that each 30 g/day serving of cheese was associated with a 20% decrease in T2D risk[113].

The evidence appears to support the concept of cheese's protective effects against T2D development. However, these data are confounded by inconsistencies among meta-analyses, and the different categorization of dairy products between studies. Furthermore, it is virtually impossible to control for every confounder, which emphasizes the need for well-controlled experimental studies. The above reasons prompted one systematic review to conclude that the reduced risk of T2D associated with cheese consumption was supported by only moderate quality evidence[24]. Researchers called for more studies to explore whether LF cheese and HF cheese are similarly associated with T2D risk[24].

1.16 Cheese Intake and T2D Risk: Experimental Studies

Unfortunately, very few experimental studies have been conducted that examine cheese feeding and T2D risk or treatment potential. In a study examining the impact of cheese ripening on glucose and lipid metabolism, Geurts et al. fed 6-week old male diabetes-prone db/db mice diets rich in cheese that had been ripened for 0 days, 15 days, or 35 days for four weeks[95]. Since vitamin K2 is exclusively synthesized by bacteria, and

is only found in fermented dairy products such as cheese[93], one would expect that cheese that had been ripened longer would have increased vitamin K2 content. This is significant, given that vitamin K2 consumption has been linked to lower diabetes incidence[94]. Following 4 weeks of feeding, it was reported that the 35-day ripened cheese diet had significantly improved glucose tolerance without affecting insulin secretion[95]. The same diet also resulted in decreased adipose tissue lipid peroxidase markers. Next, both 15-day and 35-day ripened cheese diets significantly decreased hepatic lipid content, with a more pronounced effect observed in the 35-day ripened cheese diet mice[95]. These data demonstrate that a relatively short 4-week period of cheese feeding can improve measures of glucose homeostasis, and significantly decrease hepatic liver content. Since both glucose and lipid homeostasis are largely controlled by insulin, as explained earlier, this raises the possibility that cheese consumption affects insulin action. However, despite the strength of the evidence in animals, no trials have been conducted examining cheese intake in a human population with T2D or prediabetes.

Recently, in a 12-week randomized parallel-intervention study by Raziani et al.[96], the effects of supplementing diets with low fat or regular fat cheese was examined in a population of adults with BMI 18.5-37.5 kg/m^2, waist circumference >80 cm or >94 cm for women and men, respectively, and one additional risk factor for metabolic syndrome. Participants were allocated to one of three groups: (1) Regular fat cheese (REG), (2) Reduced fat cheese (RED), and (3) a carbohydrate control (CHO). REG diet group were provided with 25% and 35% MF cheeses, while RED groups were given 13% and 16% MF cheeses. Both cheese groups substituted 80 g of cheese each day per 10 MJ (~2,390

kcal) consumed. The CHO group supplemented their diet with 90 g of bread and 25 g of jam per 10 MJ (~2390 kcal) each day. At the conclusion of the trial, there were no observed differences between REG and RED, or REG and CHO diets with respect to fasting blood glucose, insulin, or HOMA-IR[96]. These data suggest that LF (RED) and HF (REG) cheese do not perform differently from one another with regards to measures of fasting glucose homeostasis, when incorporated into the diet for a period of 12 weeks. This finding calls into question the rationale behind recommending LF over HF cheese.

1.17 Cheese and Glucose Homeostasis: Possible Mechanisms

Multiple mechanisms have been proposed to explain the protective effects of cheese on development of T2D. Cheese is rich in both magnesium and calcium, which have been implicated in the improvement of pancreatic β-cell function and insulin sensitivity[97]. The USDA food composition databases record LF cheese (7% M.F.) as containing 42% less calcium and 41% less magnesium than HF cheese[98]. Several recent studies support the direct effects of dietary calcium and magnesium on T2D and insulin resistance[97,99–101]. Furthermore, calcium suppresses hormones that promote adiposity, and can act as an anti-nutrient, inhibiting fat absorption, in the gastrointestinal tract through the formation of soaps[102]. The formation of calcium soaps results in increased fecal fatty acid excretion, and thereby increasing energy excretion[103,104]. Moreover, dairy products in Canada and the USA are fortified with vitamin D, which has been found to have an inverse relationship with T2D[105,106]. As vitamin D is a fat-soluble vitamin, quantities are higher in higher fat cheeses[98]. However, none of these studies have

examined how calcium, magnesium or vitamin D affect T2D or insulin resistance when consumed within a dairy/cheese matrix. A food matrix refers to the nutrient and non-nutrient components of food, and how they relate to one another with respect to their chemical bonds[107].

Next, the fermentation involved in the cheese-making process could confer additional health benefits. Vitamin K2 is exclusively synthesized by bacteria, meaning that it is only present in fermented dairy products, including cheese[93]. Vitamin K2 has been linked to reduced risk of T2D[94]. However, one prospective study found that while cheese and fermented dairy product consumption had beneficial effects on glucose regulation, it did not result in reduced incidence of T2D[90]. Like vitamin D, vitamin K2 is fat-soluble and found in greater quantities in high fat cheese than low fat[98].

Finally, several cheese fat components have been shown to independently have beneficial impacts on glucose homeostasis. Cheese is an excellent source of trans-vaccenic acid (VA)[108] and cis-9, trans-11 conjugated linoleic acid (CLA)[109], both of which have been shown to have numerous metabolic effects, including activation of transcription factors that regulate fat metabolism[110]. Studies in this lab have shown that 8 weeks of VA feeding results in improved insulin secretion and beta-cell growth in a rat model of T2D[111]. As a fatty component of cheese, VA is found in greater concentrations in HF cheese than LF cheese[98]. Next, dairy is one of the few dietary sources of the short chain fatty acid butyric acid. Butyric acid is thought to play a role in glucose metabolism by altering gut barrier integrity[112]. In this respect, it could reduce translocation of pathogenic bacteria across the epithelial cell layer. Following the addition of sodium, butyric acid becomes

sodium butyrate. In one study of mice, adding 5% w/w butyrate to a high fat diet prevented obesity and diet-induced insulin resistance, while in obese mice, butyrate supplementation led to improvements in insulin sensitivity and reduced adiposity[113]. Trans-palmitoleic acid (cis-16:1n-7), a fatty acid present in cheese, has been associated with a lower incidence of T2D. Mozaffarian et al. reported that those with the highest levels of circulating trans-palmitoleate had a 16.7% decreased presence of insulin resistance, and a 62% reduced rate of T2D incidence relative to those with the lowest levels[114]. In muscle cells, palmitate, a saturated fatty acid, has been shown to impair insulin signaling and insulin-stimulated glucose transport, however, palmitoleate enhances glucose uptake, oxidation, and glycogen synthesis[115]. High fat cheese has greater quantities of palmitoleate than low fat cheese[98]. Phytanic acid, which is found in dairy products, is another fatty acid of interest. Phytanic acid is produced from phytol, a branched chain fatty alcohol. This process is regulated by PPARα, with the liver acting as the main site of phytol metabolism in the body[116]. In normal BALB/c mice, adding either 0.2% or 0.5% phytol to a normal chow diet for 4 weeks resulted in a substantial decrease in hepatic TAG[117], which would be expected to improve hepatic insulin sensitivity. This is further supported by the observation that phytanic acid regulates glucose metabolism in hepatocytes *in vitro*[118].

At present time, there are very few data surrounding the metabolic effects of cheese, especially with regard to insulin-regulated glucose homeostasis. The vast majority of experimental studies focus on cardiovascular disease and its relevant endpoints, while some studies include measures of fasting glucose and insulin without a

complete characterization of diabetes-related endpoints. This gap is even more evident when examining the food matrix as opposed to individual cheese constituents. Many studies examine different cheese components and their impact on mechanisms underlying glucose homeostasis, whereas experimental studies looking at whole cheese are rather rare. Of interest is the finding that many different cheese components associated with improvements on the T2D phenotype are reportedly higher in HF cheese. This includes vitamins D and K2, magnesium and calcium, VA, and palmitoleate[98]. The studies undertaken in this thesis will provide important information that can be used to guide further analyses of samples collected in human studies in future trials.

Chapter 2: Research Plan

2.1 Rationale

Nutrition is recognized as a modifiable determinant of T2D risk, and proper nutrition following diabetes diagnosis can improve β-cell function and glucose regulation. Moreover, nutritional intervention soon after diagnosis can slow, or even reverse the progression of T2D[64-68]. Current Canadian nutrition guidelines recommend consumers choose low fat cheese over high fat cheeses, however, the science behind those recommendations is unclear. The vast majority of epidemiological evidence points towards a potential benefit of increased cheese consumption on T2D [76, 81-82], or no impact [83,84,86–88,90], while experimental studies show that cheese feeding can improve measures of glucose homeostasis[95,96], and that low fat cheese does not perform differently from high fat cheese[96]. Moreover, several cheese fat components, including VA, butyric acid, phytanic acid, and palmitoleic acid have been shown independently to have beneficial effects on glucose homeostasis[112–115,117,118], therefore it is of particular interest whether low fat cheese and high fat cheese perform differently from one another.

Two different rodent groups, a preventative cohort and a treatment cohort, will be followed *in vivo*. Since roughly 70% of individuals with prediabetes eventually progress to T2D[59], and the prevention of T2D with diet and other lifestyle factors has proven to be so effective, we have chosen to conduct a more in-depth analysis on the mechanisms underlying any observations that we make in our prediabetic cohort. We will conduct *in*

vitro experiments to further examine the serum metabolome and lipidome, as well as the liver lipidome and transcriptome.

2.2 Objectives

Overall this project has three main objectives:

Objective 1: To determine the impacts of cheese feeding on *in vivo* responses to glucose and insulin in prediabetic and type 2 diabetic rats;

Objective 2: Explore the mechanisms by which cheese affects metabolism in prediabetic rats using an untargeted metabolomic analysis (metabolomic fingerprinting) of blood serum to identify differences in metabolomic outcomes elicited by diets containing cheese;

Objective 3: Use the results of the *in vivo* studies and metabolomics assays to direct additional investigations of the effects of cheese feeding in prediabetic rats.

2.3 Research Questions

Our research questions are as follows:

1. What is the effect of cheese on the metabolic phenotype, focusing on glucose tolerance and insulin sensitivity, of prediabetic and type 2 diabetic rats *in vivo*?
    - Is there a difference between high and low fat cheese?
    - Does cheese have similar effects on the metabolic phenotype in prediabetic and type 2 diabetic rats?

2. What is the effect of cheese on the serum metabolome of prediabetic rats? Based on the outcomes of Questions 1 and 2, the third question was developed as follows:
3. What is the effect of cheese on liver lipid metabolism?
    - Is there a difference between high and low fat cheese in prediabetic rats?

2.4 Hypotheses

1. A cheese diet will improve glucose tolerance and insulin sensitivity of prediabetic and type 2 diabetic rats.
2. A cheese diet will reduce the amount of fat and change the profile of lipids accumulated in liver in response to high fat diet in the prediabetic rats.
3. In both cases, high fat cheese will elicit more pronounced outcomes than low fat cheese.

Chapter 3: Methods

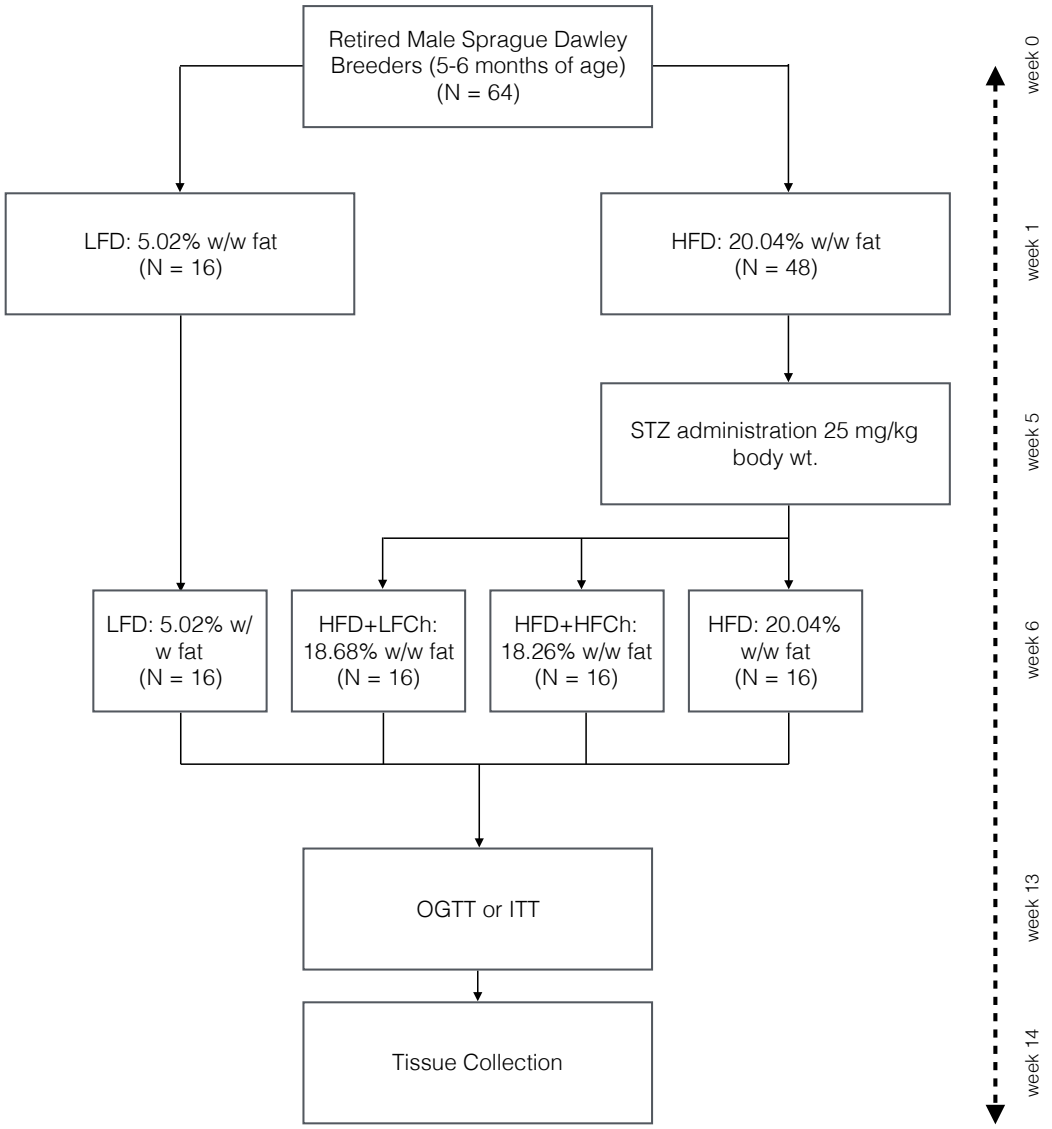
3.1 *In Vivo* Protocols

3.1.1 Animal Treatment and Protocols

All procedures involving animals were approved by the Animal Care and Use Committee at the University of Alberta (AUP #232) and were in accordance with the guidelines set forth by the Canadian Council on Animal Care. In the T2D cohort, retired male Sprague Dawley breeder rats aged 5-6 months were purchased from either the University of Alberta, or Charles River Canada (St. Constant, QC, Canada) and shipped to the University of Alberta. Animals were acclimatized to the facility for one week, and housed as they arrived, with either one or two animals per cage. For the T2D cohort, following acclimatization, one quarter of rats were randomly assigned to receive low fat control diet (LFD), with the remainder put on high fat control diet (HFD). Diet composition is described in Section 3.1.3. Rats were given *ad libitum* food and water for 4 weeks. At the beginning of week 5, a one-time low dose of STZ was administered to all rats consuming HFD (see Section 3.1.4). Animals were returned to their cages and allowed to recover for one week. At the beginning of week 6, HFD rats were randomized into one of three high fat diet groups: the HFD control, a high fat, high-fat cheese diet (HFD+HFCh), or a high fat, low-fat cheese diet (HFD+LFCh). Although these three high fat diets are identical in macronutrient distributions (Figure 3.3), they contained different percentages of fat from cheese, with highest quantities found in the HFD+HFCh, followed by HFD+LFCh, and finally no fat from cheese in the HFD. LFD group rats simply

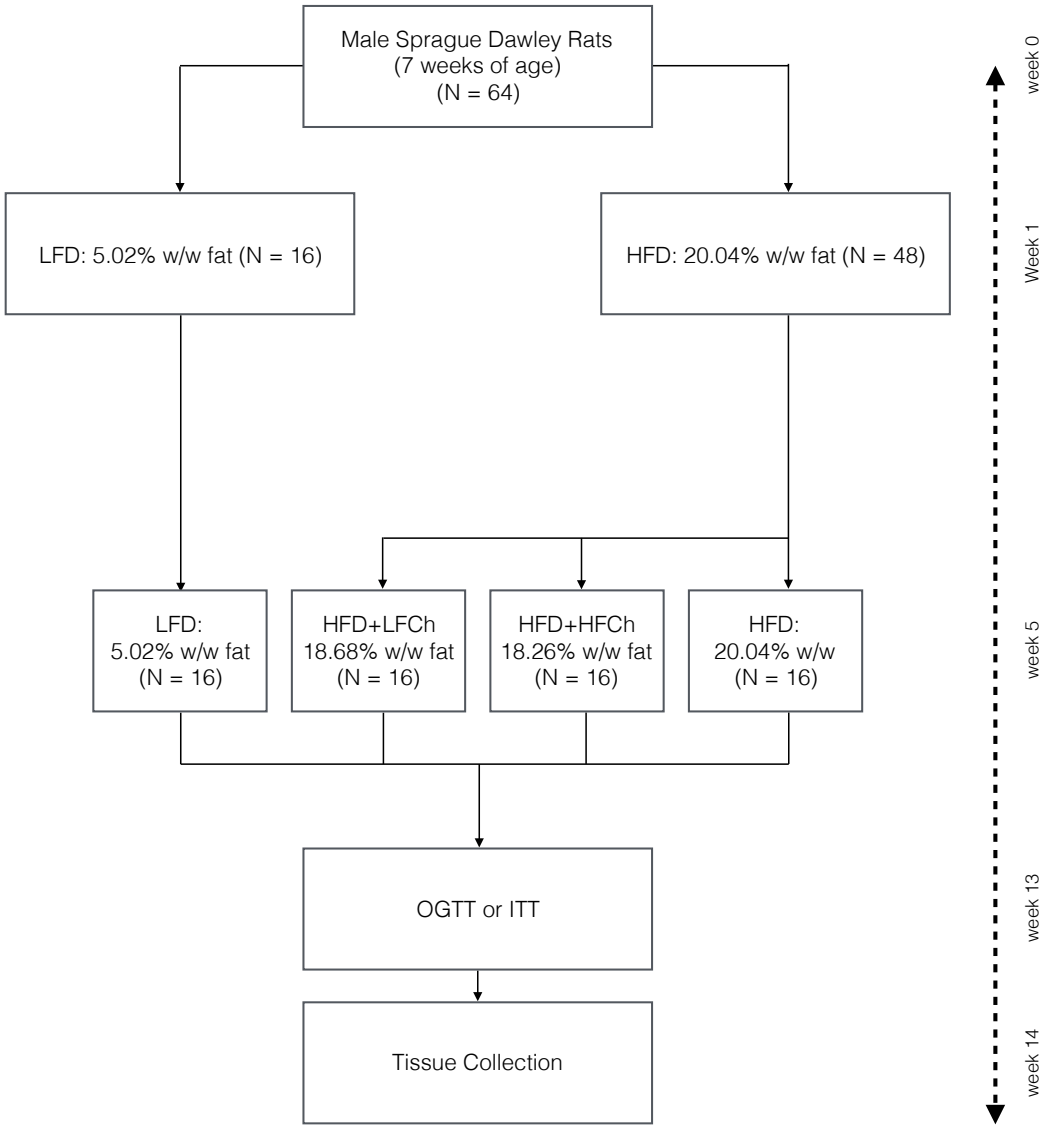
continued on the LFD and never received STZ. All rats were fed their respective diets with *ad libitum* access to food and water for 7 weeks, at which point they were randomized to undergo either an oral glucose tolerance test (OGTT) or insulin tolerance test (ITT). The following week all animals were euthanized, and tissues were collected.

Figure 3.1: Schematic Diagram Outlining Study Design (T2D Cohort)



In the prediabetic cohort, 64 7-week old Sprague Dawley rats arrived at the facility and were allowed to acclimatize for one week. As with the diabetic cohort, 16 of rats were randomized to LFD, and 48 were randomized to begin on HFD. Following 4 weeks of feeding, HFD rats were randomized to either continue on the HFD, or begin either HFD+HFCh, or HFD+LFCh diets (N = 16 per group). Rats were fed their respective diets for 8 weeks, at which point they were randomized to undergo either an OGTT or ITT. The following week rats were euthanized and tissues were collected.

Figure 3.2: Schematic Diagram Outlining Study Design (Prediabetic Cohort)



3.1.2 Animal Models

Prediabetes was modelled using 8-week-old Sprague Dawley rats fed HFD. The Sprague Dawley rat is a well-characterized model of prediabetes that spontaneously develops insulin resistance within 6 weeks of initiating HFD feeding[119]. These animals exhibit glucose intolerance, but not outright T2D.

T2D was modelled using retired breeder rats fed HFD and administered a single low-dose of streptozotocin (HFD/STZ). STZ is a toxin commonly used to induce diabetes because it is toxic to pancreatic β-cells in mammals. STZ enters β-cells via glucose transporter 2, and causes the alkylation of DNA[120]. This DNA damage induces the activation of poly ADP-ribosylation, a process which leads to the depletion of cellular NAD+ and ATP. Moreover, STZ results in increased ATP dephosphorylation, which fuels xanithine oxidase and the formation of superoxide radicals[120]. As a result, hydrogen peroxide and hydroxyl radicals are produced. Finally, STZ results in nitric oxide release, thus inhibiting aconitase activity, and promoting DNA damage[120]. Altogether, this induces β-cell necrosis. The STZ model can be used to mimic both T1D and T2D, as both have reported pancreatic β-cell loss. In human T1D, 60-80% of functional β-cell mass is lost at the time of diagnosis[121], whereas only 24% of functional β-cell mass is lost in newly diagnosed T2D patients, or 54% in those who have had T2D for 15 years[122]. Despite T1D and late-stage T2D demonstrating similar degrees of β-cell loss, the HFD feeding prior to STZ more closely mimics the pathogenesis seen in T2D. In T2D, aging and lifestyle often contributes towards disease development and progression, similar to using retired breeders (middle-aged rats) along with HFD feeding in HFD/STZ rats. Moreover, in this protocol STZ dose was titrated such that a non-ketotic hyperglycemic state was achieved. This suggests the retention of a reasonable number of β-cells. Furthermore, it has been demonstrated that dietary interventions can attenuate β-cell loss in this model[123]. However, the events that lead to β-cell failure in T2D involve lipotoxicity, insulin resistance, hyperinsulinemia, stress, and low-level inflammation[124]. As it lacks these

characteristics, the HFD/STZ model of T2D is not a perfect simulation of T2D disease progression. The combination of HFD/STZ has been studied at different concentrations of STZ. Both 35 mg/kg doses, and 2x 35 mg/kg have proven to be successful[125,126], albeit in younger rats. Nevertheless, this HFD/STZ rat model has been successfully used to reasonably represent T2D in experiments previously carried out in this lab[111].

