THE EFFECT OF SOY PROTEIN ISOLATE ON SERUM LIPIDS
IN ADULTS WITH TYPE 2 DIABETES

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of
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by
ELIZABETH ANNE PHILLIPS

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ABSTRACT

THE EFFECT OF SOY PROTEIN ISOLATE ON SERUM LIPIDS IN ADULTS WITH TYPE 2 DIABETES

Elizabeth Anne Phillips
University of Guelph 2007

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Alison M. Duncan, Ph.D., R.D.

Type 2 diabetes increases CVD risk, however serum lipids in response to dietary change have not been fully investigated in adults with type 2 diabetes. This study determined the effect of soy protein isolate (SPI) on serum lipids in adults with type 2 diabetes. In a double-blind, randomized, crossover, placebo-controlled intervention study design, 29 adults with diet-controlled type 2 diabetes consumed SPI or milk protein isolate (MPI) for 57 days each separated by a 4-week washout. Fasted blood was collected on days 1 and 57 of each treatment and analyzed for serum lipids. SPI consumption significantly ($p \leq 0.05$) reduced LDL-C, LDL-C/HDL-C and apo B/apo A-I but did not significantly affect TC, HDL-C, TG, apo B or apo A-I, when compared with MPI consumption. These data demonstrate that consumption of soy protein can modulate serum lipids in a direction beneficial for CVD risk in adults with type 2 diabetes.

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LIST OF ABBREVIATIONS

AMORIS	Apolipoprotein Related Mortality Risk
ANCOVA	Analysis of Co-Variance
ANOVA	Analysis of Variance
Apo	Apolipoprotein
BA	Bile Acids
BIA	Bioelectrical Impedance Analysis
BMI	Body Mass Index
CVD	Cardiovascular Disease
CETP	Cholesterol Ester Transfer Protein
C	Cholesterol
Chylom	Chylomicron
CM	Chylomicron
CR	Chylomicron Remnant
CV	Coefficient of Variation
DEC	Diabetes Education Centre
Extrah	Extra-hepatic
FFA	Free Fatty Acid
FFQ	Food Frequency Questionnaire
GCP	Genistein Combined Polysaccharide
GI	Gastrointestinal Tract
HbA1c	Hemoglobin A1c

HDL	High Density Lipoprotein
HDL-C	High Density Lipoprotein Cholesterol
HI	High Isoflavone
HNRU	Human Nutraceutical Research Unit
IDL	Intermediate Density Lipoprotein
LCAT	Lecithin-Cholesterol Acyl Transferase
LDL	Low Density Lipoprotein
LDL-C	Low Density Lipoprotein Cholesterol
LDLR	Low Density Lipoprotein Receptors
LI	Low Isoflavone
LPL	Lipoprotein Lipase
LRC-CPPT	Lipid Research Clinics Coronary Prevention Trial
MONICA/KORA	Multinational Monitoring of trends and determinants in Cardiovascular disease/Kooperative Gesundheitsforschung in der Region Augsburg
MPI	Milk Protein Isolate
MRFIT	Multiple Risk Factor Intervention Trial
MTP	Microsomal Transfer Protein
MUFA	Monounsaturated Fatty Acids
ODMA	O-desmethylangolensin
OGTT	Oral Glucose Tolerance Test
PPAR	Perioxisome-Proliferator Activated Receptors
PROCAM	Prospective Cardiovascular Münster Study
PUFA	Polyunsaturated Fatty Acids

SAS	Statistical Analysis System
SFA	Saturated Fatty Acids
SMR	Soy Protein Isolate Meal Replacement
SPI	Soy Protein Isolate
SR-BI	Scavenger Receptor, class B, type I
STZ	Streptozotocin
TBARS	Thiobarbituric Acid-Reactive Substances
TC	Total Cholesterol
TG	Triacylglycerides/ Triglycerides
VLDL	Very Low Density Lipoprotein

A. BACKGROUND

1. Type 2 Diabetes

1.a. Definition and Incidence

Type 2 diabetes mellitus is a metabolic disease caused by a deficiency in the production of insulin and/or by a resistance to insulin produced (1, 2). Previously referred to as adult onset diabetes, the prevalence of this disease has been on the rise for decades and the rate of diagnosis continues to be alarming. Approximately 90% of diabetes cases are type 2 diabetes (2). Worldwide, it is estimated that 30 million people had diabetes in 1985 and the numbers rose to 150 million in 2000 (3). In 2006, the World Health Organization projected that this number will rise to 333 million by the year 2030 (2). In 2006, roughly 1.8 million people were diagnosed with type 2 diabetes in Canada and 41500 Canadians die each year due to diabetes complications (1). The cost of diabetes to the Canadian health care system is estimated to be \$9 billion dollars annually and continues to rise (4).