3.1.3 Diet

The two studies involved 128 rats, randomized into one of four diet groups:

1. Low Fat Control (LFD) – 5.02% w/w fat from lard, flaxseed oil, and corn oil
2. High Fat Control (HFD) – 20.04% w/w from lard, flaxseed oil, and corn oil
3. High Fat High Cheese (HFD+HFCh) - (9.12% w/w fat from cheese) + lard, flaxseed oil and corn oil (9.14% w/w). Thus, half of the fat in the HFD was replaced with fat from cheese.
4. High Fat Low Cheese (HFD+LFCh) - (7.00% w/w fat from cheese) + lard, flaxseed oil and corn oil (11.68% w/w).

HFD, HFD+HFCh, and HFD+LFCh diets were all matched for macronutrient content in a ratio of 25:42:33 for protein, fat, and carbohydrate (Figure 3.3). These diets were isocaloric for kcal per gram of diet. The LFD, which contains significantly less fat than all other diets, was adjusted for carbohydrate quantity by adding equal amounts of starch and glucose. The LFD diet had a macronutrient distribution of 25:12:63 for protein, fat, and carbohydrate and was reduced in calories compared with HF diets (3.7 kcal/g versus 4.1 kcal/g). Corn and flaxseed oil were used to ensure that all diets were matched for polyunsaturated:saturated fat ratio and provided sufficient essential fatty acids. Diets

without cheese were adjusted for protein content with casein and methionine to ensure that all diets were isonitrogenous (Table 3.1). A detailed breakdown of different fatty acids can be found in Table 3.3.

Diets were prepared using grated cheese to ensure that texture was similar in all diets. Diets were prepared monthly, and stored at 4°C until they were put into animals' cages. Fresh diet was dispensed into cages on Mondays, Wednesdays, and Fridays of each week.

Figure 3.3 Diet Macronutrient Composition

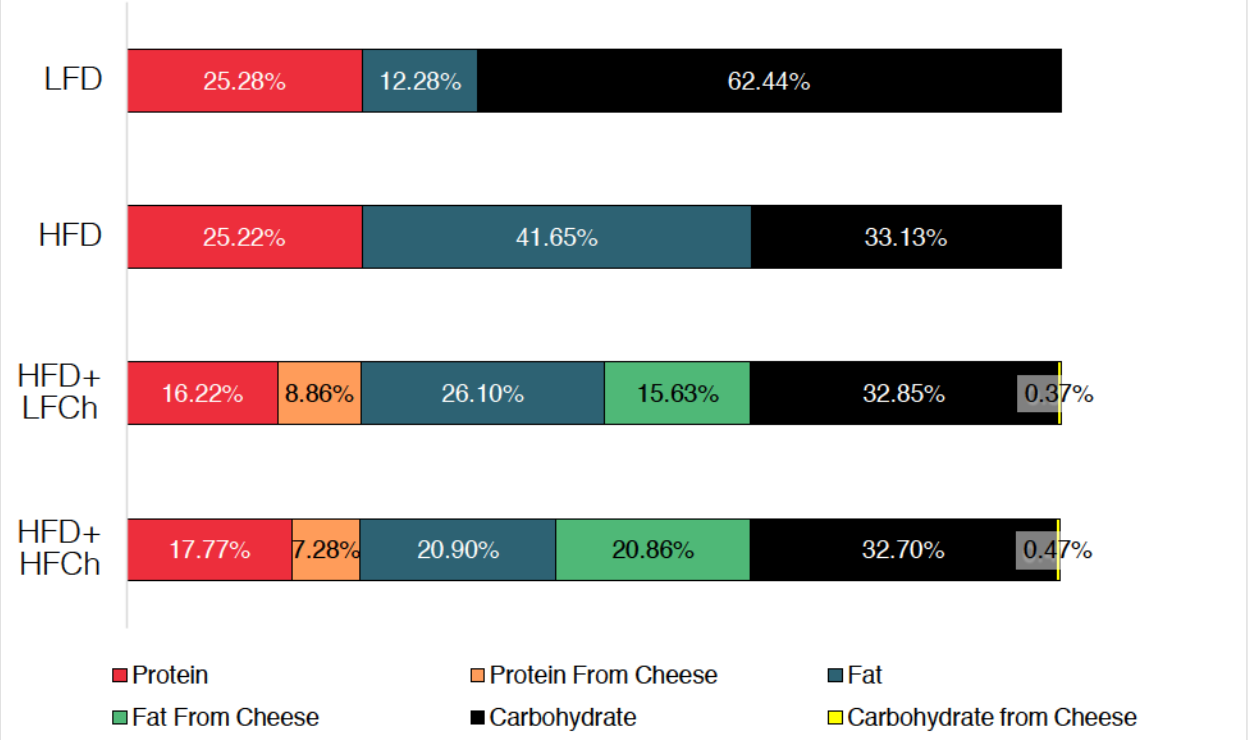


Table 3.1 Diet Composition (g in ~ 1 kg)

Ingredient	LFD	HFD	HFD+LFCh	HFD+HFCh
Flaxseed Oil †	2.0 g	2.0 g	2.0 g	2.0 g
Mazola Corn Oil	3.0 g	8.0 g	23.0 g	33.0 g
Lard	45.0 g	190.0 g	100.0 g	65.0 g
Casein §	229.5 g	270.0 g	175.2 g	190.32 g
L-Methionine ¶	2.1 g	2.5 g	0.0 g	0.0 g
Sucrose §	255.0 g	300.0 g	295.0 g	295.0 g
Corn Starch ‡	317.0 g	58.0 g	58.0 g	58.0 g
Cellulose §	84.8 g	100.0 g	100.0 g	100.0 g
Mineral Mix Bernhart & Tomarelli §	43.2 g	51.0 g	51.0 g	51.0 g
Vitamin Mix AIN-93-VX §	8.5 g	10.0 g	10.0 g	10.0 g
Inositol ¶	5.3 g	6.3 g	6.3 g	6.3 g
Choline Bitartrate ¶	2.4 g	2.8 g	2.8 g	2.8 g
Low Fat Cheese (19% MF)	0.0 g	0.0 g	394.0 g	0.0 g
Regular Fat Cheese (31% MF)	0.0 g	0.0 g	0.0 g	322.0 g
Total Weight (dry)	995.4 g	997.8 g	1070.0 g	1094.0 g

Table 3.2 Diet Nutrition Information (g/kg)

Nutrient	LFD	HFD	HFD+LFCh	HFD+HFCh
Protein (total)	231.6 g	272.5 g	270.0 g	270.0 g
Protein from Cheese	0.0 g	0.0 g	94.8 g	79.7 g
Fat (total)	50.0 g	200.0 g	199.9 g	199.8 g
Fat from Cheese	0.0 g	0.0 g	74.9 g	99.8 g
CHO (total)	572.0 g	358.0 g	358.0 g	357.1 g
CHO from Cheese	0.0 g	0.0 g	5.0 g	4.1 g
Sugar	255.0 g	300.0 g	295.0 g	295.0 g

Note: all values are adjusted to reflect weight in 1000 g of diet.

Table 3.3 Fatty Acid Breakdown

Fatty acid (g/100 g of total fatty acid)	LFD	HFD	HFD+LFCH	HFD+HFCH
C10:0	0	0	0.7	0.9
C12:0	0	0	0.5	0.8
C14:0	1.4	1.4	2.6	5.6
C16:0	27.1	27.7	28	28.9
C16:1 n-9	1.7	1.8	2.2	2.2
C18:0	15.4	16	13.3	11.8
C18:1 t11	0	0	0	0.7
C18:1 n-9	33.4	34.6	30.3	27.3
C18:2 n-6 (Linoleic Acid)	14.5	13.8	12.3	12.2
C18:3 n-3 (Linolenic Acid)	2.7	1.1	1.2	1.4

C18:3 n-6	0	0	0	0.6
Total SFA	43.8	45.2	50.1	50.7
Total MUFA	38	38.9	33.9	31.1
Total n-6 PUFA	14.5	13.8	12.3	12.8
Total n-3 PUFA	2.7	1.1	1.2	1.4
Ratio n-6:n-3	5.3	12.5	10.1	9.2
Total PUFA	17.2	14.9	13.5	13.8
Ratio PUFA:SFA	0.4	0.3	0.3	0.3

Table 3.4 Macronutrients as a Percentage of Total Kilocalories

Ingredient	LFD	HFD	HFD+LFCh	HFD+HFCh
Kcal/1000 g	3681.2	4331.5	4028.6	3936.6
% Total Protein	25.3%	25.2%	25.1%	25.1%
% Protein from Cheese	0.0%	0.0%	8.8%	7.4%
% Total Fat	12.3%	41.7%	41.7%	41.8%
% Fat from Cheese	0.0%	0.0%	15.6%	20.9%
% Total Carbohydrate	62.4%	33.1%	33.2%	33.2%
% Carbohydrate from Cheese	0.0%	0.0%	0.5%	0.4%
% Sugar	27.8%	27.8%	27.4%	27.4%

3.1.4 Streptozotocin

Prior to weighing STZ, a 5% potassium hydroxide (KOH) solution was prepared to be used for cleaning. Next, 25 mg of STZ (Sigma-Aldrich, Oakville, ON, Canada) was weighed into individual vacutainers under a fume hood, and stored at -20°C until the time at which they were to be used. Acetate buffer was prepared, adjusted to a pH of 4.5, and sterilized.

On the morning of STZ administration, all rats were weighed. Immediately before STZ administration, rats were anesthetized with isoflurane. Using a syringe and an 18 G needle, 1 mL of acetate buffer was added to each 25 mg STZ vacutainer. The vacutainers were agitated to dissolve the powdered STZ. Rats were given a dose of 25 mg of STZ per kg of body weight in a volume equivalent to 1mL/kg. The STZ solution was administered intraperitoneally via 27 G needle. Before disposal in biohazardous waste, 5% KOH solution was injected into used vacutainers to neutralize any unused STZ solution. All surfaces were wiped down with 5% KOH, and then water. Throughout the study, the order of STZ administration was alternated by diet group, to control for the time that STZ spent on ice before administration because time at room temperature can reduce the potency of STZ.

3.1.5 OGTT

A 70% glucose solution (w/v) in saline was prepared. OGTT were performed following 7-8 weeks of experimental diet feeding, after an overnight fast. Animals were fasted overnight, ~16 hours, to ensure that glycogen stores were depleted, and insulin would not be in circulation. As a result, any spike in blood glucose and insulin could be

solely attributed to the oral glucose bolus. Rats were gavaged with 2 g of glucose per kg of body weight. Blood samples were taken from a clipped tail vein at 0 (fasted), 10, 20, 30, 60, 90, and 120 minutes after gavaging. Blood glucose was analyzed using a glucometer (Contour Next, Bayer, Leverkusen, Germany). Blood samples were collected using heparinized capillary tubes (Natelson Blood Collecting Tubes, Fisher Scientific, Pittsburgh, PA, USA) into 1.5 mL microcentrifuge tubes and placed on ice. Later, samples were centrifuged for 20 minutes at 1500 RPM and blood serum was extracted and frozen at -80°C until use.

To measure OGTT insulin concentrations, rat insulin ELISA kits with a dynamic range of 0.15-5.5 ng/mL and sensitivity of 0.124 ng/mL were used (Alpco, Salem, NH, United States). Conjugate stock and wash buffer were prepared using the provided reagents. A total of 10 µL of standards, controls, and serum samples were pipetted into their respective wells. Next, 75 µL of working strength conjugate was added to each well, and the plate was covered and incubated for two hours, shaking at 700 RPM. Following incubation, the microplate was decanted and washed 6 times with washing buffer. TMB substrate (100 µL) from the kit was pipetted into each well, and the plate was incubated for 15 minutes. Finally, 100 µL of Stop solution was added to each well, and the plate was read at 450 nm. Concentrations were interpolated in GraphPad Prism (GraphPad Software Inc., CA, United States) using standards to calibrate the readings.

3.1.6 ITT

Insulin tolerance tests were conducted following four hours of fasting. This fasting period was shorter than in OGTT to ensure that while insulin would have been cleared from circulation, glycogen stores would not have been depleted by prolonged fasting. Human insulin (Sigma Aldrich, St. Louis, MO, United States) was diluted to a concentration of 68 mg/mL using saline solution. Rats received an IP injection equivalent to 1 mL of dilute insulin per kg of body weight (68 mg/kg body weight). Blood glucose was tested at time points 0 (fasted), 10, 20, 30, 60, 90, and 120 minutes following insulin injection. Blood glucose was analyzed using a glucometer (Contour Next, Bayer, Leverkusen, Germany).

3.1.7 Tissue Collection

Animals were fasted overnight prior to euthanasia. Animals were anesthetized with an IP injection of pentobarbital at 60 mg/kg of body weight. Death was induced via diaphragm puncture. Liver, soleus, colon, ileum, jejunum, and epididymal fat were collected and flash frozen in liquid nitrogen. Blood was collected and centrifuged 20 minutes at 3,000 RPM and 4°C. Serum was extracted and frozen. Samples were stored at -80°C until analysis.

3.2 *In Vitro* Protocols

3.2.1 Metabolomic Analysis

A total of 32 serum samples from the prediabetic cohort (N = 8 per diet group) were sent for metabolomic analysis at The Metabolomics Innovation Centre at the University

of Alberta. Samples were analyzed using both a direct ionization-mass spectrometry-based approach (DI-MS), and nuclear magnetic resonance spectroscopy (NMR). Results were analyzed using the MetaboAnalyst software[127].

Metabolomic analysis is an emerging tool that is incredibly useful for studying phenotype and diet-induced changes in phenotype. Two common approaches to metabolomic analysis are metabolomic profiling, and metabolomic fingerprinting[128]. This thesis focuses on metabolomic fingerprinting. This approach, also called an untargeted or unbiased approach, focuses not on specific metabolites, but compares the patterns of the metabolites that are altered in response to external stimulus.

3.2.2 Liver and Serum Lipid Concentrations

Liver and serum samples were prepared for total lipid analysis by gas-liquid chromatography (GC). First, liver samples had to be homogenized, and protein concentrations determined. Approximately 0.05 g of liver samples were weighed out and added to labelled 1.5 mL screw cap microtubes (DiaTEC, Kitchener, ON, Canada) along with ~250 µL of 1mm glass beads (BioSpec Products, United States) and 300 µL of lysis buffer. Samples were homogenized for 2 x 20 seconds at 6000 RPM. Next, samples were allowed to sit on ice for 30 minutes, then all liquid was aspirated into new microcentrifuge tubes. Tubes were centrifuged at 12,000 RPM for 15 minutes at 4°C. The protein concentration of each sample was determined using a colourimetric assay (in duplicate) and the bicinchoninic acid assay method. A microplate layout was created, and 40 µL of standards, or 0.5 µL of liver + 39.5 µL of ddH_2O was added, in duplicate, to each well. Bovine serum albumin was used as a protein standard. With a multichannel pipette, 200

µL of Lowry Reagent (Sigma, St. Louis, MO, United states) was added to each well, and incubated at room temperature for 10 minutes. Folin & Ciocalteu Phenol Reagent 2.0N (Thermo Scientific, Waltham, MA, United states) was diluted 1 in 2 with ddH_2O, and 20 µL of the dilute mixture were added to each well, and incubated for 30 minutes. Absorbance was measured at 750 nm using a spectrophotometer. Concentrations were interpolated in GraphPad Prism (GraphPad Software Inc., CA, United States) using bovine serum albumin standards to calibrate the readings.

Using the obtained protein concentrations, 2 mg of liver protein, or 100 µL of serum was used to prepare samples for GC. In 15-mL detergent-free glass tubes, samples were diluted to a total volume of 1 mL using ddH_2O. Phospholipase-C solution (Sigma, St. Louis, MO, United states) was created, and 2 mL was added to tubes, followed by the addition of 2 mL anhydrous diethyl ether (Thermo Scientific, Waltham, MA, United states). Samples were vortexed for 2 hours at 33°C, then 1 mL of tridecanoin internal standard (TD), 4 mL of chloroform, and 2 mL of methanol were added to each tube. Tubes were vortexed and centrifuged for 10 minutes at 2,500 RPM. The lower phase was removed and passed through a Pasteur pipette containing anhydrous $NaSO_4$. The effluent was evaporated under a nitrogen dryer, and 100 µL of Sylon BFT (Supelco Inc, Bellefonte, PA, USA) was added. Samples were incubated at room temperature for 1 hour, then evaporated under nitrogen. Samples were re-dissolved in 100 µL of hexane, and 50 µL were transferred to Agilent-compatible 2 mL vials with glass spring inserts.

Samples were analyzed for total lipids in the Lipid Core, Faculty of Medicine and Dentistry, University of Alberta. Samples were analyzed using a Zebron ZB-5 column

(Phenomenex Inc., Torrance, CA, USA) using an HP Agilent 6890 gas chromatograph with a flame-ionization detector (Agilent, Mississauga, Ontario). Lipid peaks were assessed using area under the curve (AUC) relative to the TD.

3.2.4 Histology

Frozen liver samples (N=2 per diet group) were sectioned, and ~1mL of oil red O (ORO) working solution was pipetted over each section. Sections were incubated for 5 minutes at room temperature, then rinsed under running water for 30 minutes. Slides were mounted using a water-soluble mounting medium, and coverslips were placed on top. Following a 10-minute incubation period at room temperature, 30 randomly selected pictures of each slide were taken using a Zeiss AxioCam HRm microscope attached to a Canon Powershot G10. Photos were taken at 20x objective lens magnification. ORO staining was quantified using ImageJ software (National Institutes of Health, Bethesda, MD, USA). Microsoft Excel (Microsoft Corporation, Redmond, WA, USA) was used to calculate percentage values. Data were analyzed in Graphpad Prism by way of one-way ANOVA, followed by post hoc analyses where $p<0.05$ was considered significant.

3.3 Statistical Analysis

Results were analyzed using GraphPad Prism (GraphPad Software Inc., CA, United States). The D'Agostino Pearson normality computes skewness and kurtosis to quantify how well the distribution matches a Gaussian distribution, with regards to asymmetry and shape. Moreover, it calculates how far each value differs from the expected Gaussian distribution, and uses these data to compute a p-value[129]. For

normally distributed data, one-way or two-way ANOVA was run, using repeated measures where appropriate. A p-value of ≤0.05 was considered significant. Data sets were tested for normality by pooling all values and running the D'Agostino Pearson normality test. The Kruskal-Wallis Test was run on data sets with one factor that was not normally distributed, while data sets with more than one factor that were not normally distributed were log transformed, and a two-way ANOVA was run. If, following log transformation, a data set failed the D'Agostino Pearson normality test, a linear regression was performed.

Chapter 4: Effect of Cheese on Metabolic Phenotype in Insulin Resistant and Type 2 Diabetic States

4.1 Diet Group Summary

In this chapter, we sought to address the question "What is the effect of cheese on the metabolic phenotype, focusing on glucose tolerance and insulin sensitivity, of prediabetic and type 2 diabetic rats *in vivo*?". A summary of *in vivo* data is given in Table 4.1. At baseline and the conclusion of the trial body weights were similar among groups in both prediabetic and T2D cohorts. There was no significant effect of diet on mean food intake, fasting blood glucose, insulin, HOMA-IR, or Glucose AUC x Insulin AUC ($_{G}AUCx_{I}AUC$). In the T2D cohort there was no difference between groups with regards to epididymal fat as a % of total body weight, however LFD had the lowest amount of epididymal fat compared with all other groups in the prediabetic cohort (p=0.1196).

Throughout the trials, a total of 4 rats were euthanized prior to their expected termination date. In the prediabetic cohort, N=1 rat in the HFD+LF-Ch cohort died suddenly before gavaging in an OGTT, during week 13 of diet feeding. In the T2D cohort, three animals were euthanized early: N=2 rats in the HFD+HFCh diet group, and N=1 animal in the HFD+LFCh group. The criteria for early euthanasia were: persistent elevated blood glucose (above 20.0 mmol/L), and a loss of >10% of body weight after STZ administration. In the HFD+HFCh group, tissue was collected from the 2 rats at the end of week 9 and week 11, respectively while the HFD+LFCh rat was terminated at the beginning of week 7 of the trial. All data from these animals were included in analyses to

ensure that those animals that showed the least benefit from cheese diets were represented in the data presented in this thesis.

Table 4.1 – Metabolic Profile of Rats in Prediabetic and Type 2 Diabetic Cohorts

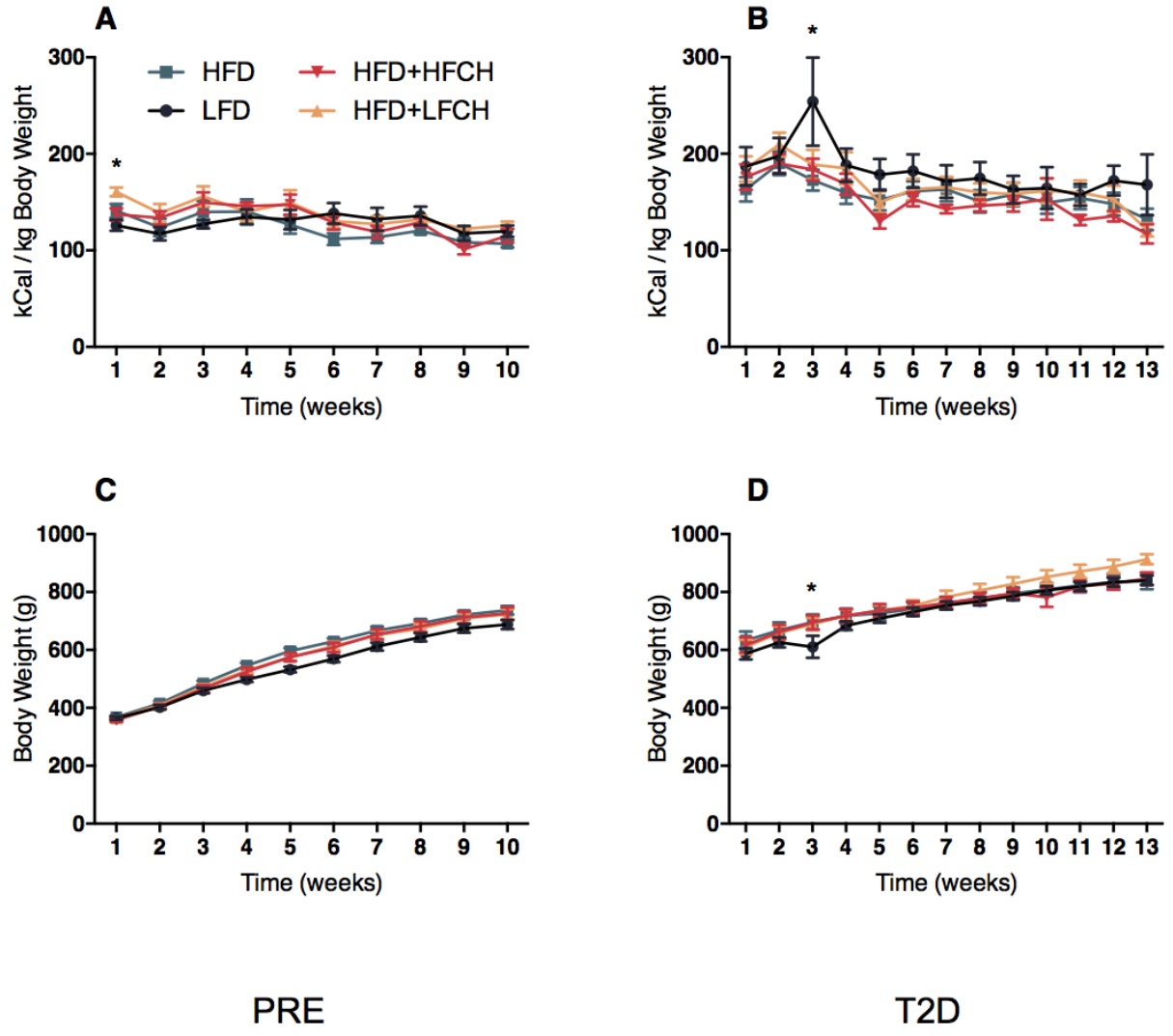
Diet Group	LFD	HFD	HFD+LFCh	HFD+HFCh
PREDIABETIC COHORT (Mean ± SEM)				
BW, Baseline (g)	365 ± 5.6	366 ± 5.8	363 ± 5.1	358 ± 7.1
BW, Final (g)	688 ± 15.2	737 ± 15.1	723 ± 18.1	726 ± 21.4
Change (% Baseline)	88.5	101.4	99.2	102.8
Food Intake (kcal/day)	128 ± 8	123 ± 7	138 ± 7	131 ± 7
Fasting BG (mmol/L)	6.50 ± 0.19	6.63 ± 0.13	6.89 ± 0.28	7.25 ± 0.30
Fasting Insulin (ng/mL)	1.34 ± 0.30	0.86 ± 0.19	1.09 ± 0.36	0.89 ± 0.26
HOMA-IR (mM x μU/mL)	1.92 ± 0.45	1.21 ± 0.28	1.70 ± 0.51	1.43 ± 0.44
$_{G}AUCx_{I}AUC$	280150 ± 75329	289543 ± 67790	390355 ± 151850	301969 ± 94540
Epididymal Fat as a % of Total BW	2.50 ± 0.15	3.21 ± 0.16*	3.39 ± 0.16**	3.46 ± 0.22**
TYPE 2 DIABETIC COHORT (Mean ± SEM)				
BW, Baseline (g)	587 ± 19.7	633 ± 30.7	610 ± 25.1	617± 26.1
BW, Final (g)	841 ± 16.5	838 ± 27.7	913 ± 17.0	846 ± 21.8
Change (% Baseline)	43.27	32.34	49.62	37.11
Food Intake (kcal/day)	181 ± 20	161 ± 13	162 ± 12	148 ± 9
Fasting BG (mmol/L)	5.61 ± 0.23	6.34 ± 0.51	5.77 ± 0.26	6.22 ± 0.60
Fasting Insulin (ng/mL)	1.14 ± 0.17	0.89 ± 0.18	1.54 ± 0.24	0.96 ± 0.30
HOMA-IR (mM x μU/mL)	1.39 ± 0.22	1.20 ± 0.25	1.92 ± 0.32	1.25 ± 0.41
Glucose AUC x Insulin AUC	325633 ± 75359	261550 ± 68744	377169 ± 69453	374870 ± 106837
Epididymal Fat as a % of Total BW	2.68% ± 0.11	3.14% ± 0.14	3.18% ± 0.19	2.88% ± 0.16

* $p<0.05$, ** $p<0.01$ compared with LFD

4.2 Food Intake and Body Weight

Energy intake was normalized for body weight (kcal/kg of body weight). In both prediabetic and T2D cohorts, there was a significant effect of diet on food intake. For the prediabetic cohort, diet [$F(3,600)=1.078$, $p=0.0003$] and time [$F(9,600)=7.304$, $p<0.0001$] were significant with, no significant interaction of diet x time [$F(27,600)=1.078$, $p=0.3605$]. In the T2D cohort, diet [$F(3,761)=9.662$, $p<0.0001$] and, time [$F(12,761)=6.165$, $p<0.0001$] were significant but not the interaction [$F(36,761)=0.5687$, $p=0.9811$] (Figure 4.1.A, and 4.1.B). There was no effect of diet group on body weight in the prediabetic cohort [$F(3,60)=2.283$, $p=0.0881$], although time was significant [$F(9,540)=1532$, $p<0.0001$], as was the interaction between the two [$F(27,540)=2.283$, $p=0.0003$] (Figure 4.1.C). There was an effect of diet [$F(3,761)=7.048$, $p=0.0.001$] and time [$F(12,761)=47.52$, $p<0.0001$], on body weight in the T2D, while the interaction between the two was not significant [$F(36,761)=0.7252$, $p=0.8838$](Figure 4.1.D). A pattern appears in week 3 for the LFD group in the T2D cohort (Figure 4.1.B, and 4.1.D). Energy intake drastically increased relative to other groups ($p<0.01$, $p<0.05$, $p<0.01$ for LFD relative to HFD, HFD+LFCh, HFD+HFCh, respectively), while body weight decreases ($p<0.01$ for LFD relative to HFD, HFD+LFCh, and HFD+HFCh). This could not be explained by any one outlying value nor by any experimental manipulations. While there is a significant effect of diet on food intake in the prediabetic cohort, post hoc analysis revealed that differences only exist between LFD and HFD+LFCh at week 1 ($p<0.05$).

Figure 4.1: Effects of Diet Feeding on Caloric intake and Body Weight

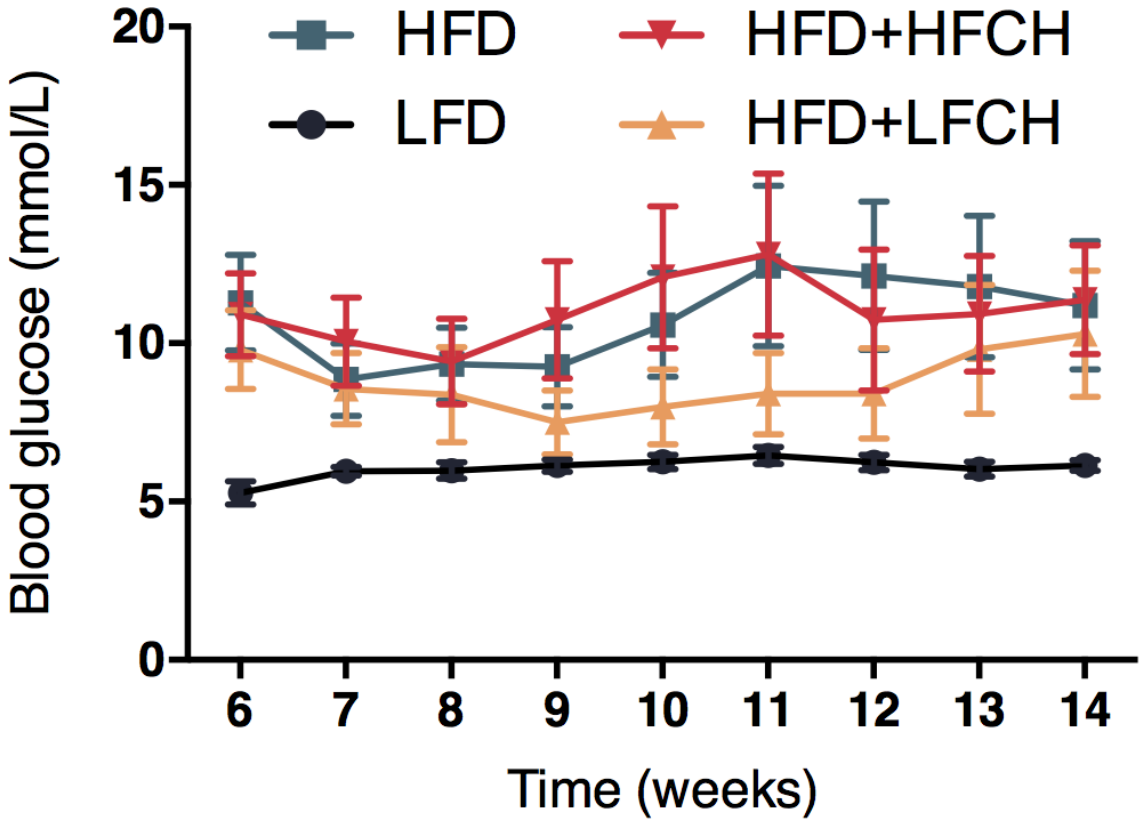


Caloric intake of rats fed HFD, HFD+LFCh, HFD+HFCh, or LFD diets at specified time points in Prediabetic (A) and T2D (B) cohorts; bodyweight at indicated time points in Prediabetic (C) and T2D (D) cohorts. Data is presented as mean ± SEM. Data collected around the body weight of the T2D cohort (D) failed normality testing, and was log transformed [Y = log(Y)]. Two-way ANOVA followed by Bonferroni's test was performed on all data (A, B, C), and log transformed data that failed normality testing (D). *n* per group (identical in both cohorts): HFD=16, HFD+LFCh=16, HFD+HFCh=16, LFD=16.

4.3 Ambient Blood Glucose

Ambient blood glucose data were gathered only for the T2D cohort following STZ administration (Figure 4.2) as a means of monitoring progression to diabetes as well as blood glucose concentrations that exceeded the criteria for a humane endpoint. The data set was not normally distributed, and so a log transformation [Y=log(Y)] was performed. However, log-transformed data failed the D'Agostino Pearson Normality Test, and so a linear regression was performed. Overall ambient blood glucose was significantly different in rats receiving STZ when compared to the LFD group that did not undergo STZ administration. Blood glucose levels in HFD, HFD+LFCh, and HFD+HFCh diet groups were significantly higher ($p<0.001$, $p=0.012$, and $p=0.001$, respectively) when compared to LFD. No other significant differences were observed between diet groups. A linear mixed methods regression was conducted, and it was revealed that HFD was significantly different from LFD at weeks 7-12 and 14, HFD+HFCh was significantly different from LFD at weeks 7-10, and HFD+LFCh was significantly different from LFD at weeks 7-12. At week 6, blood glucose in all groups that underwent STZ administration were elevated relative to the LFD control, however the HFD+LFCh group appears to return closer to normoglycemia than either the HFD or HFD+HFCh groups.

Figure 4.2: Ambient Blood Glucose Levels in T2D Cohort



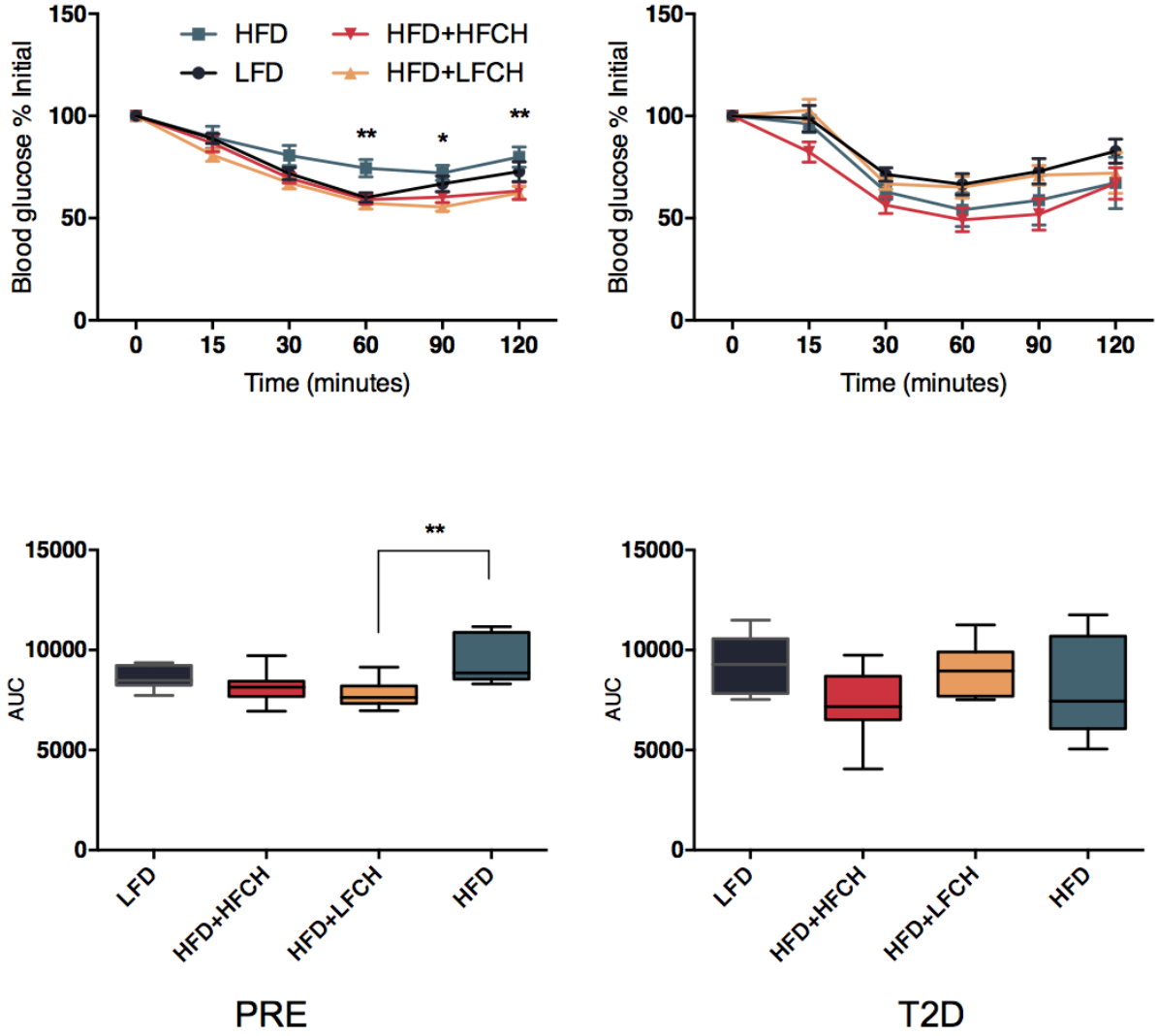
Effects of 8 weeks of HFD, HF-LCh, or HF-HCh diet feeding on ambient blood glucose (mmol/L) in T2D rats following STZ administration (25 mg/kg body weight). Data are presented as mean ± SEM. Following log transformation [y=log(y)], data failed normality testing. Linear regression was performed on log-transformed data. Overall, HFD, HFD+LFCh, and HFD+HFCh were significantly different from the LFD group ($p<0.001$, $p=0.012$, and $p=0.001$, respectively). *n* per group: HFD=16, HFD+LFCh=16, HFD+HFCh=16, LFD=16.

4.4 Insulin Tolerance Tests

An ITT was performed on half of both cohorts to determine treatment effects on insulin sensitivity. ITTs are shown as responses over 120 minutes (Figure 4.3.A and 4.3.B) for prediabetic and T2D cohorts, respectively, and area under the curve (Figure 4.3.C and 4.3.D) for prediabetic and T2D cohorts. In the prediabetic cohort, there was a significant effect of diet on both the time-dependent changes in blood glucose, expressed as % initial values (Figure 4.3.A), and AUC (Figure 4.3.A). Diet group as a source of variation had $F(3,27)=5.363$, with $p=0.0050$ while time had $F(5,135)=117$ with $p<0.0001$, and the interaction had $F(15,135)=2.443$ with $p=0.0035$. Further analysis revealed that beginning at T=60, blood glucose in HFD was higher than LFD ($p<0.01$), HFD+LFCh ($p<0.001$), and HFD+HFCh ($p<0.01$) diet groups. At T=90, HFD remained higher than HFD+LFCh ($p<0.001$), and HFD+HFCh ($p<0.05$) diet groups. HFD+LFCh was also higher than LFD group ($p<0.05$). At T=120, HFD was higher than HFD+LFCh ($p<0.01$), and HFD+HFCh ($p<0.01$) diet groups. When examining the AUC data for the prediabetic cohort, the HFD was significantly increased when compared to the HFD+LFCh diet group ($p<0.01$).

No differences were observed in the ITT between diet groups in the T2D cohort [$F(3,28)=2.022$, $p=0.1336$ for blood glucose % initial, and $F(2.172,15.20)=1.843$, $p=0.1904$ for AUC] (Figure 4.3.B and 4.3.D).

Figure 4.3: ITT Blood Glucose Levels in Prediabetic and T2D Cohorts



Effects of 7-8 weeks of cheese diet feeding on insulin tolerance in HFD, HFD+LFCh, HFD+HFCh, and LFD groups. Insulin tolerance tests were performed on rats following a 4-hour fast. Blood glucose values are shown as mean % of basal glucose ± SEM for prediabetic (A) and type 2 diabetic (B) cohorts, * $p<0.05$, ** $p<0.01$ compared with LFD. Data are shown as mean, min and max of area under the curve (AUC) for prediabetic (C) and T2D (D) cohorts. There was a significant effect of diet on AUC for glucose in the prediabetic cohort ($p=0.0058$). Post-hoc analysis revealed that the HF-LCh had improved relative to the HFD. All significant interactions are summarized in text. Data for both prediabetic and T2D cohorts for ITT blood glucose % initial (A, B) were log transformed to ensure Gaussian distribution before two-way ANOVA followed by Bonferroni's test was performed. The Kruskal-Wallis Multiple comparisons test was performed on AUC data in the prediabetic cohort (C), as it failed normality testing, and one-way ANOVA followed by Bonferroni's test was performed on the T2D cohort. *n* per group (identical in both cohorts): HFD=8, HFD+LFCh=8, HFD+HFCh=8, LFD=8.

4.5 Oral Glucose Tolerance Tests

OGTT glucose values are shown as responses over 120 minutes (Figure 4.4.A and 4.4.B) for prediabetic and T2D cohorts, respectively, and incremental area under the curve (Figure 4.4.C and 4.4.D) for prediabetic and T2D cohorts. In the prediabetic cohort, there were no statistically significant differences in blood glucose concentrations (Figure 4.4.A) or iAUC [$F(3,27)=0.6789$, $p=0.5726$ for blood glucose, and $p=0.3988$ for iAUC](Figure 4.4.C). However, in the T2D cohort, differences were observed. Overall, HFD and HFD+HFHCh were significantly higher than LFD ($p=0.010$, and $p=0.031$, respectively), with post-hoc analyses revealing significantly elevated blood glucose values relative to LFD at T=20 ($p=0.009$), T=30 ($p<0.001$), T=60 ($p<0.001$), T=90 ($p=0.002$), T=120 ($p=0.042$) in the HFD group, and T=20 ($p=0.047$), T=30 ($p<0.001$), T=60 ($p<0.001$), T=90 ($p=0.001$), T=120 ($p=0.012$) in the HFD+HFCh group (Figure 4.4.B). The effect of diet was significant for iAUC blood glucose values ($p=0.0022$), with the LFD being significantly decreased relative to both HFD ($p<0.01$) and HFD+HFCh ($p<0.01$) groups, but not the HFD+LFCH group (Figure 4.4.D).

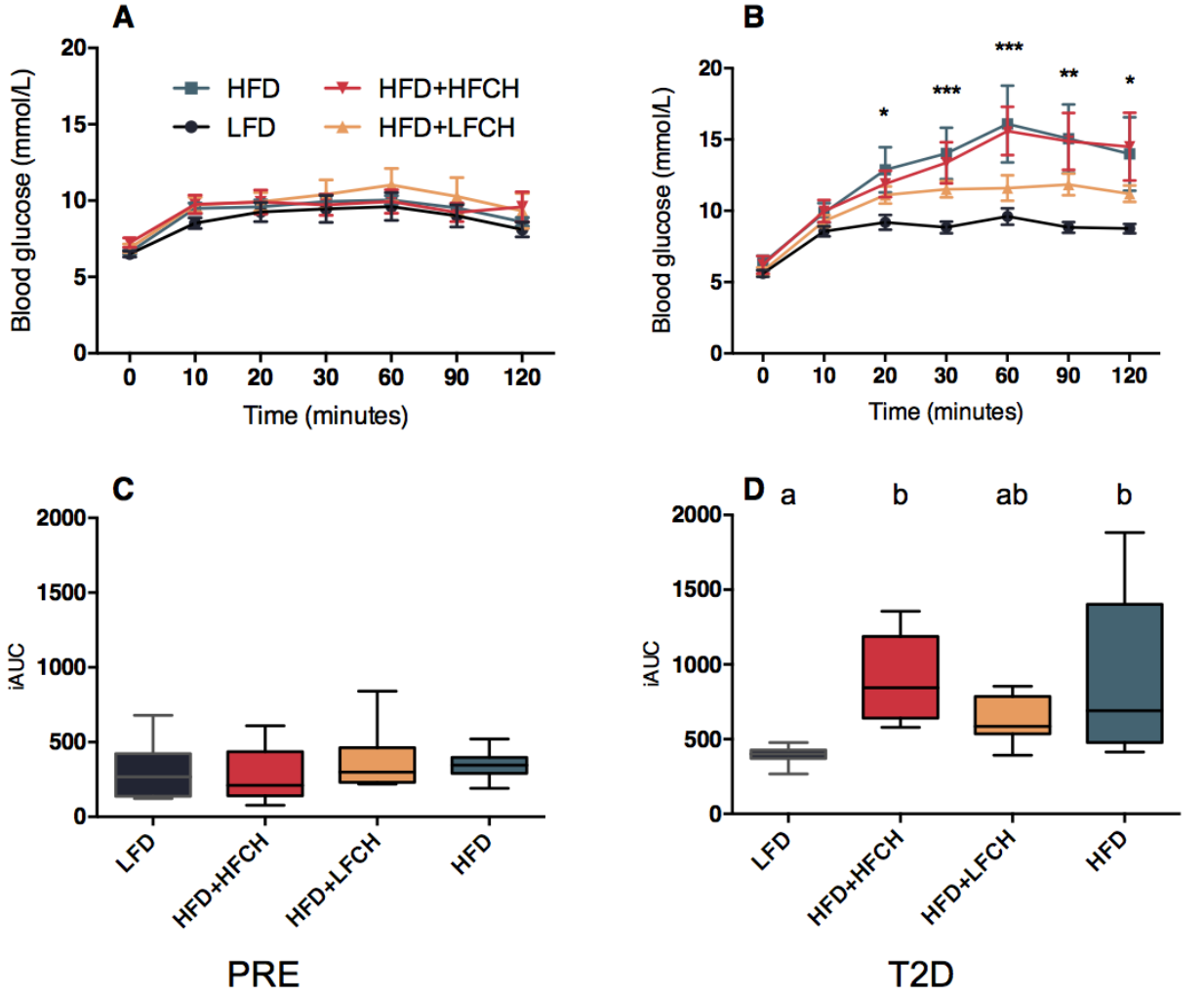
OGTT insulin values are shown as responses over 120 minutes (Figure 4.5.A and 4.5.B) for prediabetic and T2D cohorts, respectively, and area under the curve (Figure 4.5.C and 4.5.D) for prediabetic and T2D cohorts. In the prediabetic group, there was no effect of diet group on insulin concentration ($p=0.4526$) (Figure 4.5.A), or area under the curve ($p=0.7131$) (Figure 4.5.C). However, in the T2D cohort there was a significant effect of diet and time on insulin concentration [$F(3,172)=6.534$, $p=0.0003$, and $F(6,172)=3.075$, $p=0.0069$, respectively], with post-hoc analyses revealing that at T=10, both HFD and

HFD+HFCh were significantly lower than the LFD ($p<0.01$ for both interactions)(Figure 4.5.B). Despite this, the differences between AUC means were not statistically significant ($p=0.1613$).