1.b. Risk Factors

The risk factors for type 2 diabetes can be either non-modifiable or modifiable. Non-modifiable risk factors are those for which the individual has no control over and include: family history of diabetes, previous incidence of gestational diabetes and/or given birth to a baby greater than 4 kg, age over 40 years and part of a group considered at higher risk for type 2 diabetes (e.g. Aboriginal, Hispanic, Asian, south Asian or African Decent) (5). There are also a host of related conditions that increase risk of developing type 2 diabetes such as impaired glucose tolerance, polycystic ovary syndrome, acanthosis nigricans (darkened patches of skin) which are associated with

insulin resistance (5). Schizophrenia is another condition that increases the risk of type 2 diabetes as the medications for this condition can promote obesity and insulin resistance (6). The Canadian Diabetes Association (CDA) broadens the category of people at risk for type 2 diabetes by stating that anyone over the age of 40 has a risk of developing type 2 diabetes and should be tested for the disease at least every three years (1). The CDA also states that those with greater risk of developing the disease should be tested earlier and/or more frequently (7).

Modifiable risk factors for type 2 diabetes are those for which the individual is able to control and include things such as high blood pressure, high serum lipids and obesity (especially abdominal obesity) (8). Diet and physical activity are also important modifiable factors that can influence the risk of type 2 diabetes (1). For example, a diet low in saturated fat and refined carbohydrates, and high in fibre and unsaturated fats can reduce risk of type 2 diabetes (9). On the other hand, a diet high in saturated fats has been shown to reduce insulin sensitivity which can lead to impaired glucose metabolism and increased risk of type 2 diabetes (10, 11). Finally, increased physical activity, such as vigorous activity 5 times a week has been shown to improve insulin sensitivity which decreases insulin resistance and risk of type 2 diabetes (10).

1.c. Diagnosis

Type 2 diabetes can be diagnosed with a fasting plasma glucose level ≥ 7.0 mmol/L (1). A non-fasted casual blood sample (anytime of day) can also diagnose type 2 diabetes if plasma glucose is ≥ 11.1 mmol/L and accompanied with symptoms of type 2 diabetes (unusual thirst and frequent urination, blurred vision, weight change, extreme fatigue, numbness in hands or feet, frequent infections) (12). A third way to diagnose

type 2 diabetes is if plasma glucose is ≥ 1.1 mmol/L at any time point of an oral glucose tolerance test (OGTT) (12). An OGTT begins with a fasting blood sample followed by consumption of a 75 g glucose solution and subsequent blood samples over a two hour period (12).

1.d. Pathophysiology

The pathophysiology of type 2 diabetes relates to a breakdown in the control of blood glucose which is largely influenced by hormones. Insulin is an anabolic hormone that is produced by the beta cells of the islets of Langerhans in the pancreas and plays a key role in glycemic control and therefore the pathophysiology of type 2 diabetes (13). When blood glucose levels begin to rise, insulin is secreted into the bloodstream by these pancreatic cells. Insulin circulates in the blood throughout the body and signals to the adipose and muscle cells to take up glucose from the bloodstream (14). This helps decrease blood glucose levels. The signaling begins when insulin binds to, and activates, the tyrosine-specific protein kinase insulin receptor on the cell surface. The activated enzyme triggers autophosphorylation of tyrosine residues of the receptor on the cytosolic side (15). This action triggers a cascade of further phosphorylation leading to the increased transcription of glucose transporters. The glucose transporters are then translocated to the cell membrane surface to import glucose into the cells and out of the bloodstream (15) (Figure 1). If the production of insulin is impaired, the body will have an elevated blood glucose level, called hyperglycemia, for a longer duration than what normally occurs in the healthy individual (15).