4.6 Fasting Blood Glucose and Insulin

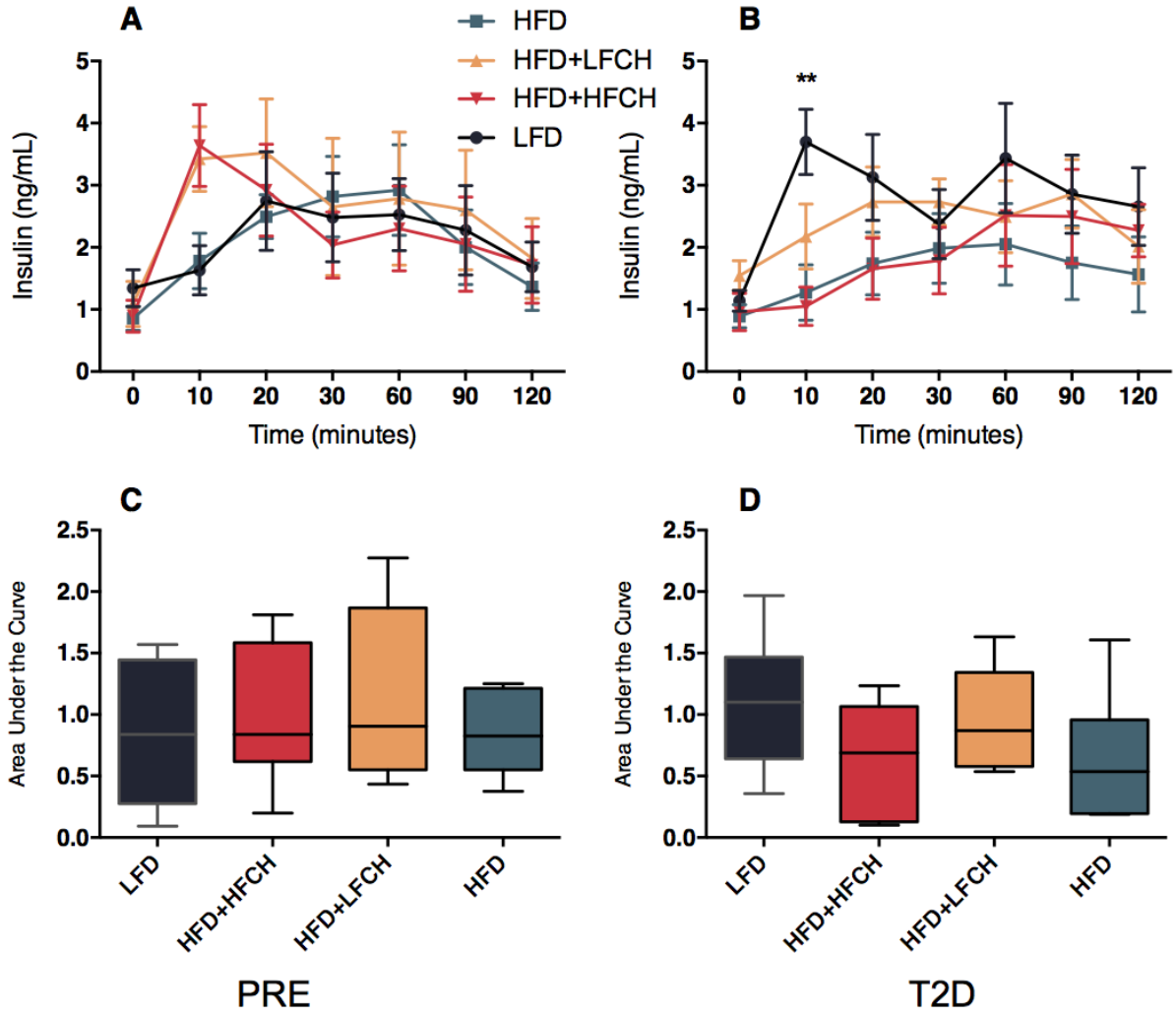
Fasting blood glucose and fasting insulin were obtained in rats following 16 hours of overnight fasting prior to OGTT (Figure 4.6). In both prediabetic and T2D cohorts, diet group did not have a statistically significant effect on fasting glucose (Figures 4.6.A and 4.6.B, respectively), or fasting insulin (Figures 4.6.C and 4.6.D), respectively.

Figure 4.4: OGTT Blood Glucose Levels and iAUC in Prediabetic and T2D Cohorts



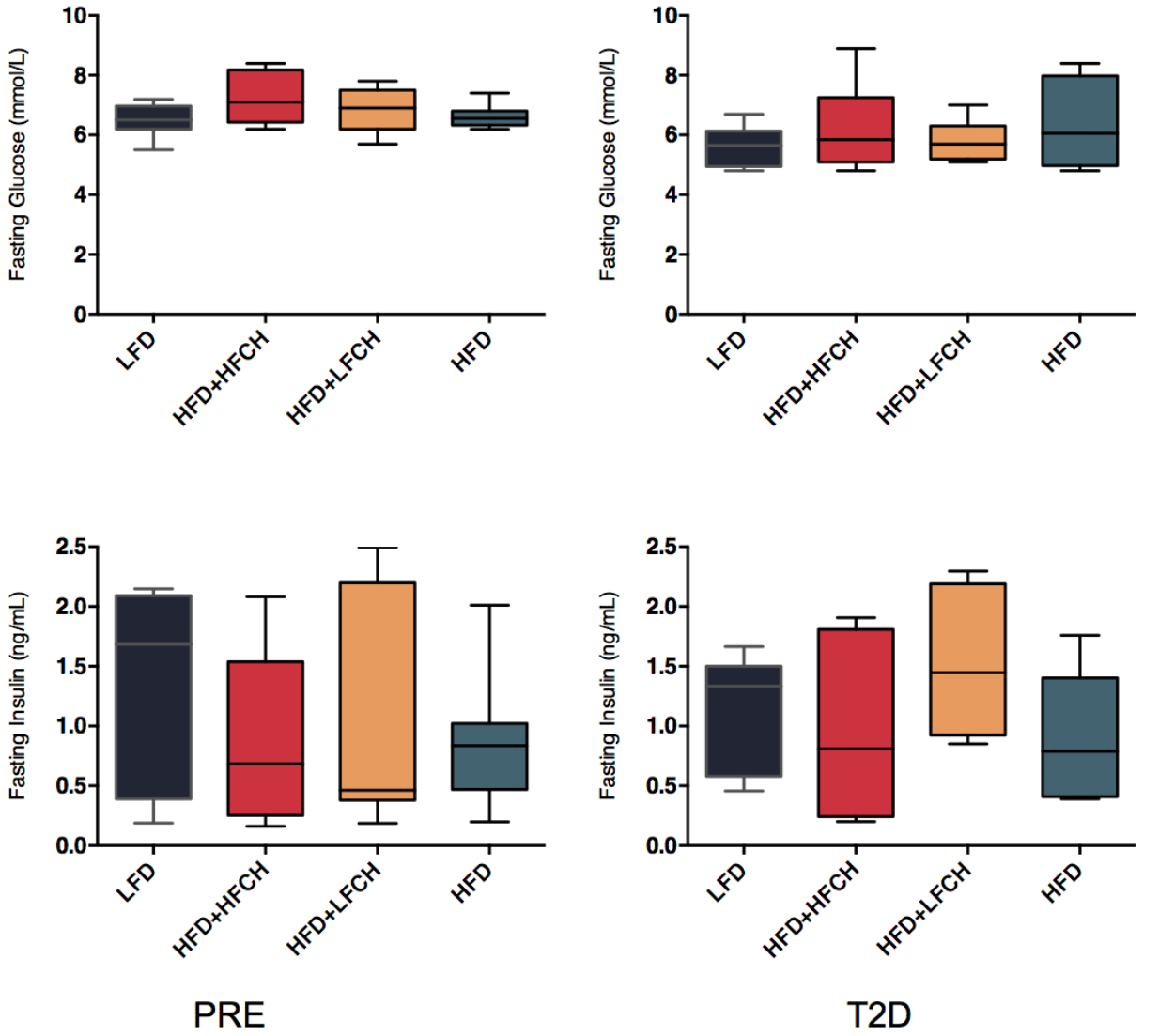
Effects of 7-8 weeks of cheese diet feeding on oral glucose tolerance in HFD, HFD+LFCh, HFD+HFCh, and LFD groups. Data are presented as mean ± SEM (A and B), or mean, min, and max (C and D). In D, significant differences among diet groups are denoted using subscript. Oral glucose tolerance tests were performed on rats following a 16-hour overnight fast. Blood glucose values are shown for prediabetic (A) and T2D (B) cohorts, and area under the curve (AUC) for prediabetic (C) and T2D (B) cohorts. There was a significant effect of diet on OGTT glucose in the T2D cohort ($p<0.001$), with post-hoc analysis revealing improvements in the glucose tolerance of HFD+LFCh rats, when compared HFD rats. From T=30 until the end of the OGTT, both HFD and HFD+HFCh rats were significantly different from the LFD, while the HFD+LFCh was not. When iAUC was calculated, both HFD and HFD+HFCh groups were significantly different from the LFD ($p<0.001$). No other statistically significant differences were observed. No data were normally distributed, and so the Kruskal-Wallis Test was performed on iAUC values (C, D), and blood glucose values were log transformed, and a two-way ANOVA was performed (A, B). Data from the T2D cohort's blood glucose values remained skewed even after log transformation, so linear regression was performed on log-transformed data (B). *n* per group (identical in both cohorts): HFD=8, HFD+LFCh=8, HFD+HFCh=8, LFD=8. *$p<0.05$, **$p<0.01$, ***$p<0.001$, compared to LFD group.

Figure 4.5 OGTT Serum Insulin and AUC in Prediabetic and T2D Cohorts



Effects of 7-8 weeks of cheese diet feeding on serum insulin during an oral glucose tolerance test in HFD, HFD+LFCh, HFD+HFCh, and LFD groups. Oral glucose tolerance tests were performed on rats following an 8-hour fast. Insulin concentration is shown for prediabetic (A) and T2D (B) cohorts, and area under the curve (AUC) for prediabetic (C) and T2D (D) cohorts. Data are shown as mean ± SEM (A and B), or mean, min, and max (C and D). In the T2D rats, there was a significant effect of diet during the OGTT ($p=0.0003$), with the LFD diet group being significantly different (**) from both HFD ($p<0.005$) and HFD+HFCh ($p<0.005$) at T=10 (B). However, this effect was not observed in the AUC data (D). No other statistically significant differences were observed. *n* per group (identical in both cohorts): HFD=8, HFD+LFCh=8, HFD+HFCh=8, LFD=8.

Figure 4.6: Fasting Blood Glucose and Insulin in Prediabetic and T2D Cohorts



Effects of 7-8 weeks of cheese diet feeding on fasting blood glucose and insulin in HFD, HFD+LFCh, HFD+HFCh, and LFD groups. Blood glucose and insulin were measured following an overnight 16-hour fast. Mean fasting blood glucose shown, by diet group, for prediabetic cohort (A) and T2D cohort (B). Mean fasting insulin, by diet group, shown for prediabetic (C) and T2D (D) cohorts. The data are mean, min, and max. There was no significant difference observed between diet groups for any of these measures. Fasting glucose data in the T2D cohort (A), and fasting insulin data in the prediabetic cohort (D) failed normality testing, and the nonparametric Kruskal-Wallis test was run, rather than the one-way ANOVA and Bonferroni test performed on normally distributed data (B, C). *n* per group (identical in both cohorts): HFD=8, HFD+LFCh=8, HFD+HFCh=8, LFD=8.

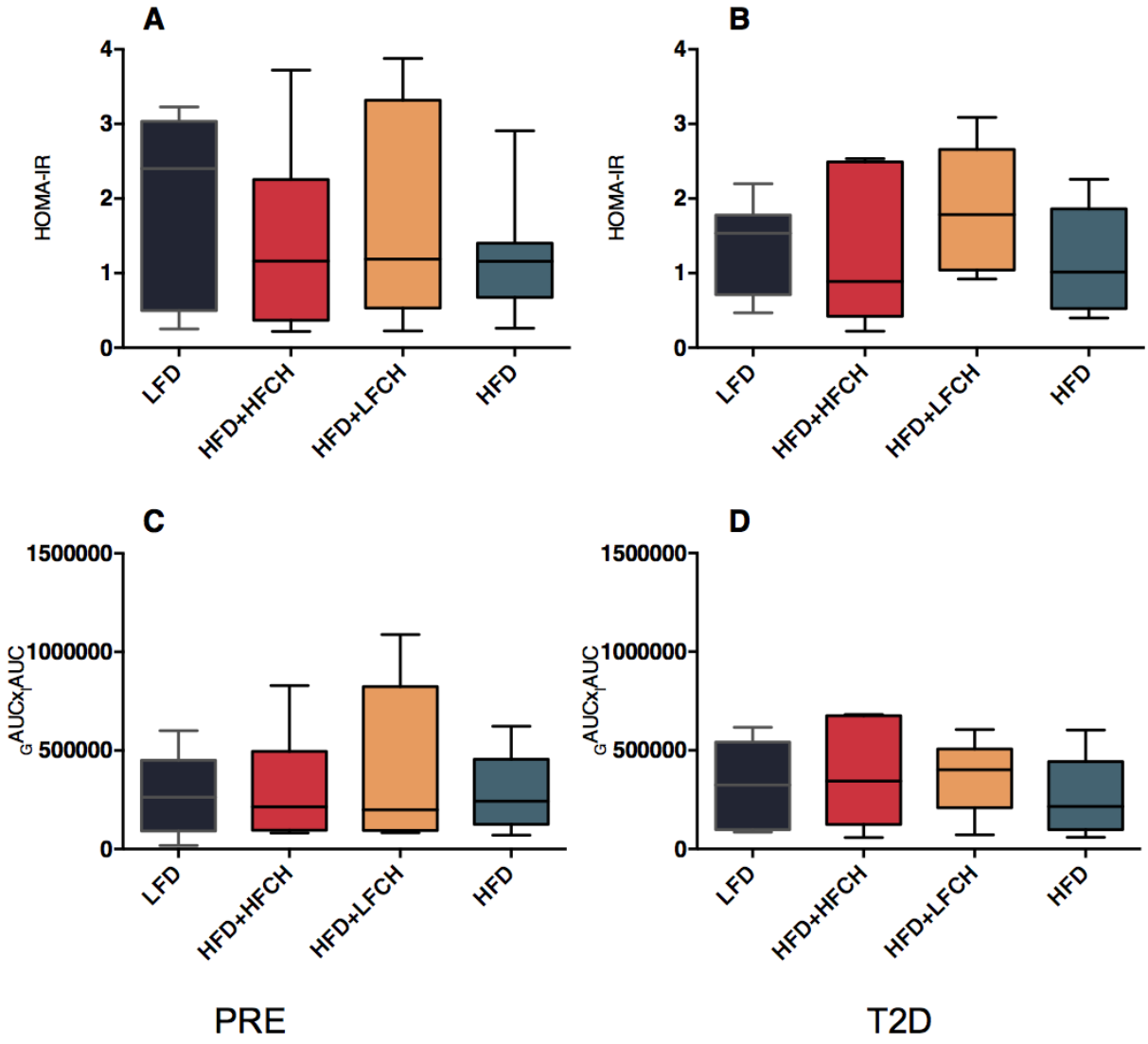
4.7 Insulin Sensitivity

HOMA-IR was calculated as a fasted measure of insulin sensitivity for both prediabetic (Figure 4.7.A) and T2D cohorts (Figure 4.7.B). The $_{G}AUCx_{I}AUC$ was calculated from AUC values for glucose and insulin during OGTTs for both prediabetic (Figure 4.7.C) and T2D cohorts (Figure 4.7.D). This is used as a marker of whole body insulin sensitivity in the fed state[130]. HOMA-IR, homeostatic model assessment insulin resistance was obtained by multiplying fasting glucose (mmol/L) x fasting insulin (μU/mL)/22.5. No statistically significant differences were observed between diet group means for either HOMA-IR or glucose-insulin AUC index ($_{G}AUCx_{I}AUC$) in either the prediabetic, or T2D cohorts.

4.8 Epididymal Fat Weight

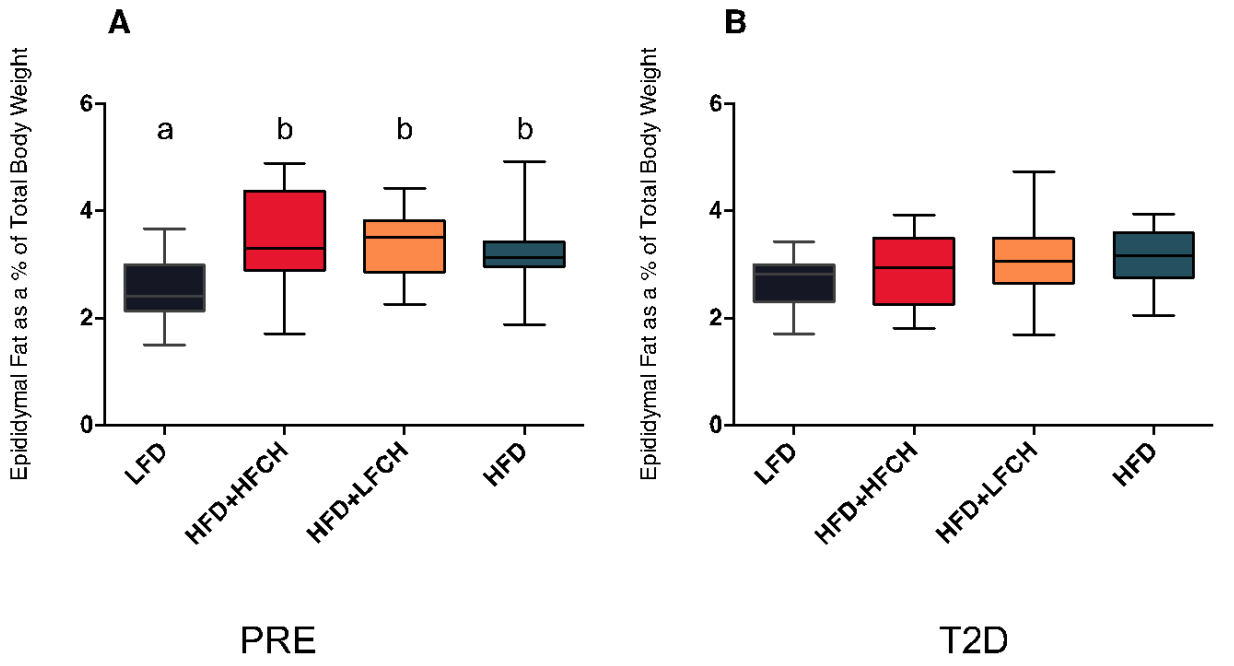
At time of death, epididymal fat and body fat weight data was collected. Epididymal fat as a % of total body fat data was obtained by dividing epididymal fat weight by body weight and multiplying by 100. In the prediabetic cohort (Figure 4.8), there was a significant effect of diet on the outcome measure ($p=0.0013$). Differences were observed between LFD and HFD ($p<0.05$), LFD and HFD+LFCh ($p<0.01$), and LFD and HFD+HFCh ($p<0.01$). No such differences were observed in the T2D cohort (Figure 4.8.B).

Figure 4.7: HOMA-IR and $_{G}AUCx_{I}AUC$ in Prediabetic and T2D Cohorts



Effects of 7-8 weeks of cheese diet feeding on fasted and fed insulin sensitivity in HFD, HFD+LFCh, HFD+HFCh, and LFD groups. Data are shown as mean, min, and max. HOMA-IR, homeostatic model assessment insulin resistance was obtained by multiplying fasting glucose (mmol/L) x fasting insulin (μU/mL)/22.5. HOMA-IR of the prediabetic cohort is seen in (A), and data from the T2D cohort is seen in (B). A measure of fed insulin sensitivity, $_{G}AUCx_{I}AUC$ was obtained by multiplying fasting glucose AUC x fasting insulin AUC for prediabetic (C) and T2D (D) cohorts. one-way ANOVA followed by Bonferroni's test was run on all data sets. There was no significant effect of diet on any outcome measures. *n* per group (identical in both cohorts): HFD=8, HFD+LFCh=8, HFD+HFCh=8, LFD=8.

Figure 4.8: Epididymal Fat % Total Body Weight in Prediabetic and T2D Cohorts



Epididymal fat weight as a % of total body weight at time of death in HFD, HFD+LFCh, HFD+HFCh, and LFD groups. Data are presented as mean, min, and max, with subscript letters denoting significant differences observed amongst groups. Values were obtained by dividing epididymal fat weight by body weight at time of death. Data is shown for both prediabetic (A) and T2D (B) cohorts. There was a significant effect of diet on Epididymal fat % in the prediabetic cohort ($p=0.013$), and it was revealed in post-hoc analyses that all HFD+HFCh, HFD+LFCh, and HFD groups were all significantly different from the LFD ($p<0.05$, $p<0.001$, $p<0.001$, respectively), but not from one another. There were no significant effects of diet in the T2D cohort. one-way ANOVA followed by Bonferroni's test was performed on all data. *n* per group (identical in both cohorts): HFD=16, HFD+LFCh=16, HFD+HFCh=16, LFD=16.

Chapter 5: Effect of Cheese on Serum and Hepatic Lipid Phenotype in Prediabetic Rats

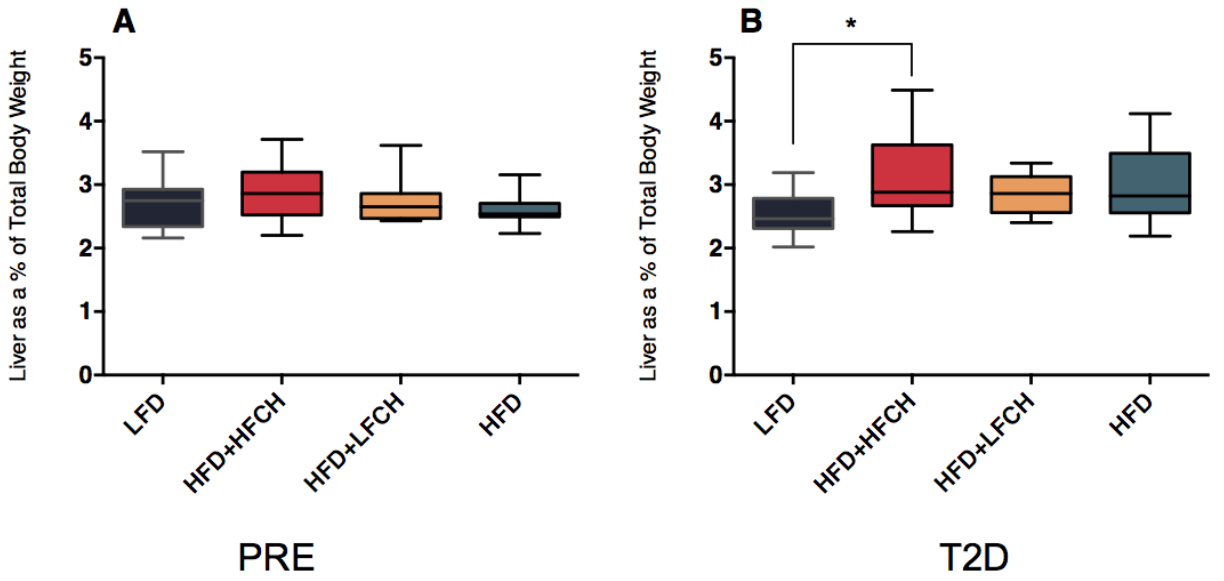
5.1 Introduction

In this chapter, we examined the mechanisms behind the effects of cheese observed *in vitro* in our prediabetic rat model. This was done by analyzing the serum metabolome, the serum and liver lipidome, as well as looking at liver histology. We first sought to answer the question, "What is the effect of cheese on the serum metabolome of prediabetic rats?". In earlier *in vivo* experiments it was revealed that insulin sensitivity, and more specifically hepatic insulin sensitivity was improved in prediabetic rats fed cheese diets. Since liver lipid accumulation plays a role in insulin sensitivity, we further sought to answer the question, "What is the effect of feeding a diet high in cheese on liver lipid metabolism?" Moreover, we aimed to determine whether there was a difference between animals assigned to HFD+HFCh and HFD+LFCh diets with regard to these questions.