Figure 1: Illustration of Glucose Uptake From the Signaling of Insulin Binding to its Receptor (16).

Insulin resistance occurs when cells do not respond correctly to insulin or become less sensitive to the signal of insulin (14). As a result, the body tries to overcome the lack of signaling by producing more insulin which contributes to becoming even more insulin resistant (14). This situation can cause both hyperglycemia and hyperinsulinemia (17). In extreme situations, the beta cells of the pancreas become exhausted and can no longer produce adequate insulin to help compensate for the insulin resistance and control glucose levels (Figure 2). At this point hyperglycemia and hypoinsulinemia can occur together.

Natural History of Type 2 Diabetes

Figure 2: A Comparison of Insulin Production, Blood Glucose Levels and Level of Insulin Resistance in Normal, Impaired Glucose Tolerance and Type 2 Diabetes States. Adapted from (17).

The symptoms of type 2 diabetes include unusual thirst, frequent urination, weight change (gain or loss), extreme lack of energy, blurred vision, frequent or recurring infections, cuts and bruises that are slow to heal and tingling or numbness in the hands or feet (1). It is possible for these symptoms to not manifest themselves until there is significant advancement of the disease (1).

Many secondary complications can arise due to the chronic hyperglycemia that can occur with type 2 diabetes. These complications include nephropathy which can severely compromise kidney function (13), retinopathy which can lead to blindness (1), neuropathy which can lead to circulation problems and risk of amputation (1), problems with erection (impotence) (1), and finally, cardiovascular disease (CVD) (1).

Although CVD may not immediately lead to an obvious acute physical health problem like the other secondary complications, it is one of the most significant chronic

complications of type 2 diabetes (18). Evidence of this is that CVD is the leading cause of death for people living with type 2 diabetes and 80% of those diagnosed with diabetes will die from CVD complications (1). Further evidence is that the risk of developing CVD is 4-6 times greater in adults with type 2 diabetes compared to adults without type 2 diabetes (18). Diabetic hyperglycemia can promote oxidative stress which can oxidize blood lipids and also damage the endothelial layer of the artery (19). In extreme cases, macrovascular problems such as heart disease and stroke are brought on by diabetes (20). These complications cause serious limitations to individuals, elevate health care costs and affect tens of thousands of people with diabetes each year (13). As such, reducing CVD risk is an important goal for creating prevention strategies for those living with type 2 diabetes.

2. Cardiovascular Disease (CVD)

2.a. Definition, Incidence and Pathophysiology

CVD refers to any disease or injury to the cardiovascular system, which includes the heart, the blood vessels of the heart, and the system of blood vessels (veins and arteries) throughout the body and within the brain (21, 22). Heart disease and stroke, two of the most common forms of CVD, are major causes of death, illness and disability (21, 22). In 1999, more than one third of deaths in Canada were attributed to CVD (23). The impact of this chronic disease ranges from individual limitations and discomfort to high personal, community and health care costs. A 1998 analysis of the economic burden of disease in Canada showed that CVD is costing the Canadian economy \$18 billion a year (24).

CVD develops over many years and most often manifests itself in the form of atherosclerosis (25, 26). Atherosclerosis involves the thickening and hardening of the arteries due to plaque formation in the intima, the inner most layer of the artery wall (27). The process of atherosclerosis is initiated following damage to the endothelial layer of the artery. Endothelial damage can cause numerous processes to occur including smooth muscle cell proliferation and migration, production of connective tissue, infiltration and migration of T-lymphocytes and macrophages (most of which develop into lipid containing foam cells) and blood coagulation (26, 28). Foam cell formation from macrophages occurs and a fatty streak begins to develop in the artery leading to plaque formation in the intima (29). As plaque accumulates it alters vascular tone and structure and can result in a narrowed vessel and decreased blood flow (30) (Figure 3). When plaque accumulates to the point that substantial obstruction of the artery occurs, it can cause damage the tissues to which the artery is supplying blood. Ultimately a heart attack or stroke can result if complete obstruction of the artery occurs (29).

Of most concern is when the plaque ruptures, possibly dislodging from the artery wall (26). If the ruptured plaque travels the circulatory system as an embolism and lodges, it can block blood flow and cause a myocardial infarction (blood flow blocked to the heart) or a stroke (blood flow blocked to the brain) (26). Both of these conditions can result in morbidity or mortality (30). Therefore, atherosclerotic plaque accumulation is a central problem of CVD and creates an increased risk of myocardial infarction and stroke.

Figure 3: Cross Section Illustration of Advanced Atherosclerosis (29).

2.b. Risk Factors

As with type 2 diabetes, there are both non-modifiable and modifiable risk factors for developing CVD (31). Non-modifiable risk factors include family history of CVD, ethnicity (African, South Asian, and First Nation populations are at higher risk), increasing age (women >55 years old and for men >45 years old are at higher risk) and gender, such that before menopause, women are at lower risk whereas after menopause, women experience a decrease in cardio-protective estrogen thereby equalizing the risk between men and women (32).

There are numerous modifiable risk factors for CVD (31), many of which are similar to the modifiable risk factors for type 2 diabetes (33). These factors include medical issues such as dyslipidemia, hypertension and diabetes, particularly diabetes with

poor glycemic control. These factors also include lifestyle issues such as low physical activity, smoking, excess alcohol, stress, body mass index (BMI) greater than 27 kg/m² (31) and abdominal or visceral obesity (34).

To emphasize the greater risk of CVD in those with diabetes compared to those without diabetes, the National Education Program-Adult Treatment Program III, from the United States, has stated that the risk of a myocardial infarction in patients with type 2 diabetes, with no previous cardiovascular problems, is equivalent to the risk of a myocardial infarction in patients without type 2 diabetes who already have CVD (35). This suggests the importance for prevention and screening for CVD early in individuals with type 2 diabetes. One of the most routine and accepted methods of screening for CVD risk involves testing for serum lipids.

3. Serum Lipids

As previously mentioned, dyslipidemia is among the modifiable risk factors for CVD, which is a major complication of type 2 diabetes. Imbalances in serum lipids can promote oxidative stress and lipid deposition on the artery wall (26). This can damage the endothelial layer of the artery and encourage plaque formation and atherosclerosis (26). There are several different kinds of serum lipids and keeping them at the appropriate levels and ratios is important for the management and assessment of CVD risk (26, 33, 36).

3.a. Structure and Function

Serum lipids are lipids found in the bloodstream and include triglycerides (TG), phospholipids, cholesterol and cholesterol esters and free fatty acids (15, 37). TG are made of a glycerol backbone with three fatty acids covalently bonded to the backbone.

TG are non-polar, water-insoluble substances that are used by the body as very efficient energy reserves. TG are the most abundant lipids consumed in the human diet and are the main storage form of fat in the body (15, 37).

Phospholipids are similar to TG except that one of the three fatty acid molecules is replaced by a phosphate group (15, 37). The phosphate group of the phospholipid makes the molecule polar with the phosphate group being hydrophilic and the fatty acids being hydrophobic. The phospholipid molecule is therefore said to be amphiphilic with one end of the molecule being water soluble and the other being water insoluble (15, 37).

Cholesterol is a four ringed steroidal structure and, like TG, is insoluble in water (15, 37). Cholesterol can be obtained from the diet or produced by the hepatocytes of the liver. Cholesterol has important roles in the structural stability and fluidity of cell membranes, and is also a precursor for the synthesis of vitamin D, steroid hormones, corticosteroids and bile acids (15, 37). In order for cholesterol to be transferred and stored in the body it must be converted to a cholesterol ester to be more hydrophilic. Cholesterol esters are formed by esterification of a fatty acid chain to the hydroxyl group of the cholesterol molecule, a reaction catalyzed by acyl-CoA-cholesterol acyl transferase (15, 37). The esterification occurs in the liver where the cholesterol esters can then be stored or transported throughout the body in lipoproteins (15, 37).

Free fatty acids are not abundant in the bloodstream since most fatty acids in the body, whether being stored or transported, are usually part of a TG molecule or a protein, such as albumin, in order to be more hydrophilic (15, 37). In the event that fatty acid stores in the adipose tissue need to be accessed for energy, the stored TG are acted upon by hormone sensitive lipase. This enzyme catalyzes the breakdown of TG into a glycerol

molecule and three free fatty acids. The free fatty acids are then transported across the adipose membrane and quickly become associated with an albumin protein to become more stable in the aqueous environment of the blood. The free fatty acid-albumin complex is transported to the liver where it can be metabolized for energy or repackaged into a lipoprotein for further transport (15, 37).