5.2 Liver Weight

At time of death, liver and body fat weight data were collected. Liver weight as a percentage of total body fat data was obtained by dividing liver weight by body weight. In the T2D cohort (Figure 5.1.B), there was a significant effect of diet on the outcome measure ($p=0.0253$). Differences were observed between LFD and HFD+HFCh ($p<0.05$). No differences were observed in the prediabetic cohort (Figure 5.1.A).

Figure 5.1: Liver Weight in Prediabetic and T2D Cohorts



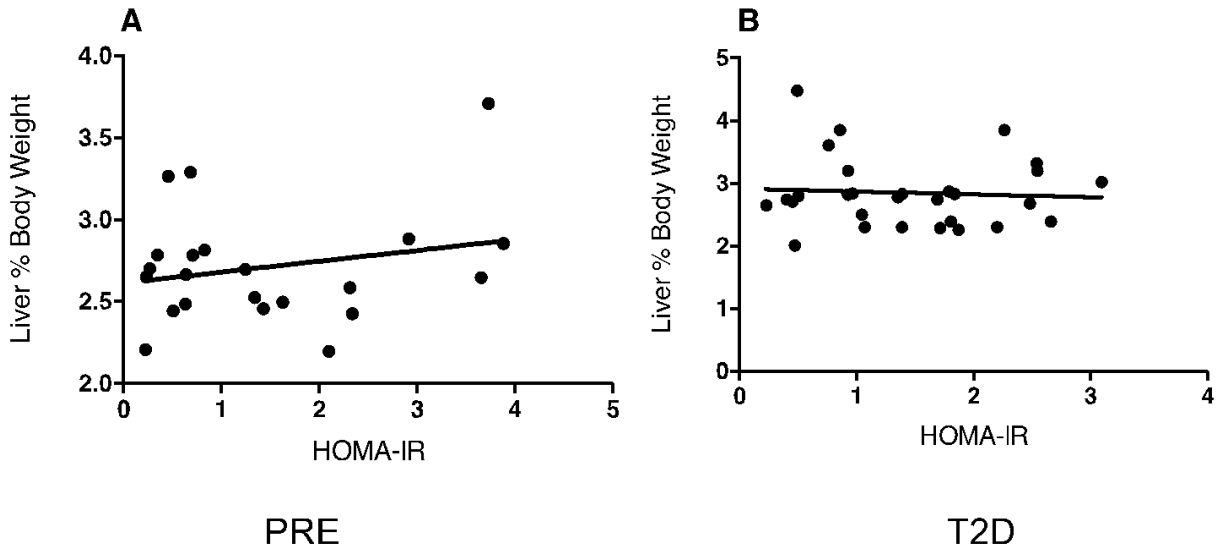
Liver weight as % of total body weight following 8 weeks of HFD, HFD+LFCh, HFD+HFCh, or LFD feeding. Data are presented as mean, min, and max. Values were obtained by dividing liver weight by body weight at time of death. Data are shown for both prediabetic (A) and T2D (B) cohorts. There was a significant effect of diet on liver weight in the T2D cohort ($p=0.0253$), and it was revealed in post-hoc analyses that the HFD+HFCh was significantly different from the LFD group ($p<0.05$). No significant effect of diet was observed in the prediabetic cohort. Data were analyzed by one-way ANOVA, followed by Bonferroni's test. *n* per group: HFD=16, HFD+LFCh=16, 15 in PRE cohort, HFD+HFCh=16, LFD=16.

5.3 Relationship between HOMA-IR vs. Liver Weight

A linear regression was run to determine the correlation between HOMA-IR and liver weight as a % of total body weight at time of death. Liver weight may reflect liver lipid storage, which is known to be associated with insulin sensitivity. We aimed to examine whether there was an association between insulin sensitivity and liver weight. All diet groups were pooled into one column comparing liver weight values with HOMA-IR values. No significant relationship was found between the two measures in either the prediabetic

cohort [F(1.0,20.0)=1.078], p=0.3116, r^2=0.05113] or T2D cohort [F(1.0, 27.0)=0.1189, p=0.7329, r^2=0.004385].

Figure 5.2 Linear Regression of HOMA-IR vs. Liver % BW



Effect of liver weight as a % of total body weight on insulin sensitivity following 8 weeks of HFD, HFD+LFCh, HFD+HFCh, or LFD feeding. Data are presented as points on a graph with a line of best fit. All diet groups were pooled (N=63 in the prediabetic cohort, N = 64 in the T2D cohort), and a regression line was generated considering each y-value as an individual point. Results were nonsignificant with p=0.3116 in the PRE cohort, and p=0.7329 in the T2D cohort. R^2 values were 0.05113 in the prediabetic cohort, and 0.004385 in the T2D cohort.

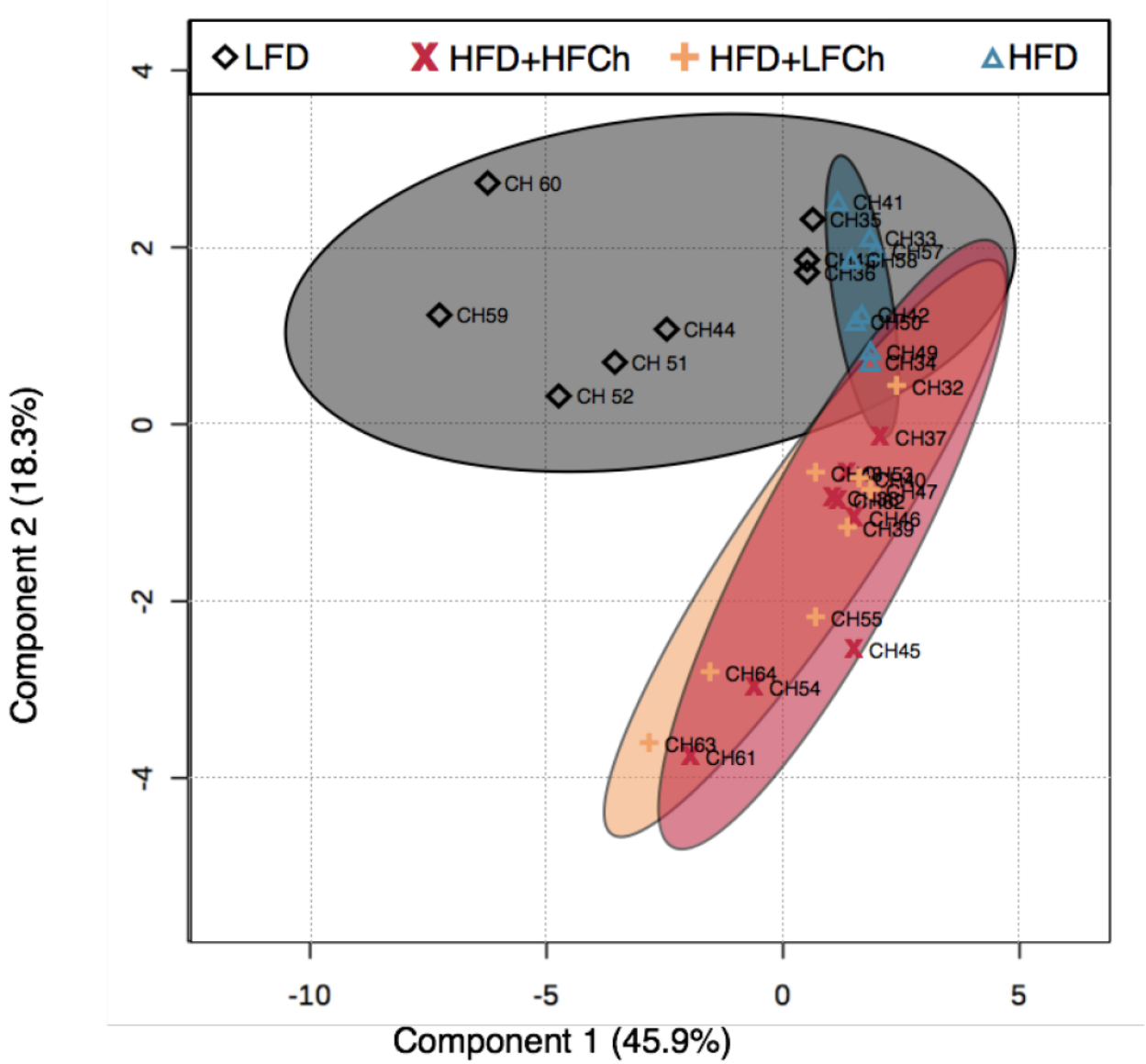
5.4 Serum Metabolome in Prediabetic Rats

5.4.1 Determination of Metabolomic Profile

A total of 32 samples from the prediabetic cohort were sent for metabolomic analysis (N=8 per diet group). Samples underwent NMR and GC analysis. A partial least squares discriminant analysis (PLS-DA) was conducted on metabolomics results. To assess the significance of class discrimination, a permutation test was performed. In each

permutation, a PLS-DA model was built, with the data as x-value, and permutated class label as y-value. Two variable importance measures are used: (1) a weighted sum of squares of partial least squares (PLS) loadings. This considers (a) the amount of explained Y-variation in each dimension, and (b) a factor based on the weighted sum of PLS-regression. (2) a function of the reduction of the sums of squares across the number of PLS components. Figure 5.3. shows the 2-D scores plot between selected components. This visualization shows the 95% confidence interval of the points in different diet groups. The 95% confidence interval of LFD points take up a large area, while the HFD points cluster tightly, albeit within the confidence interval of the LFD. A large plot area indicates greater variability in responses, while a smaller area indicates consistency between samples. This implies greater variability in prediabetic rats' serum metabolomic response to LFD than to HFD. Both HFD+HFCh and HFD+LFCh behave similarly, their 95% confidence intervals almost entirely overlapping with one another, while only slightly overlapping the HFD and LFD areas. This suggests that cheese diets are similar in their metabolomics profile, but dissimilar to either HFD or LFD.

Figure 5.3 2D Scores Plot of Prediabetic Cohort's Serum Metabolites

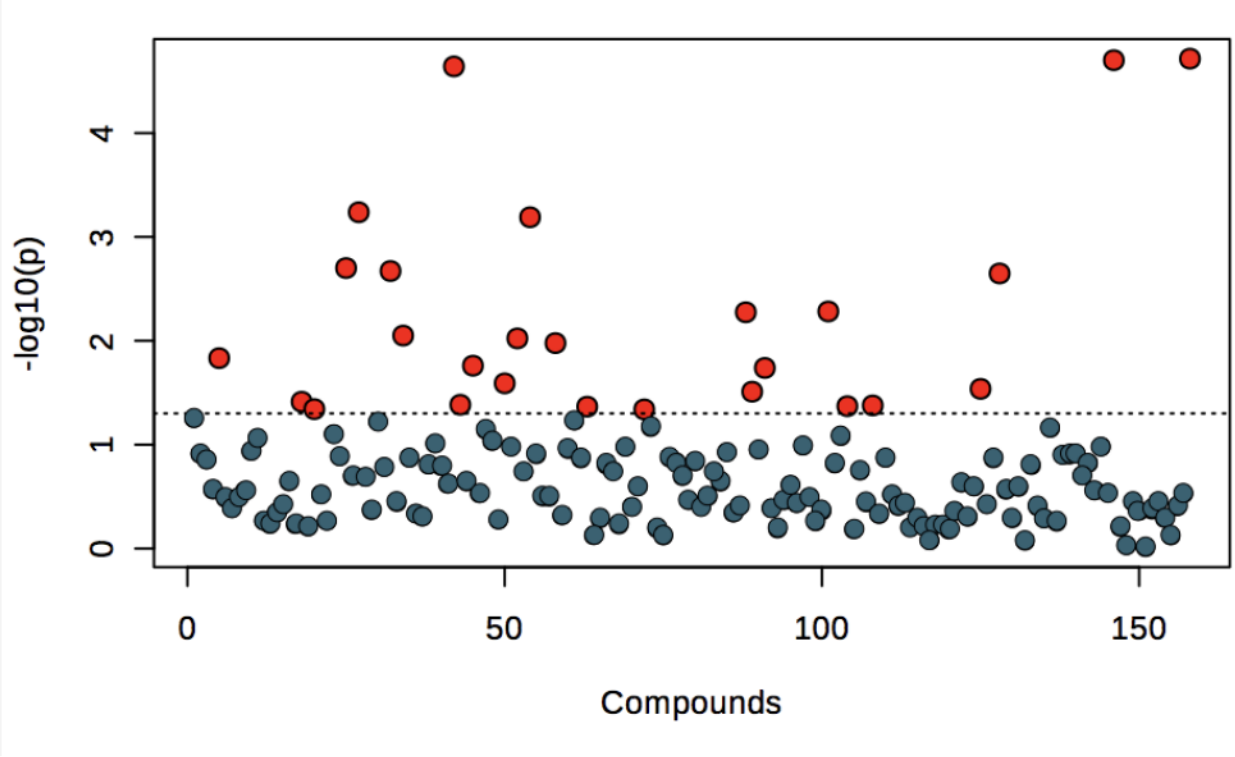


Partial least squares discriminate analysis 2-D score plots of the effects of HFD, HFD+LFCh, HFD+HFCh, or LFD in serum. Coloured areas represent 95% confidence intervals. LFD is seen as a large cluster with high variability, while HFD overlaps with LFD but remains in a tight area. Finally, both cheese diets overlap slightly with LFD and HFD confidence intervals, yet spread out over different areas. This suggests that overall, pattern of metabolites differs between LFD, HFD, and cheese groups, although HFD+HFCh and HFD+LFCh behave similarly to one another. Explained variance is described in brackets. *n* per group: HFD=8, HFD+LFCh=8, HFD+HFCh=8, LFD=8.

5.4.2 Identification of Important Features

Data obtained from metabolomic analysis underwent row-wise normalization and range scaling. Next, important features were identified by one-way ANOVA of log-transformed data with a p-value threshold of 0.05 (Figure 5.4). This revealed statistically significant differences ($p < 0.05$) in 26 compounds. Fisher's Least Significant Difference (LSD), a post hoc test, was conducted to determine which diet groups were significantly different from one another. These compounds and the results of the one-way ANOVA are summarized in Table 5.1.

Figure 5.4 One-Way ANOVA of Important Features in Metabolomic Analysis



N=26 Important features selected by ANOVA plot of log-transformed data with a p-value <0.05. Important features (which represent individual compounds, see Table 5.1) are displayed in red.

Table 5.1 Important Features Identified by One-Way ANOVA and Post-Hoc Analysis

Compounds	F-Value	P-Value	Fisher's LSD*
Biogenic Amines			
Acetylornithine	12.72	<0.01	HFD+HFCh - HFD; HFD+LFCh - HFD; HFD+LFCh - HFD+HFCh; HFD+LFCh - LFD
Taurine	12.78	<0.01	HFD+HFCh - HFD; HFD+LFCh - HFD; HFD+HFCh - LFD; HFD+LFCh - LFD
Amino Acids			
Alanine	3.48	0.03	HFD+HFCh - HFD; HFD+HFCh - HFD+LFCh
Aspartic acid	6.22	<0.01	HFD+HFCh - HFD; HFD+LFCh - HFD; HFD+HFCh - LFD
Acylcarnitines			
C14:1-OH	4.17	0.01	HFD+HFCh - HFD; HFD+LFCh - HFD; HFD+HFCh - LFD
C3	3.2	0.04	HFD+HFCh - HFD; HFD+HFCh - HFD+LFCh
C4	3.04	0.05	LFD - HFD
Glycerophospholipids			
LysoPC a C14:0	6.36	<0.01	HFD+HFCh - HFD; HFD+LFCh - HFD; HFD+HFCh - LFD
LysoPC a C16:1	7.88	<0.01	LFD - HFD; LFD - HFD+HFCh; LFD - HFD+LFCh
LysoPC a C20:3	6.29	<0.01	LFD - HFD; LFD - HFD+HFCh; LFD - HFD+LFCh
LysoPC a C24:0	4.69	0.01	LFD - HFD; LFD - HFD+HFCh; LFD - HFD+LFCh
PC aa C32:1	12.5	<0.01	LFD - HFD; LFD - HFD+HFCh; LFD - HFD+LFCh
PC aa C32:2	3.13	0.04	HFD+HFCh - HFD; HFD+LFCh - HFD; LFD - HFD

PC aa C34:1	3.99	0.02	LFD - HFD; LFD - HFD+HFCh; LFD - HFD+LFCh
PC aa C36:1	3.6	0.03	LFD - HFD; LFD - HFD+HFCh; LFD - HFD+LFCh
PC aa C36:3	4.63	0.01	LFD - HFD; LFD - HFD+HFCh; LFD - HFD+LFCh
PC aa C36:5	7.74	<0.01	LFD - HFD; LFD - HFD+HFCh; LFD - HFD+LFCh
PC aa C38:3	4.51	0.01	LFD - HFD; LFD - HFD+HFCh; LFD - HFD+LFCh
PC aa C40:3	3.09	0.04	LFD - HFD; LFD - HFD+HFCh; LFD - HFD+LFCh
PC aa C42:6	3.03	0.05	LFD - HFD; LFD - HFD+HFCh; LFD - HFD+LFCh
PC ae C38:0	5.25	0.01	LFD - HFD; LFD - HFD+HFCh; LFD - HFD+LFCh
PC ae C38:1	3.42	0.03	LFD - HFD
PC ae C38:3	3.94	0.02	LFD - HFD; LFD - HFD+HFCh
PC ae C42:0	5.27	0.01	LFD - HFD; LFD - HFD+HFCh; LFD - HFD+LFCh
PC ae C42:3	3.1	0.04	LFD - HFD; LFD - HFD+HFCh
PC ae C44:5	3.12	0.04	LFD - HFD; LFD - HFD+HFCh

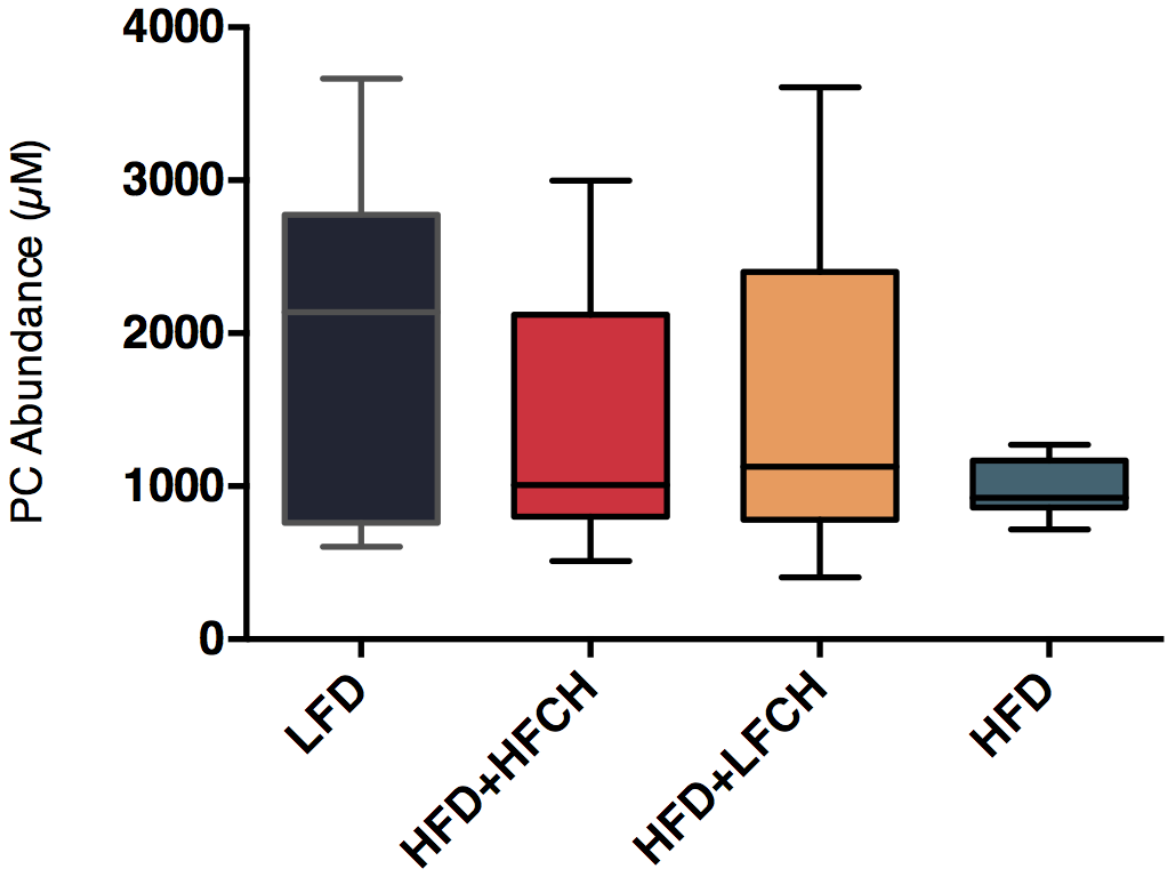
*Fisher's LSD compared each group to each other group. The notation indicates differences between groups, eg. LFD – HFD means that these 2 groups were significantly ($p<0.05$) different from each other.

5.4.3 Abundance of Phosphatidylcholine Species in Serum

Of 26 important features, 19 species of phosphatidylcholine (PC) or their metabolites (lysoPC) were identified in metabolomic analysis. PC species are a class of phospholipids characterized by a choline headgroup. They are a major component of biological membranes, playing a role in the maintenance of membrane permeability, structural integrity, and membrane-mediated cell signaling[131]. PCs can be synthesized in the liver or obtained from diet[131]. In mammals, phosphatidylcholine is synthesized from choline through the Kennedy, or CDP-choline, pathway. First, choline kinase catalyzes the conversion of choline to phosphocholine[132,133]. Next, in the rate limiting step, the phosphocholine is converted to CDP-choline, catalyzed by CTP:phosphocholine cytidyltransferase[134]. The final step involves the action of CDP-choline:1,2-diacylglycerol cholinephosphotransferase to catalyze the conversion of CDP-choline to phosphatidylcholine[132,133]. A parallel arm of the Kennedy pathway can use ethanolamine to synthesize phosphatidylcholine[132]. Up to 95% of choline obtained in the diet goes towards PC synthesis[135]. Interestingly, PC biosynthesis is necessary for the normal secretion of VLDL secretion from hepatocytes[135]. Choline is recycled in the liver[135]. Given our earlier findings that the many PC and LysoPC species were altered by cheese feeding, and since it was suspected that hepatic insulin sensitivity, and possibly hepatic lipid accumulation, were altered by cheese, a more in-depth analysis was conducted examining the abundance of PC species in serum of prediabetic rats. Total abundance of PC in serum was calculated (Figure 5.5), and a one-way ANOVA was conducted, followed by Bonferroni's test. Diet group did not affect total PC abundance ($p=0.0695$),

although a trend emerged (p=0.0695) with LFD having the greatest mean PC abundance (1938 ± 394.4 μM), followed by HFD+LFCh (1521 ± 387.0 μM), HFD+HFCH (1348 ± 312.6μM), and finally HFD (983.9 ± 387.0 μM). Mean PC abundance was increased by ~97.0% in the LFD relative to HFD, while mean HFD+LFCh was only 12.8% higher than mean HFD+HFCh. The concentration of the 19 different significant PC species or their metabolites is summarized in Table 5.2. Data is presented as max, min, and mean, with significance determined by either one-way ANOVA, or Kruskal-Wallis' test when data failed the D'Agostino & Pearson normality test. These tests were followed by Bonferroni's test comparing means among groups. Although most differences existed only between LFD and HFD, several other significant differences between diet group means were observed. In the PC aa C32:1, HFD+LFCh and HFD were both significantly different from the LFD, while HFD+HFCh was not. In LysoPC a C14:0, HFD+HFCh was different from both HFD and LFD, but not from HFD+LFCh. In PC aa C32:2, HFD was different from all other diets, while LFD, HFD+HFCh, and HFD+LFCh were not significantly different from one another.

Figure 5.5 Abundance of Phosphatidylcholine Species in Serum



Effect of diet on abundance of PC species in serum of prediabetic rats. Data are presented as mean, min, and max. Serum underwent metabolomic analysis, and the total concentration of all PC species was calculated. Data were analyzed by way of one-way ANOVA, followed by Bonferroni's test. No significant differences were observed among diet groups. *n* per group: HFD=8, HFD+LFCh=8, HFD+HFCh=8, LFD=8.

Table 5.2 Abundance of Different PC Species by Diet Group

Lipid Species (μM)	LFD	HFD	HFD+LFCh	HFD+HFCh
PC aa C32:1	7.51 ± 1.23	1.49 ± 0.13***	2.76 ± 0.70*	2.57 ± 0.52
PC aa C32:2	0.62 ± 0.13	0.17 ± 0.01**	0.55±0.16 ¶	0.65 ± 0.14 ¶¶
PC aa C34:1	89.33 ± 17.82	32.83 ± 2.70 *	50.48 ± 12.66	46.21 ± 10.24
PC aa C36:1	32.19 ± 7.08	11.86 ± 1.01	17.81 ± 4.96	15.88 ± 3.38
PC aa C36:3	30.32 ± 8.25	6.06 ± 0.68**	13.53 ± 3.99	12.97 ± 2.73
PC aa C36:5	5.31 ± 0.96	1.35 ± 0.14 ***	2.56 ± 0.62	2.08 ± 0.47
PC aa C38:3	45.47 ± 11.64	11.01 ± 1.38	20.16 ± 7.31	15.80 ± 4.30
PC aa C40:3	0.52 ± 0.11	0.22 ± 0.02	0.29 ± 0.08	0.28 ± 0.06
PC aa C42:6	0.48 ± 0.10	0.23 ± 0.02	0.28 ± 0.06	0.29 ± 0.05
PC ae C38:0	1.49 ± 0.28	0.55 ±0.07*	0.82 ± 0.15	0.73 ± 0.16
PC ae C38:1	0.46 ± 0.10	0.14 ± 0.02*	0.30 ± 0.08	0.27 ± 0.05
PC ae C38:3	1.12 ± 0.22	0.36 ± 0.02**	0.69 ± 0.17	0.65 ± 0.16
PC ae C42:0	1.07 ± 0.15	0.64 ± 0.02	0.71 ± 0.06	0.67 ± 0.05
PC ae C42:3	1.09 ± 0.25	0.43 ± 0.05	0.64 ± 0.16	0.55 ± 0.14
PC ae C44:5	0.16 ± 0.03	0.08 ± 0.01	0.10 ± 0.02	0.09 ± 0.02
LysoPC a C14:0	9.30 ± 0.75	8.81 ± 0.77	11.04 ± 0.55	13.03 ± 0.92*¶¶
LysoPC a C16:1	7.82 ± 1.57	1.53 ± 0.07***	3.3 ± 0.85	3.2 ± 0.7
LysoPC a C20:3	8.18 ± 2.19	1.31 ± 0.22	2.59 ± 0.91	2.44 ± 0.64
LysoPC a C24:0	2.40 ± 0.08	2.00 ± 0.02**	2.15 ± 0.10	2.17 ± 0.08

Effect of 8 weeks of diet feeding on PC serum metabolites in prediabetic rats. Data are presented as Mean ± SEM. Significance was calculated using one-way ANOVA followed by Bonferroni's test. If data failed D'Agostino & Pearson normality testing, a nonparametric Kruskal-Wallis' test was performed. N=8 per diet group. *p<0.05, **p<0.005, ***p<0.001, ****p<0.0005 compared to LFD; ¶p<0.05, ¶¶p<0.01 compared to HFD.

5.5 Serum Lipids

To further explore the mechanisms of lipid metabolism in prediabetic rats fed cheese diets, the serum and liver lipidome were examined. First, N=32 serum samples were prepared for gas liquid chromatography (GC) analysis; N=8 samples per diet group. Total serum cholesterol, phospholipid, cholesterol ester, triglyceride, and total lipid were calculated. Data are presented in Table 5.3 as Means ± SEM. Data were analyzed by one-way ANOVA followed by Bonferroni's test to determine differences between group means. No significant differences were observed. Total lipid concentration was highest in the HFD+LFCh, followed by LFD, HFD, and finally HFD+HFCh, with ~57% difference between means of the HFD+LFCh and HFD+HFCh groups. HFD+LFCh had the highest triglyceride, cholesterol ester, and phospholipid content. LFD had the highest cholesterol content. HFD+HFCh had the lowest serum lipid concentration on all species, in addition to being lowest overall.

Table 5.3 Serum Lipid Profile

Lipid Species (µg/100µL serum)	LFD	HFD	HFD+LFCh	HFD+HFCh
Cholesterol	21.95 ± 1.66	21.61 ± 1.64	21.75 ± 2.89	16.29 ± 1.47
Phospholipid	106.6 ± 4.25	95.77 ± 11.86	117.5 ± 16.89	80.39 ± 6.70
Cholesterol Ester	26.09 ± 0.93	23.56 ± 2.03	26.57 ± 3.79	21.16 ± 1.54
Triglyceride	108.9 ± 11.12	75.73 ± 8.93	121.0 ± 25.90	65.25 ± 12.62
Total Lipid	267.9 ± 15.34	219.8 ± 21.93	291.1 ± 48.35	184.6 ± 20.74

Effect of diet on serum lipids. N=32 samples (N=8 per diet group) were analyzed by GC. The abundance of lipid species is presented as means ± SEM. Data were analyzed by one-way ANOVA or the nonparametric Kruskal-Wallis test if data failed normality testing. Data sets for total lipid and phospholipid underwent nonparametric tests. There was no effect of diet on serum lipid content.

5.6 Liver Lipidome

In addition to serum samples, N=32 liver samples were prepared for gas liquid chromatography (GC) analysis; N=8 samples per diet group. Total liver cholesterol, phospholipid, cholesterol ester, triglyceride, and total lipid per mg of liver tissue were calculated. Data are presented in Table 5.4 as Means ± SEM. Data were analyzed by one-way ANOVA followed by Bonferroni's test to determine differences between group means. There was a significant effect of diet on liver triglyceride content ($p<0.05$), with post-hoc analysis revealing that HFD+LFCh had significantly increased triglyceride content relative to HFD. No other statistically significant differences occurred. However, hepatic cholesterol ester content came close to achieving statistical significance ($p=0.0554$), with HFD+LFCh having the highest mean concentration, and HFD the lowest.

As with serum, there was a trend (p=0.6752) towards highest total liver lipid found in the HFD+LFCh group, followed by HFD, LFD, and HFD+HFCh again having the lowest total lipid. However, the difference between groups was less pronounced than in plasma, with ~12% difference between the means of the cheese groups. HFD+LFCh had the highest cholesterol ester, and triglyceride. HFD+HFCh had the lowest phospholipid. HFD had the lowest cholesterol ester and triglyceride, while having the highest cholesterol.

Table 5.4 Liver Lipid Profile

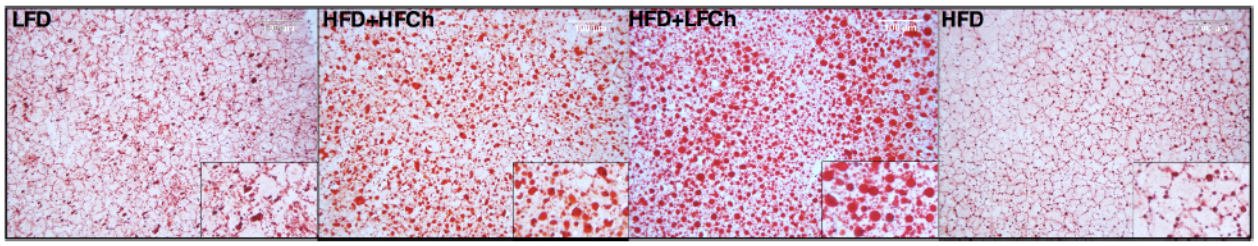
Lipid Species (µg/mg liver)	LFD	HFD	HFD+LFCh	HFD+HFCh
Cholesterol	5.31 ± 0.38	5.39 ± 0.32	4.61 ± 0.24	4.67 ± 0.21
Phospholipid	81.64 ± 6.00	84.23 ± 4.88	77.76 ± 3.22	71.24 ± 4.25
Cholesterol Ester	1.27 ± 0.24	1.13 ± 0.15	2.08 ± 0.25	1.47 ± 0.27
Triglyceride	12.20 ± 1.61	8.91 ± 1.54	22.35 ± 3.75*	16.43 ± 4.11
Total Lipid	100.60 ± 6.78	104.70 ± 8.86	105.30 ± 6.35	93.82 ± 7.51

Effect of diet on liver lipid content. N=32 samples (N=8 per diet group) were analyzed by GC. The abundance of lipid species is presented as means ± SEM. Data were analyzed by one-way ANOVA. The nonparametric Kruskal-Wallis test was used on hepatic triglyceride, as it failed normality testing. There was an effect of diet on hepatic triglyceride content, with post-hoc analyses revealing significant differences between HFD+LFCh and HFD groups ($p<0.05$). No other significant differences were observed between means. *$p<0.05$

5.7 Liver Histology

A small sample of preliminary data (N=2 per diet group) was gathered examining the liver lipid content of prediabetic rats. Samples were stained with ORO to visualize lipid droplets (Figure 5.5).

Figure 5.6 Oil Red O Staining in Prediabetic Cohort Livers



Effect of diet on hepatic lipid accumulation. N=8 (N = 2 per diet group) prediabetic rat liver samples were stained with ORO. 30 randomly selected pictures of each slide were taken with 20x objective lens magnification, and were quantified using ImageJ software. There was a significant effect of diet on liver lipid accumulation (p=0.012), with both HFD+LFCh and HFD+HFCh different from both LFD and HFD. ORO Staining magnification 20x of HFD, HFD+HFCH, HFD+LFCH, and LFD.

Chapter 6: Discussion and Conclusions

There is very little information surrounding the impact of cheese feeding on the diabetic or pre-diabetic (insulin resistant) phenotype in humans. What little evidence exists is by association in large cohort studies. Therefore, in this study the first objective was to conduct an experimental trial examining the impact of cheese on glucose tolerance and insulin sensitivity in rodent models of pre-diabetes and diabetes. Few studies compare LF and HF cheese. This is of particular importance, as Canadian food guide recommendations promote LF cheese over HF cheese. However, numerous cheese fat components having been shown to benefit the maintenance of glucose homeostasis, bringing into question the rationale behind this recommendation. Thus, a sub-objective was to compare effects of LF versus HF cheese on glucose tolerance and insulin sensitivity. Since the mechanisms through cheese may confer metabolic benefits are not well elucidated, in our second objective we used an untargeted metabolomic analysis of serum from the prediabetic rats to identify compounds of interest that could point to modulation of specific processes by cheese. After examining the outcomes of objectives 1 and 2, it was determined that alterations in lipid metabolism were of interest; therefore, in the third objective we chose to further examine hepatic lipid metabolism in prediabetic rats.

6.1 Objective 1

To test the hypothesis that cheese will improve glucose tolerance and insulin sensitivity in prediabetic and diabetic rats, a battery of *in vivo* measures were done including glucose tolerance tests, insulin tolerance tests, assays of fasting blood glucose, and fasting plasma insulin. The results are first presented for the prediabetic rat cohort followed by the diabetic rat cohort. Overall, for prediabetic rats, an increase in hepatic insulin sensitivity was demonstrated during the insulin tolerance test and this was the same for both LF and HF cheese. In contrast, in diabetic rats, LF but not HF cheese improved glucose tolerance. No other marked effects were seen in either cohort, as summarized below, leading to the conclusion that feeding a high cheese diet has minor effects on glucose homeostasis in both prediabetic and diabetic models.

6.2 Prediabetic Cohort: Effects of Cheese on Indicators of Glucose Homeostasis

An insulin tolerance test (ITT) is a procedure that involves injecting insulin into the subject's peritoneum, and observing the resulting change in blood glucose. The more profound or lengthy the response, the more insulin-sensitive the subject is determined to be. During an ITT, there was a significant effect of diet on both glucose removal from plasma and AUC. Up until T=60, LFD and animals fed both cheese diets responded to insulin with rapid decreases in serum glucose, while in rats fed the HFD there was a less pronounced plasma glucose response. This better response to insulin indicates improved insulin sensitivity of peripheral tissues such as skeletal muscle[136]. However, after T=60,

blood glucose in the LFD group began to increase, while this did not occur in either of the cheese diets. This suggests that rats fed cheese may be experiencing increased hepatic insulin sensitivity relative to both HFD and LFD. Insulin suppresses hepatic gluconeogenesis[19], and here the effects of this suppression appears to last longer in animals fed cheese. HFD+LFCh experienced a significant decreased in serum glucose AUC relative to HFD, dropping ~22%, while HFD+HFCh experienced a ~17% decrease relative to HFD, although this was not significant. Overall, this observation suggests that feeding a HF cheese diet increases insulin sensitivity in prediabetic rats, while there appears to be a modest benefit of consuming LF over HF cheese. Unfortunately, no studies were found to have completed ITT while feeding HF cheese diets, and therefore no direct evidence could be compared. Given our findings, we can conclude that in prediabetic rats, diets rich in LF cheese can cause improvements in insulin sensitivity.

To test prediabetic rats' response to a glucose challenge, an OGTT was conducted. While there was no effect of diet on either time-dependent glucose, or iAUC, mean iAUC was ~42% increased in the HFD+HFCh rats relative to HFD+LFCh rats. Mean HFD+HFCh time-dependent glucose peaked at levels ~11% higher than HFD+LFCh. There was no effect of diet on serum insulin during the OGTT, either time-dependent, or AUC. However, the mean HFD+LFCh AUC was ~12% increased relative to HFD+HFCh, with both cheese diets having higher mean insulin AUC than either controls. Overall this points towards there being no effect of diet upon serum glucose or insulin during an OGTT. Contrary to our findings, Tholstrup et al.[137] reported that three weeks of consuming a diet supplemented with HF cheese resulted in elevated post-prandial glucose when

compared to an isocaloric milk diet. However, cheese feeding did not result in significant differences when compared to a butter diet[137].

Fasting insulin and glucose are important data to obtain, as they reflect how well a system maintains blood glucose levels when the system is not stressed. Elevated fasting serum blood glucose indicates a more profound dysfunction, as it demonstrates that the system is not able to maintain blood glucose in the least taxing of situations. Elevated fasting serum insulin indicates that even at rest, the body must provide larger quantities of insulin to maintain serum glucose. This implies serious strain, as even when not challenged by a food bolus, the pancreatic beta cells must still produce large volumes of insulin to maintain glucose homeostasis, which is likely unsustainable over a long period of time. In this trial, there was no effect of the experimental diets on either fasting blood glucose or fasting insulin concentrations. This implies that adding cheese to the diet did not aid or hinder systems maintaining blood glucose in a fasted state. In accordance with our findings, Raziani et al. (2016)[96] reported no significant effect of LF or HF cheese diet feeding on fasting glucose or insulin. This study was completed in a population of adults with 2 or more risk factors for metabolic syndrome. Another study completed in older overweight adults reported no changes in fasting glucose or insulin when isocaloric cheese or butter diets were compared[40]. These experiments were comparable to our own, as 20% of total energy came from dairy fat, while 16% and 21% of total energy came from cheese fat in our HFD+LFCh and HFD+HFCh diets, respectively. Further, a high cheese diet did not influence either fasting glucose or insulin in a randomized crossover trial of overweight post-menopausal women, when compared

to a carbohydrate or meat diet control[38]. One small trial completed in healthy young men found that fasting glucose was not impacted by cheese consumption when compared to an isocaloric non-dairy control. However, it was also reported that individuals consuming the cheese diet had significantly lower fasting serum insulin compared to controls[138]. Finally, in a population of healthy adults, higher fasting plasma glucose was observed following cheese consumption, when compared to butter consumption[139]. Overall, the evidence surrounding the impact of cheese consumption on fasting insulin and glucose is contradictory, and requires further elucidation. Metabolic status could affect outcomes, as studies conducted in healthy individuals tended to report effects of cheese on fasting insulin and glucose, while those conducted in individuals who were overweight or had MetS risk factors trended towards cheese having no effect on fasting serum insulin and glucose.

Data from fasting blood glucose, fasting plasma insulin and glucose and insulin responses during the OGTT can be used as surrogate measures of insulin sensitivity. HOMA-IR is a measure of fasted insulin sensitivity, while the $_{G}AUCx_{I}AUC$ measures insulin sensitivity following a food bolus, when the system is being challenged. It is important to measure both, as HOMA-IR alone does not reflect how a system may perform when challenged, while $_{G}AUCx_{I}AUC$ shows only a challenged system, and not how it may perform when at rest. This trial found no effect of diet on fasted or fed insulin sensitivity computed using standard formulas. Analysis of both HOMA-IR and $_{G}AUCx_{I}AUC$ found no effect of diet. This finding suggests that insulin sensitivity is not affected by cheese feeding, which contradicts earlier findings in the insulin tolerance test,

where both cheese diets showed improved responsiveness to insulin, when compared to HFD. However, an ITT is a larger challenge to biological systems, as it is in a partially fasted state and responding to an insulin challenge. Therefore, such conditions may show a response that is not evident under conditions where the system is not comparably challenged. Nevertheless, these findings are consistent with 2 trials done in adults with 2 or more symptoms of metabolic syndrome[96], as well as a randomized crossover trial of overweight post-menopausal women[38].

6.3 T2D Cohort: Effects of Cheese on Indicators of Glucose Homeostasis

Diabetes was confirmed in the T2D cohort by measuring ambient BG weekly following STZ administration. One week after STZ, BG was >10 mM in HFD, HFD+HFCh and HFD+LFCh groups compared with ~6 mM in LFD group, which did not receive STZ. HFD group maintained a similar elevated BG for the rest of the trial whereas BG of HFD+LFCh trended as much as 48% lower than HFD. Contrary to our hypotheses, the HFD+HFCh diet group performed similarly to the HFD. This suggests that, when added to the diet, LF cheese can assist in attenuating the effects of a diet high in fat on ambient blood glucose. However, the addition of HF cheese does not appear to confer any benefit but neither did it worsen BG.

When subjected to a glucose challenge in the OGTT, there was a significant effect of diet on both time-dependent blood glucose, and iAUC. Both HFD and HFD+HFCh groups performed similarly, demonstrating impaired glucose tolerance. HFD+LFCh had intermediate glucose tolerance, while the healthy LFD group does not ever attain a mean

blood glucose value that would indicate prediabetes. While it did not reach significance, mean OGTT blood glucose iAUC was 30% and 29% lower in the HFD+LFCh compared to HFD and HFD+HFCh, respectively. Yet, HFD and HFD+HFCh were significantly higher than LFD while HFD+LFCh was not, which indicates that HFD+LFCh fed animals had improved oral glucose tolerance compared to HFD and HFD+HFCh diet groups. Although findings were not statistically significant, HFD+LFCh rats were secreting more insulin during the OGTT than either HFD or HFD+HFCh. Given these findings, we can conclude that in T2D rats, diets rich in LF cheese may be beneficial in improving oral glucose tolerance, and that these improvements may be to increased insulin secretion. A diet rich in HF cheese did not show any benefits when compared to a regular HF diet. In accordance with our findings, Tholstrup et al. reported that a diet with HF (45% MF) cheese resulted in elevated post-prandial glucose when compared to an isocaloric milk diet[137]. This experiment was completed in a population of healthy young men. No LF cheese was examined.

Improvements in glucose tolerance might be a result of increased insulin sensitivity of peripheral tissues. An ITT was performed to determine whether there were differences in response to insulin following a 4-hour fast. There was no effect of diet upon either time-dependent glucose, or AUC glucose. Analyses of both HOMA-IR and $_{G}AUCx_{I}AUC$ also found no effect of diet. In a trial of adults who are overweight, or have risk factors of metabolic syndrome, HOMA-IR was reported to be unaffected by diets containing cheese[38,96] This finding is in accordance with earlier findings that insulin sensitivity during and ITT was unaffected by cheese diets.

6.4 Summary and Comparison of Cheese Effects in Two Models of Impaired Glucose Homeostasis *In Vivo*

Overall, cheese feeding had modest effects on glucose tolerance or sensitivity but the effects depended upon both the fat content of the cheese and the severity of glucose intolerance. In a cohort of prediabetic rats, which exhibited mild insulin resistance, both LF and HF cheese elicited improvement in the second phase of the insulin tolerance test. This is interpreted as enhanced insulin sensitivity of the liver, manifesting as reduced gluconeogenesis despite hypoglycemia. In contrast, in the HFD/STZ model of T2D, which exhibits a more severe phenotype due to both insulin resistance and loss of insulin-secreting capacity, only HFD+LFCh exerted a beneficial effect on glucose tolerance. Neither cheese affected insulin sensitivity, which suggests that LF cheese may increase insulin-secreting capacity. Although not significantly different, rats in the HFD+LFCh group secreted more insulin than either HFD+HFCh and HFD. In the T2D cohort, blood glucose followed a similar trend to that observed during the OGTT, with elevated blood glucose observed in HFD and HFD+HFCh groups, while HFD+LFCh served as an intermediate between them and the healthy LFD control. Additional experiments to determine glucose-stimulated insulin secretion from isolated islets and measurement of β-cell mass in the pancreas could be done to provide additional information about the effects of LF cheese on insulin secretion. These findings are illustrated in Figure 6.1.

6.5 Objectives 2 and 3

To address the objective of identifying metabolites of interest that were modulated by feeding high cheese diets, a metabolomics analysis of serum from rats in the prediabetic cohort was conducted. Based on those results, Objective 3 was articulated as the specific research question: what is the effect of cheese on liver lipid metabolism, and is there a difference between LF and HF cheese?

Metabolomics is a new technique that analyzes the relative abundance of low-molecular-weight endogenous metabolites found in biological samples at a point in time. In this study, serum was analyzed. This comprehensive measurement of small molecules allows researchers to see an overview of any changes elicited by experimental conditions[140]. The metabolomic approach used here is known as an untargeted or unbiased approach. Specific metabolites were not studied; rather, an overview of metabolite abundance was generated, and one-way ANOVAs were conducted to determine which metabolites were different by diet. While metabolomic analysis can be quite costly, the two different methods, DI-MS and NMR, have several strengths. DI-MS has greater sensitivity than NMR, however both have moderate-high reproducibility. With NMR, quantitation is possible, although there is the possibility of sample bias. In DI-MS, unknown metabolites can be identified, and data analysis can be automated[140].

Overall, a 2-D scores plot of metabolomic analysis outcomes indicate that HFD+HFCh and HFD+LFCh elicit similar metabolomic outcomes to one another, yet are different from either HFD or LFD. Although very few trials have examined the differential effects of LF and HF cheese, and none examined metabolomic outcomes, Raziani et al.

(2016) reported that participants fed diets supplemented with HF and LF cheese exhibited similar fasting glucose, insulin, and HOMA-IR, indicating similar metabolic response induced by LF and HF cheeses[96].

Metabolomic analysis identified a total of 26 species as having been significantly impacted by the experimental diets. Of these, 19 were related to PC or lysoPC. In this experiment, total PC abundance was not significantly affected by diet. Nevertheless, total PC abundance was 49%, 35%, and 27% higher in LFD, HFD+LFCh, and HFD+HFCh, respectively, relative to HFD, while there was only an 11% difference observed between cheese diet groups. Total PC abundance trended towards being highest in LFD, with cheese diet groups having intermediate levels, and HFD having the lowest abundance. Serum lipids were unaffected by diet, which suggests that any differences in PC species observed in the serum metabolome were not due to increased overall serum lipid content. This could not be explained by phosphatidylcholine or choline in the diet. Cheese diets and HFD had identical quantities of choline bitartrate added, and while cheese and casein are sources of choline, casein protein was used in HFD to ensure that diets were isonitrogenous. There is approximately 16.4 and 14.0 mg of choline per 100 g serving of HF and LF cheeses, respectively. Of this choline, 6.8 and 4.5 mg are PC[141]. Meanwhile, casein and choline added to LFD were lower than in any other diet, yet these animals demonstrated the greatest PC species abundance in serum. Therefore we can conclude that the differences observed in serum PC were not due to choline intake. Interestingly, the gut microbiota has been shown to alter the abundance of serum PC metabolites in mice, which may point to a possible explanation of our findings[142]. In this trial, male germ-

free mice were conventionalized using the cecal contents of donor mice, and fed an autoclaved chow diet. However, the mechanisms through which this occurs were not elucidated, pointing only to the notion that the gut microbiota can modulate lipid metabolism in mice. Butyric acid, a saturated fatty acid found in cheese, has been implicated in altering gut barrier integrity and preventing the translocation of pathogenic microorganisms across the epithelial cell layer[112]. This could prove a possible link between cheese feeding and the altered PC metabolite abundance in serum observed in this trial. However, were a cheese fat component solely responsible for the observed effects, we would expect to see a greater PC abundance in HFD+LFCh than in HFD+HFCh, which is not the case.

Recently, there has been much interest in the gut microbiota, and how different foods can affect it. As a fermented food, cheese is an especially promising vector for the modulation of intestinal microorganisms. One trial investigating camembert cheese found that regular consumption of 80 g of camembert cheese/day for four weeks was able to significantly increase fecal contents of *Lactococcus lactis*, and *Leuconostoc mesenteroides,* although the significance that such changes could have on human health remains to be tested[143]. Butyric acid, a fatty acid found in cheese, is thought to play a role in glucose metabolism through its ability to alter gut barrier integrity, thereby reducing the translocation of pathogenic bacteria across the epithelial cell layer[112]. Butyrate is especially important, as the gut microbiota of individuals with T2D is characterized by a distinct reduction in butyrate-producing bacteria[144].

Identification of altered PC metabolism prompted a fuller investigation of liver lipid metabolism because the liver is the main site of PC synthesis. In the liver, triglyceride content was 151% higher in the HFD+LFCh relative to HFD, while cholesterol ester content, which was 83% higher, was close to achieving significance. Although HFD+HFCh did not achieve statistical significance, it followed the pattern observed in HFD+LFCh, with an 84% increase in triglyceride and a 29% increase in cholesterol ester content relative to HFD. Interestingly, liver cholesterol content was also modestly decreased in both cheese diets relative to LFD and HFD, which had comparable cholesterol content. However, this finding was not significant. In contrast with our findings, one study examining phytanic acid, a fatty acid abundant in cheese, reported that diets containing 0.2% and 0.5% phytanic acid, by weight, significantly reduced hepatic triglyceride accumulation[117]. Preliminary histology results support the evidence that lipid content is increased in both HFD+HFCh and HFD+LFCh diet animals. ORO staining area was 303% and 231% increased in HFD+LFCh and HFD+HFCh livers, respectively, relative to HFD, while cheese diets were not significantly different from one another. However, like the triglyceride and cholesterol ester results, HFD+LFCh lipid content was slightly higher than the HFD+HFCh (roughly 18% higher).

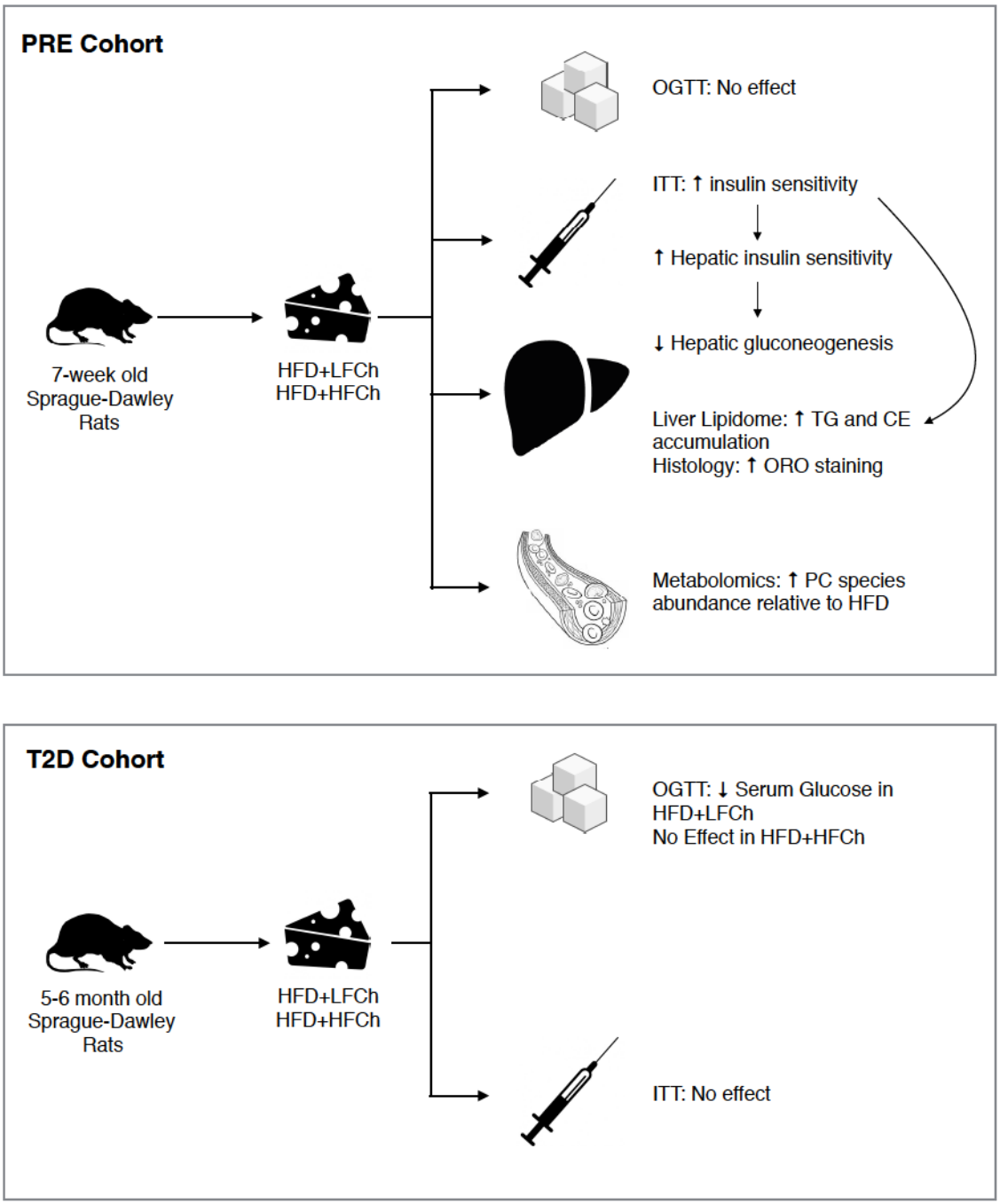
The finding that cheese feeding resulted in higher content of hepatic triglyceride and cholesterol ester contradicts earlier research done by Geurts et al., who reported that rats fed diets rich in cheese significantly lowered hepatic lipid content[95]. While in this trial certain species of lipid were increased in the livers of cheese-fed rats, others, such as cholesterol and phospholipid, were moderately decreased. Overall, total hepatic lipid

accumulation remained similar among all diet groups. One possible explanation for the increased hepatic triglyceride and cholesterol ester accumulation in cheese diet animals would be diet methionine content. In HFD there is 2.5 g of methionine added per kg, while none is added to either cheese diet. Both HF and LF cheeses have 0.558 g of methionine per 100g (Health Canada, 2016), and a total of 322 g of HF cheese, and 394 g of LF cheese are added to ~1 kg of diet. This equals 1.80 and 2.20 g, respectively, of methionine per ~1 kg. It is plausible that this difference in methionine content could result in increased hepatic fat accumulation, as low methionine diets are often used to induce hepatic steatosis[146]. Hepatic steatosis is the state in which hepatocytes accumulate triglyceride[147]. Nevertheless, experimental trials induce steatosis using diets containing only 0.17% of total energy coming from methionine[148], while cheese alone supplies 0.18-0.22%, with additional methionine found in casein. Therefore, cheese diets would not be considered low in methionine. Moreover, if methionine were the sole contributor to hepatic steatosis, we would expect that the HFD+HFCh would have increased fat accumulation relative to the HFD+LFCh, as it is poorer in methionine. Yet, HFD+LFCh demonstrates the highest hepatic lipid abundance, indicating that methionine content alone could not account for the observed differences. This finding is cause for concern, as hepatic lipid accumulation typically precedes several diseased states. In this trial, muscle lipid was not assessed. It is plausible that lipid is being stored in the liver as a compensatory mechanism, and that this hepatic lipid accumulation is serving to spare muscle. Future experiments could be done examining the histology of muscle samples gathered to determine whether cheese feeding is also affecting lipid accumulation in skeletal muscle.

6.6 Summary of *In Vitro* Findings: Prediabetic Cohort

From this trial, we can conclude that cheese may exert its benefits through phosphatidylcholine metabolism, or altered hepatic lipid metabolism and accumulation of triglycerides or cholesterol ester in the liver. Despite increased hepatic triglyceride and cholesterol ester accumulation, insulin sensitivity was improved during an ITT in prediabetic rats. These data suggest that while LF cheese may have a slight benefit over HF cheese, both have beneficial impacts on glucose homeostasis in a prediabetic population. Findings are illustrated in Figure 6.1.

Figure 6.1 Summary of Findings



Findings of both PRE and T2D cohort. In the PRE cohort, both cheese diets induced increased insulin sensitivity during an ITT, while increased triglyceride (TG) and cholesterol ester (CE) accumulation, as well as oil red-O (ORO) staining were observed in the liver. Metabolomic analysis revealed increased phosphatidylcholine (PC) species in serum. In the T2D cohort, HFD+LFCh diet resulted in decreased ambient blood glucose, and decreased blood glucose during an OGTT.

6.7 Strengths and Limitations

As this is an animal study, the findings cannot be directly extrapolated to a human population. However, animal studies confer the advantage of total control over diet content and do not rely on self-reported dietary intake, which has many limitations. In addition, this study has the strength of comparing the effects of cheese in essentially isocaloric diets. This experiment was designed to prove that any improvements observed were due to the source of fat, rather than changes in total fat content, as fat was ~42% of total calories in both cheese diets and HFD. However, some weaknesses do exist in the diet design. We did not control for the additional micronutrients found in cheese. In ~1 kg of diet, there was 394 g of LF cheese or 322 g of HF cheese. Mineral and vitamin content is adjusted in the LFD, where 43.24 g and 8.48 g of mineral and vitamin mix, respectively were added to ~1 kg of diet. However, HFD and both cheese diets all had identical quantities of mineral mix and vitamin mix added, at 51.00 g and 10.0 g per ~1 kg, respectively. Moreover, there were slight differences in methionine content. While 2.5 g was added to ~1 kg of HFD, and 2.05 g to LFD, different quantities of cheese added to each cheese diet resulted in roughly 1.80 g and 2.20 g of methionine in HFD+HFCh, and HFD+LFCh, respectively. This value does not account for any methionine contained in the casein powder added to HFD and LFD. Although these differences are small, they could have effects on experimental outcomes, especially hepatic lipid accumulation. Finally, this experiment was designed to be relevant to Canadian consumers. As such, two cheddar cheeses, one 19% MF and one 31% MF, that are available on grocery store shelves were selected to be used as part of experimental cheese diets. This has the

strength of resulting in an experiment that examines realistic patterns of cheese consumption, however, it is plausible that more pronounced effects could have been observed if much lower and higher fat cheeses had been used instead. As only cheddar cheeses were used, the findings in this study cannot be generalized to other cheeses that may have lower or higher fat contents, phosphatidylcholine content, different ripening methods and durations, or different bacterial species within the cheese matrix.

6.8 Future Directions

In the future, experiments could be conducted with a moderately altered version of the diet in this study. Most importantly, a study could be conducted with a diet that takes micronutrient content of cheese into account, thereby removing micronutrient content as a confounder. It would be interesting to compare findings from our current study and see if they hold true after micronutrient intake has been controlled. Next, this study aimed to compare cheeses that any consumer would be able to purchase from a grocery store. As a result, the cheeses chosen were 19% MF and 31% MF. However, there exist cheeses with much higher and lower fat contents. To further examine the impact of cheese fat on glucose homeostasis, future experiments could examine cheeses with much higher, and much lower fat contents, and examine whether: (1) the findings in this study remain true and (2) whether a greater difference in cheese fat content reveals greater differences between cheese diet groups. Finally, the limitation of any animal research is that it cannot be extrapolated to humans, or serve as evidence towards the creation of dietary guidelines. Therefore, we hope that the evidence presented in this thesis may one day

serve as rationale for human trials, wherein either cheese overall, or high fat and low fat cheese are compared to controls to determine whether cheese consumption can benefit a prediabetic or T2D phenotype.

6.9 Conclusions

From this trial, we can conclude that T2D rats that consumed HFD+LFCh demonstrated improvements in either responsiveness to ambient blood glucose, or glucose tolerance during an OGTT, while HFD+HFCh did not. However, prediabetic rats demonstrated improvements in responsiveness to insulin during an ITT, after consuming either HFD+LFCh or HFD+HFCh. These findings could not be explained by effects on body weight or food intake. In prediabetic rats, cheese may exert its benefits through phosphatidylcholine synthesis. Of concern is the finding that cheese feeding resulted the accumulation of triglycerides or cholesterol ester in the liver. Paradoxically, animals who exhibited increased triglyceride or cholesterol ester accumulation demonstrated improved insulin sensitivity during an ITT. Future studies should focus on this finding, exploring whether cheese diet feeding can harm the liver when continued for extended periods of time, and whether skeletal muscle lipid accumulation is similarly affected. This study is one of the first of its kind, comparing and contrasting LF and HF cheeses, as well as overall cheese consumption in an experimental design. Further studies comparing the effects of high fat dairy products with their low fat versions are desperately needed to further inform nutrition policy related to dairy consumption.

Sources Cited

1. Martha, H. & Stipanuk, W. B. Biochemical and Physiological Aspects of Human Nutrition. *Saunders Co.* 45–62 (2000).
2. Daneman, D. Type 1 diabetes. *Lancet* **367,** 847–858 (2011).
3. Colagiuri, R., Brown, J. & Dain, K. *Global Diabetes Plan 2011-2021*. (International Diabetes Federation, 2011).
4. Hramiak, I., Leiter, L. A., Paul, T. L. & Ur, E. Assessment of obesity and its complications in adults. *Can. Med. Assoc. J.* **178,** 36–39 (2007).
5. Pelletier, C. *et al. Diabetes in Canada: Facts and figures from a public health perspective*. *Chronic Diseases and Injuries in Canada* **33,** (2012).
6. Hu, F. B. *et al.* Diet, lifestyle, and the risk of type 2 diabetes mellitus in women. *N. Engl. J. Med.* **345,** 790–797 (2001).
7. Groop, L. *et al.* Metabolic consequences of a family history of NIDDM (the Botnia study): evidence for sex-specific parental effects. *Diabetes* **45,** 1585–1593 (1996).
8. Cheng, A. Y. Y. & Lau, D. C. W. The Canadian Diabetes Association 2013 clinical practice guidelines—raising the bar and setting higher standards! *Can. J. Diabetes* **37,** 137–138 (2013).
9. Goldenberg, R. & Punthakee, Z. Definition, classification and diagnosis of diabetes, prediabetes and metabolic syndrome. *Can. J. Diabetes* **37, Supple,** S8–S11 (2013).
10. Nathan, D. M. *et al.* Translating the A1C assay into estimated average glucose values. *Diabetes Care* **31,** 1473–1478 (2008).

11. Ekoé, J.-M. & Punthakee, Z. Screening for type 1 and type 2 diabetes. *Can. J. Diabetes* **37, Supple,** S295–S297 (2013).

12. Pelletier, C., Dai, S., Roberts, K. C. & Bienek, A. Report summary Diabetes in Canada: facts and figures from a public health perspective. *Chronic Dis. Inj. Can.* **33,** (2012).

13. Young, T. K., Reading, J. & Elias, B. Type 2 diabetes mellitus in Canada's First Nations: status of an epidemic in progress. *Can. Med. Assoc. J.* **163,** 561–566 (2000).

14. FNIGC. *FIRST NATIONS REGIONAL HEALTH SURVEY (RHS) 2008 / 10 National Report on Adults, Youth and Children living in First Nations Communities*. (2008).

15. Whiting, D. R., Guariguata, L., Weil, C. & Shaw, J. IDF diabetes atlas: global estimates of the prevalence of diabetes for 2011 and 2030. *Diabetes Res. Clin. Pract.* **94,** 311–321 (2011).

16. Doucet, G. & Beatty, M. The cost of diabetes in Canada: the economic tsunami. *Can. J. Diabetes* **34,** 27–29 (2010).

17. Alberti, K. G. M. M., Zimmet, P. & Shaw, J. International Diabetes Federation: a consensus on Type 2 diabetes prevention. *Diabet. Med.* **24,** 451–463 (2007).

18. Stratton, I. M. *et al.* Association of glycaemia with macrovascular and microvascular complications of type 2 diabetes (UKPDS 35): prospective observational study. *BMJ* **321,** 405–412 (2000).

19. Saltiel, A. R. & Kahn, C. R. Insulin signalling and the regulation of glucose and lipid metabolism. *Nature* **414,** 799–806 (2001).

20. Mooradian, A. D. Dyslipidemia in type 2 diabetes mellitus. *Nat. Clin. Pract. Endocrinol. Metab.* **5,** 150–159 (2009).

21. Genest, J. *et al.* 2009 Canadian Cardiovascular Society/Canadian guidelines for the diagnosis and treatment of dyslipidemia and prevention of cardiovascular disease in the adult–2009 recommendations. *Can. J. Cardiol.* **25,** 567–579 (2009).

22. Björnholm, M. & Zierath, J. R. Insulin signal transduction in human skeletal muscle: identifying the defects in Type II diabetes. (2005).

23. Simpson, F., Whitehead, J. P. & James, D. E. GLUT4–at the cross roads between membrane trafficking and signal transduction. *Traffic* **2,** 2–11 (2001).

24. Drouin-Chartier, J.-P. *et al.* Systematic review of the association between dairy product consumption and risk of cardiovascular-related clinical outcomes. *Adv. Nutr. An Int. Rev. J.* **7,** 1026–1040 (2016).

25. Drouin-Chartier, J.-P. *et al.* Comprehensive review of the impact of dairy foods and dairy fat on cardiometabolic risk. *Adv. Nutr. An Int. Rev. J.* **7,** 1041–1051 (2016).

26. Alexander, D. D. *et al.* Dairy consumption and CVD: a systematic review and meta-analysis. *Br. J. Nutr.* **115,** 737–750 (2016).

27. O'Sullivan, T. A., Hafekost, K., Mitrou, F. & Lawrence, D. Food sources of saturated fat and the association with mortality: a meta-analysis. *Am. J. Public Health* **103,** e31–e42 (2013).

28. Qin, L.-Q. *et al.* Dairy consumption and risk of cardiovascular disease: an updated meta-analysis of prospective cohort studies. *Asia Pac. J. Clin. Nutr.* **24,** 90–100 (2015).

29. Elwood, P. C., Pickering, J. E., Givens, D. I. & Gallacher, J. E. The consumption of milk and dairy foods and the incidence of vascular disease and diabetes: an overview of the evidence. *Lipids* **45,** 925–939 (2010).

30. Hu, D., Huang, J., Wang, Y., Zhang, D. & Qu, Y. Dairy foods and risk of stroke: a meta-analysis of prospective cohort studies. *Nutr. Metab. Cardiovasc. Dis.* **24,** 460–469 (2014).

31. Soedamah-Muthu, S. S., Verberne, L. D. M., Ding, E. L., Engberink, M. F. & Geleijnse, J. M. Dairy consumption and incidence of hypertension. *Hypertension* HYPERTENSIONAHA-112 (2012).

32. Ralston, R. A., Lee, J. H., Truby, H., Palermo, C. E. & Walker, K. Z. A systematic review and meta-analysis of elevated blood pressure and consumption of dairy foods. *J. Hum. Hypertens.* **26,** 3–13 (2012).

33. Pereira, M. A. *et al.* Dairy consumption, obesity, and the insulin resistance syndrome in young adults: the CARDIA Study. *JAMA* **287,** 2081–2089 (2002).

34. Fumeron, F. *et al.* Dairy consumption and the incidence of hyperglycemia and the metabolic syndrome. *Diabetes Care* **34,** 813–817 (2011).

35. Samara, A. *et al.* Dairy product consumption, calcium intakes, and metabolic syndrome-related factors over 5 years in the STANISLAS study. *Nutrition* **29,** (2013).

36. Wang, H. *et al.* Longitudinal association between dairy consumption and changes of body weight and waist circumference: the Framingham Heart Study. *Int. J. Obes. (Lond).* **38,** (2014).

37. Livingstone, K. M. *et al.* Does dairy food intake predict arterial stiffness and blood pressure in men? *Hypertension* HYPERTENSIONAHA-111 (2012).

38. Thorning, T. K. *et al.* Diets with high-fat cheese, high-fat meat, or carbohydrate on cardiovascular risk markers in overweight postmenopausal women: a randomized crossover trial. *Am. J. Clin. Nutr.* **102,** 573–581 (2015).

39. de Goede, J., Geleijnse, J. M., Ding, E. L. & Soedamah-Muthu, S. S. Effect of cheese consumption on blood lipids: a systematic review and meta-analysis of randomized controlled trials. *Nutr. Rev.* **73,** 259–275 (2015).

40. Nestel, P. J., Chronopulos, A. & Cehun, M. Dairy fat in cheese raises LDL cholesterol less than that in butter in mildly hypercholesterolaemic subjects. *Eur. J. Clin. Nutr.* **59,** 1059–1063 (2005).

41. Samuel, V. T., Petersen, K. F. & Shulman, G. I. Lipid-induced insulin resistance: unravelling the mechanism. *Lancet* **375,** 2267–2277 (2010).

42. Schwarz, J.-M., Linfoot, P., Dare, D. & Aghajanian, K. Hepatic de novo lipogenesis in normoinsulinemic and hyperinsulinemic subjects consuming high-fat, low-carbohydrate and low-fat, high-carbohydrate isoenergetic diets. *Am. J. Clin. Nutr.* **77,** 43–50 (2003).

43. Brown, M. S. & Goldstein, J. L. Selective versus total insulin resistance: a pathogenic paradox. *Cell Metab.* **7,** 95–96 (2008).

44. Utzschneider, K. M. & Kahn, S. E. The role of insulin resistance in nonalcoholic fatty liver disease. *J. Clin. Endocrinol. Metab.* **91,** 4753–4761 (2006).

45. Kim, J. K. *et al.* Redistribution of substrates to adipose tissue promotes obesity in

mice with selective insulin resistance in muscle. *J. Clin. Invest.* **105,** 1791–1797 (2000).

46. Petersen, K. F., Dufour, S., Befroy, D., Garcia, R. & Shulman, G. I. Impaired mitochondrial activity in the insulin-resistant offspring of patients with type 2 diabetes. *N. Engl. J. Med.* **350,** 664–671 (2004).

47. Chitturi, S. *et al.* NASH and insulin resistance: insulin hypersecretion and specific association with the insulin resistance syndrome. *Hepatology* **35,** 373–379 (2002).

48. Chalasani, N., Deeg, M. A., Persohn, S. & Crabb, D. W. Metabolic and anthropometric evaluation of insulin resistance in nondiabetic patients with nonalcoholic steatohepatitis. *Am. J. Gastroenterol.* **98,** 1849–1855 (2003).

49. Musso, G. *et al.* Dietary habits and their relations to insulin resistance and postprandial lipemia in nonalcoholic steatohepatitis. *Hepatology* **37,** 909–916 (2003).

50. Cassader, M. *et al.* Postprandial triglyceride-rich lipoprotein metabolism and insulin sensitivity in nonalcoholic steatohepatitis patients. *Lipids* **36,** 1117–1124 (2001).

51. Tiikkainen, M. *et al.* Liver-fat accumulation and insulin resistance in obese women with previous gestational diabetes. *Obesity* **10,** 859–867 (2002).

52. Sanyal, A. J. *et al.* Nonalcoholic steatohepatitis: association of insulin resistance and mitochondrial abnormalities. *Gastroenterology* **120,** 1183–1192 (2001).

53. Bays, H., Mandarino, L. & Defronzo, R. A. Role of the adipocyte, free fatty acids, and ectopic fat in pathogenesis of type 2 diabetes mellitus: peroxisomal proliferator-activated receptor agonists provide a rational therapeutic approach. *J. Clin.*

Endocrinol. Metab. **89,** 463–478 (2004).

54. Klein, S. *et al.* Absence of an effect of liposuction on insulin action and risk factors for coronary heart disease. *N Engl J Med* **2004,** 2549–2557 (2004).

55. Tamura, Y. *et al.* Effects of diet and exercise on muscle and liver intracellular lipid contents and insulin sensitivity in type 2 diabetic patients. *J. Clin. Endocrinol. Metab.* **90,** 3191–3196 (2005).

56. Petersen, K. F. *et al.* Reversal of nonalcoholic hepatic steatosis, hepatic insulin resistance, and hyperglycemia by moderate weight reduction in patients with type 2 diabetes. *Diabetes* **54,** 603–608 (2005).

57. Dworatzek, P. D. *et al.* Nutrition therapy. *Can. J. diabetes* **37,** S45–S55 (2013).

58. Association, A. D. Executive summary: standards of medical care in diabetes—2014. (2014).

59. Tabák, A. G., Herder, C., Rathmann, W., Brunner, E. J. & Kivimäki, M. Prediabetes: a high-risk state for diabetes development. *Lancet* **379,** 2279–2290 (2012).

60. de Vegt, F. *et al.* Relation of impaired fasting and postload glucose with incident type 2 diabetes in a Dutch population: The Hoorn Study. *JAMA* **285,** 2109–2113 (2001).

61. Pan, X.-R. *et al.* Effects of diet and exercise in preventing NIDDM in people with impaired glucose tolerance: the Da Qing IGT and Diabetes Study. *Diabetes Care* **20,** 537–544 (1997).

62. Group, D. P. P. (DPP) R. The diabetes prevention program (DPP). *Diabetes Care* **25,** 2165–2171 (2002).

63. McInnes, N. *et al.* Piloting a remission strategy in type 2 diabetes: Results of a randomized controlled trial. *J. Clin. Endocrinol. Metab.* (2017).

64. Gregg, E. W. *et al.* Association of an intensive lifestyle intervention with remission of type 2 diabetes. *JAMA* **308,** 2489–2496 (2012).

65. Mottalib, A., Sakr, M., Shehabeldin, M. & Hamdy, O. Diabetes remission after nonsurgical intensive lifestyle intervention in obese patients with type 2 diabetes. *J. Diabetes Res.* **2015,** (2015).

66. Ades, P. A., Savage, P. D., Marney, A. M., Harvey, J. & Evans, K. A. Remission of recently diagnosed type 2 diabetes mellitus with weight loss and exercise. *J. Cardiopulm. Rehabil. Prev.* **35,** 193 (2015).

67. Esposito, K., Maiorino, M. I., Petrizzo, M., Bellastella, G. & Giugliano, D. The effects of a Mediterranean diet on the need for diabetes drugs and remission of newly diagnosed type 2 diabetes: follow-up of a randomized trial. *Diabetes Care* **37,** 1824–1830 (2014).

68. Steven, S. *et al.* Very low-calorie diet and 6 months of weight stability in type 2 diabetes: pathophysiological changes in responders and nonresponders. *Diabetes Care* **39,** 808–815 (2016).

69. Health Canada, H. P. and F. B. Eating well with Canada's food guide. HC Pub.: 4651 (2011). Available at: http://www.hc-sc.gc.ca/fn-an/food-guide-aliment/order-commander/eating_well_bien_manger-eng.php. (Accessed: 20th February 2017)

70. Government of Alberta, A. H. S. Alberta Nutrition Guidelines for Adults. (2012). Available at: http://www.health.alberta.ca/documents/Nutrition-Guidelines-AB-

Adults.pdf. (Accessed: 20th February 2017)

71. Parodi, P. W. Dietary guidelines for saturated fatty acids are not supported by the evidence. *Int. Dairy J.* **52,** 115–123 (2016).

72. Lamarche, B. & Couture, P. It is time to revisit current dietary recommendations for saturated fat. *Appl. Physiol. Nutr. Metab.* **39,** 1409–1411 (2014).

73. Mozaffarian, D. & Ludwig, D. S. The 2015 US dietary guidelines: lifting the ban on total dietary fat. *JAMA* **313,** 2421–2422 (2015).

74. Carnethon, M. R. *et al.* Association of weight status with mortality in adults with incident diabetes. *JAMA* **308,** 581–590 (2012).

75. Grundy, S. M. *et al.* Diagnosis and management of the metabolic syndrome: An American Heart Association/National Heart, Lung, and Blood Institute scientific statement. *Curr. Opin. Cardiol.* **21,** (2006).

76. Ericson, U. *et al.* Food sources of fat may clarify the inconsistent role of dietary fat intake for incidence of type 2 diabetes. *Am. J. Clin. Nutr.* ajcn103010 (2015).

77. Oh, K., Hu, F. B., Manson, J. E., Stampfer, M. J. & Willett, W. C. Dietary fat intake and risk of coronary heart disease in women: 20 years of follow-up of the Nurses' Health Study. *Am. J. Epidemiol.* **161,** 672–679 (2005).

78. Kratz, M., Baars, T. & Guyenet, S. The relationship between high-fat dairy consumption and obesity, cardiovascular, and metabolic disease. *Eur. J. Nutr.* **52,** 1–24 (2013).

79. Risérus, U., Arner, P., Brismar, K. & Vessby, B. Treatment with dietary trans10cis12 conjugated linoleic acid causes isomer-specific insulin resistance in obese men with

the metabolic syndrome. *Diabetes Care* **25,** 1516–1521 (2002).

80. Niu, K. *et al.* Low-fat dairy, but not whole-/high-fat dairy, consumption is related with higher serum adiponectin levels in apparently healthy adults. *Eur. J. Nutr.* **52,** 771–778 (2013).

81. Sluijs, I. *et al.* The amount and type of dairy product intake and incident type 2 diabetes: results from the EPIC-InterAct Study. *Am. J. Clin. Nutr.* **96,** 382–390 (2012).

82. Yakoob, M. Y. *et al.* Circulating Biomarkers of Dairy Fat and Risk of Incident Diabetes Mellitus Among US Men and Women in Two Large Prospective Cohorts. *Circulation* (2016). doi:10.1161/CIRCULATIONAHA.115.018410

83. Grantham, N. M. *et al.* The association between dairy food intake and the incidence of diabetes in Australia: the Australian Diabetes Obesity and Lifestyle Study (AusDiab). *Public Health Nutr.* **16,** 339–345 (2013).

84. Choi, H. K., Willett, W. C., Stampfer, M. J., Rimm, E. & Hu, F. B. Dairy consumption and risk of type 2 diabetes mellitus in men: a prospective study. *Arch. Intern. Med.* **165,** 997–1003 (2005).

85. Díaz-López, A. *et al.* Dairy product consumption and risk of type 2 diabetes in an elderly Spanish Mediterranean population at high cardiovascular risk. *Eur. J. Nutr.* **55,** (2016).

86. Eussen, S. J. P. M. *et al.* Consumption of dairy foods in relation to impaired glucose metabolism and type 2 diabetes mellitus: the Maastricht Study. *Br. J. Nutr.* (2016). doi:10.1017/S0007114516000313

87. Kirii, K. *et al.* Calcium, vitamin D and dairy intake in relation to type 2 diabetes risk in a Japanese cohort. *Diabetologia* **52,** 2542–2550 (2009).

88. Liu, S. *et al.* A prospective study of dairy intake and the risk of type 2 diabetes in women. *Diabetes Care* **29,** 1579–1584 (2006).

89. O'Connor, L. M. *et al.* Dietary dairy product intake and incident type 2 diabetes: a prospective study using dietary data from a 7-day food diary. *Diabetologia* **57,** 909–917 (2014).

90. Struijk, E. A. *et al.* Dairy product intake in relation to glucose regulation indices and risk of type 2 diabetes. *Nutr. Metab. Cardiovasc. Dis.* **23,** 822–828 (2013).

91. Chen, M. *et al.* Dairy consumption and risk of type 2 diabetes: 3 cohorts of US adults and an updated meta-analysis. *BMC Med.* **12,** (2014).

92. Aune, D., Norat, T., Romundstad, P. & Vatten, L. J. Dairy products and the risk of type 2 diabetes: a systematic review and dose-response meta-analysis of cohort studies. *Am. J. Clin. Nutr.* ajcn-059030 (2013).

93. Schurgers, L. J. & Vermeer, C. Determination of phylloquinone and menaquinones in food. *Pathophysiol. Haemost. Thromb.* **30,** 298–307 (2001).

94. Beulens, J. W. J., Grobbee, D. E., Sluijs, I., Spijkerman, A. M. W. & Van Der Schouw, Y. T. Dietary phylloquinone and menaquinones intakes and risk of type 2 diabetes. *Diabetes Care* **33,** 1699–1705 (2010).

95. Geurts, L., Everard, A., Le Ruyet, P., Delzenne, N. M. & Cani, P. D. Ripened dairy products differentially affect hepatic lipid content and adipose tissue oxidative stress markers in obese and type 2 diabetic mice. *J. Agric. Food Chem.* **60,** 2063–

2068 (2012).

96. Raziani, F. *et al.* High intake of regular-fat cheese compared with reduced-fat cheese does not affect LDL cholesterol or risk markers of the metabolic syndrome: a randomized controlled trial. *Am. J. Clin. Nutr.* **104,** 973–981 (2016).

97. Belin, R. J. & He, K. Magnesium physiology and pathogenic mechanisms that contribute to the development of the metabolic syndrome. *Magnes. Res.* **20,** 107–129 (2007).

98. USDA. USDA Food Composition Databases. *United States Department of Agriculture Agricultural Research Service* (2017).

99. Kirii, K., Iso, H., Date, C., Fukui, M. & Tamakoshi, A. Magnesium intake and risk of self-reported type 2 diabetes among Japanese. *J. Am. Coll. Nutr.* **29,** 99–106 (2010).

100. Lopez-Ridaura, R. *et al.* Magnesium intake and risk of type 2 diabetes in men and women. *Diabetes Care* **27,** 134–140 (2004).

101. Dong, J.-Y., Xun, P., He, K. & Qin, L.-Q. Magnesium intake and risk of type 2 diabetes meta-analysis of prospective cohort studies. *Diabetes Care* **34,** 2116–2122 (2011).

102. Zemel, M. B. Proposed role of calcium and dairy food components in weight management and metabolic health. *Phys. Sports Med.* **37,** 29–39 (2009).

103. Papakonstantinou, E., Flatt, W. P., Huth, P. J. & Harris, R. High dietary calcium reduces body fat content, digestibility of fat, and serum vitamin D in rats. *Obesity* **11,** 387–394 (2003).

104. Jacobsen, R., Lorenzen, J. K., Toubro, S., Krog-Mikkelsen, I. & Astrup, A. Effect of short-term high dietary calcium intake on 24-h energy expenditure, fat oxidation, and fecal fat excretion. *Int. J. Obes.* **29,** 292–301 (2005).

105. Khan, H., Kunutsor, S., Franco, O. H. & Chowdhury, R. Vitamin D, type 2 diabetes and other metabolic outcomes: a systematic review and meta-analysis of prospective studies. *Proc. Nutr. Soc.* **72,** 89–97 (2013).

106. Song, Y. *et al.* Blood 25-hydroxy vitamin D levels and incident type 2 diabetes a meta-analysis of prospective studies. *Diabetes Care* **36,** 1422–1428 (2013).

107. USDA National Agriculture Library. *United States Department of Agriculture* (2017). Available at: https://agclass.nal.usda.gov/mtwdk.exe?k=default&l=60&n=1&s=5&t=2&w=17240 .

108. Sommerfeld, M. Trans unsaturated fatty acids in natural products and processed foods. *Prog. Lipid Res.* **22,** 221–233 (1983).

109. Mushtaq, S., Mangiapane, E. H. & Hunter, K. A. Estimation of cis-9, trans-11 conjugated linoleic acid content in UK foods and assessment of dietary intake in a cohort of healthy adults. *Br. J. Nutr.* **103,** 1366–1374 (2010).

110. Azadbakht, L., Mirmiran, P., Esmaillzadeh, A. & Azizi, F. Dairy consumption is inversely associated with the prevalence of the metabolic syndrome in Tehranian adults. *Am. J. Clin. Nutr.* **82,** 523–530 (2005).

111. Wang, X. *et al.* Trans-11 vaccenic acid improves insulin secretion in models of type 2 diabetes in vivo and in vitro. *Mol. Nutr. Food Res.* (2016).

112. Lewis, K. *et al.* Enhanced translocation of bacteria across metabolically stressed epithelia is reduced by butyrate. *Inflamm. Bowel Dis.* **16,** 1138–1148 (2010).

113. Gao, Z. *et al.* Butyrate improves insulin sensitivity and increases energy expenditure in mice. *Diabetes* **58,** 1509–1517 (2009).

114. Mozaffarian, D. *et al.* Trans-palmitoleic acid, metabolic risk factors, and new-onset diabetes in US adults: a cohort study. *Ann. Intern. Med.* **153,** 790–799 (2010).

115. Dimopoulos, N., Watson, M., Sakamoto, K. & Hundal, H. S. Differential effects of palmitate and palmitoleate on insulin action and glucose utilization in rat L6 skeletal muscle cells. *Biochem. J.* **399,** 473–481 (2006).

116. Gloerich, J. *et al.* Metabolism of phytol to phytanic acid in the mouse, and the role of PPARα in its regulation. *J. Lipid Res.* **48,** 77–85 (2007).

117. Hellgren, L. I. Phytanic acid—an overlooked bioactive fatty acid in dairy fat? *Ann. N. Y. Acad. Sci.* **1190,** 42–49 (2010).

118. Heim, M. *et al.* Phytanic acid, a natural peroxisome proliferator-activated receptor (PPAR) agonist, regulates glucose metabolism in rat primary hepatocytes. *FASEB J.* **16,** 718–720 (2002).

119. Whitlock, K. A. *et al.* Assessment of the mechanisms exerting glucose-lowering effects of dried peas in glucose-intolerant rats. *Br. J. Nutr.* **108,** S91–S102 (2012).

120. Szkudelski, T. The mechanism of alloxan and streptozotocin action in B cells of the rat pancreas. *Physiol. Res.* **50,** 537–546 (2001).

121. Di Gialleonardo, V. *et al.* Imaging of β-cell mass and insulitis in insulin-dependent (type 1) diabetes mellitus. *Endocr. Rev.* **33,** 892–919 (2012).

122. Rahier, J., Guiot, Y., Goebbels, R. M., Sempoux, C. & Henquin, J.-C. Pancreatic β-cell mass in European subjects with type 2 diabetes. *Diabetes, Obes. Metab.* **10,** 32–42 (2008).

123. Fernández-Millán, E. *et al.* Cocoa-rich diet attenuates beta cell mass loss and function in young Zucker diabetic fatty rats by preventing oxidative stress and beta cell apoptosis. *Mol. Nutr. Food Res.* **59,** 820–824 (2015).

124. Prentki, M. & Nolan, C. J. Islet β cell failure in type 2 diabetes. *J. Clin. Invest.* **116,** 1802–1812 (2006).

125. Srinivasan, K., Viswanad, B., Asrat, L., Kaul, C. L. & Ramarao, P. Combination of high-fat diet-fed and low-dose streptozotocin-treated rat: a model for type 2 diabetes and pharmacological screening. *Pharmacol. Res.* **52,** 313–320 (2005).

126. Zhang, M., Lv, X.-Y., Li, J., Xu, Z.-G. & Chen, L. The characterization of high-fat diet and multiple low-dose streptozotocin induced type 2 diabetes rat model. *Exp. Diabetes Res.* **2008,** (2009).

127. Xia, J. & Wishart, D. S. Using MetaboAnalyst 3.0 for Comprehensive Metabolomics Data Analysis. *Curr. Protoc. Bioinforma.* 10–14 (2016).

128. Dettmer, K., Aronov, P. A. & Hammock, B. D. Mass spectrometry-based metabolomics. *Mass Spectrom. Rev.* **26,** 51–78 (2007).

129. D'Agostino, R. B. Tests for the normal distribution. *Goodness-of-fit Tech.* **68,** 576 (1986).

130. Sutherland, L. N., Capozzi, L. C., Turchinsky, N. J., Bell, R. C. & Wright, D. C. Time course of high-fat diet-induced reductions in adipose tissue mitochondrial proteins:

potential mechanisms and the relationship to glucose intolerance. *Am. J. Physiol. Metab.* **295,** E1076–E1083 (2008).

131. Exton, J. H. Signaling through phosphatidylcholine breakdown. *J. Biol. Chem.* **265,** 1–4 (1990).

132. Gibellini, F. & Smith, T. K. The Kennedy pathway—de novo synthesis of phosphatidylethanolamine and phosphatidylcholine. *IUBMB Life* **62,** 414–428 (2010).

133. Jacobs, R. L., Lingrell, S., Dyck, J. R. B. & Vance, D. E. Inhibition of hepatic phosphatidylcholine synthesis by 5-aminoimidazole-4-carboxamide-1-β-4-ribofuranoside is independent of AMP-activated protein kinase activation. *J. Biol. Chem.* **282,** 4516–4523 (2007).

134. Sundler, R. & Akesson, B. Regulation of phospholipid biosynthesis in isolated rat hepatocytes. Effect of different substrates. *J. Biol. Chem.* **250,** 3359–3367 (1975).

135. Li, Z. & Vance, D. E. Thematic review series: glycerolipids. Phosphatidylcholine and choline homeostasis. *J. Lipid Res.* **49,** 1187–1194 (2008).

136. Akinmokun, A., Selby, P. L., Ramaiya, K. & Alberti, K. The short insulin tolerance test for determination of insulin sensitivity: a comparison with the euglycaemic clamp. *Diabet. Med.* **9,** 432–437 (1992).

137. Tholstrup, T., Høy, C.-E., Andersen, L. N., Christensen, R. D. K. & Sandström, B. Does fat in milk, butter and cheese affect blood lipids and cholesterol differently? *J. Am. Coll. Nutr.* **23,** 169–176 (2004).

138. Soerensen, K. V, Thorning, T. K., Astrup, A., Kristensen, M. & Lorenzen, J. K. Effect

of dairy calcium from cheese and milk on fecal fat excretion, blood lipids, and appetite in young men. *Am. J. Clin. Nutr.* **99,** 984–991 (2014).

139. Hjerpsted, J., Leedo, E. & Tholstrup, T. Cheese intake in large amounts lowers LDL-cholesterol concentrations compared with butter intake of equal fat content. *Am. J. Clin. Nutr.* **94,** 1479–1484 (2011).
140. Claudino, W. M. *et al.* Metabolomics: available results, current research projects in breast cancer, and future applications. *J. Clin. Oncol.* **25,** 2840–2846 (2007).
141. Richard, C. *et al.* Measurement of the total choline content in 48 commercial dairy products or dairy alternatives. *J. Food Compos. Anal.* **45,** 1–8 (2016).
142. Velagapudi, V. R. *et al.* The gut microbiota modulates host energy and lipid metabolism in mice. *J. Lipid Res.* **51,** 1101–1112 (2010).
143. David, L. A. *et al.* Diet rapidly and reproducibly alters the human gut microbiome. *Nature* **505,** 559–563 (2014).
144. Cotillard, A. *et al.* Dietary intervention impact on gut microbial gene richness. *Nature* **500,** 585–588 (2013).
145. Canada, H. Canadian Nutrient File. *Government of Canada* (2016). Available at: https://food-nutrition.canada.ca/cnf-fce/serving-portion.do?id=119. (Accessed: 12th May 2017)
146. Rinella, M. E. *et al.* Mechanisms of hepatic steatosis in mice fed a lipogenic methionine choline-deficient diet. *J. Lipid Res.* **49,** 1068–1076 (2008).
147. Browning, J. D. & Horton, J. D. Molecular mediators of hepatic steatosis and liver injury. *J. Clin. Invest.* **114,** 147 (2004).

148. Orentreich, N., Matias, J. R., DeFelice, A. & Zimmerman, J. A. Low methionine ingestion by rats extends life span. *J. Nutr.* **123,** 269 (1993).