Overall, all serum lipids are relevant when discussing CVD risk, however serum TG and cholesterol are most identified as major risk factors for the disease (21, 35) and will therefore be emphasized in the following sections.

3.b. Transport

Since TG and cholesterol are required by all cells (15, 37), it is essential to have a transport mechanism for their distribution throughout the body. Further, lipids cannot be transported freely in the blood since they are not water soluble. The body overcomes this issue by surrounding the lipids with hydrophilic molecules to increase their water solubility. The TG and cholesterol are first surrounded with a phospholipid monolayer, arranged in such a way that the phospholipid fatty acid tails are towards the TG and cholesterol core and the phospholipid hydrophilic phosphate group are facing outward. This creates a spherical mass with a hydrophilic surface and hydrophobic interior. On the surface of the phospholipid monolayer, or integrated within it, are apolipoproteins (15, 37). Apolipoproteins are lipid binding proteins that bind the lipid complex with their hydrophobic amino acids facing inward and their hydrophilic amino acids facing outward (15, 37). These proteins increase the solubility of the complex in the blood. Apolipoprotein B (apo B) and apolipoprotein A-I (apo A-I) are of specific interest in CVD risk because their individual measurements can represent the proportion of

atherogenic (apo B) or athero-protective (apo A-I) lipoproteins in the blood (38). In its entirety, this complex of serum lipids surrounded by the phospholipid monolayer and apolipoproteins is called a lipoprotein (Figure 4). Serum lipids and apolipoproteins combine in different ways to form several classes of lipoproteins (15, 37). The major classes of human lipoproteins include chylomicrons, very-low-density lipoproteins (VLDL), low-density lipoproteins (LDL), and high-density lipoproteins (HDL) (15, 37) (Table 1).

3.c. Metabolism

The metabolism of serum lipids is related to the metabolism of their corresponding lipoproteins. Chylomicrons, VLDL, LDL and HDL have similarities in how they are metabolized however their differences lead to distinct outcomes of how and where their constituent lipids are deposited and metabolized.

General Structure of a Plasma Lipoprotein

Figure 4: Basic Structure and Composition of a Lipoprotein (39).

Table 1 : Summary of the Characteristics of the Different Classes of Lipoproteins (40-42).¹

LIPOPROTEINS						
	Diameter (nm)	Density (g/ml)	Protein %	TG %	Major Associated Apo²	Function
Chylomicron	90-1000	<0.95	2	86	Apo B-48 , Apo CII, Apo E	transports TG and cholesterol from intestine to other tissues
VLDL	30-90	0.95-1.006	8	55	Apo E , Apo B-100, Apo CII	transports TG from liver to tissues
IDL	25-35	1.006-1.019	15	31	Apo E , Apo B-100, Apo CII	converted from VLDL by lipase
LDL	20-25	1.019-1.063	22	6	Apo B100 , Apo CII, Apo E	converted from IDL by lipase- transports cholesterol to tissues and liver
HDL	5-25	1.063-1.210	40-55	4	Apo A-I , Apo CII, Apo E	scavenges cholesterol from extra-hepatic tissue for transport back to liver

¹Abbreviations used: VLDL=Very-low-density lipoprotein; IDL=intermediate-density lipoprotein; LDL=low-density lipoprotein; HDL=high-density lipoprotein; TG=triglyceride; Apo=apolipoprotein.

²Bolded Apos indicate the major apolipoprotein of the lipoprotein.

3.c.i. Chylomicrons

When dietary TG and cholesterol are consumed, they are emulsified and digested in the small intestine by bile acids and pancreatic lipase (15, 37). These processes result in the release of free fatty acids and cholesterol which can cross over the mucosal endothelial layer of the intestine. In the mucosal cell endoplasmic reticulum, the free fatty acids are assembled back into TG and with cholesterol are arranged into the TG-rich lipoproteins called chylomicrons (15, 37). Chylomicrons leave the small intestine cells through the lymphatic system and travel through the left subclavian vein to enter the

bloodstream and travel to the capillaries. In the capillaries of tissues, such as adipose, an extracellular enzyme lipoprotein lipase (LPL) is activated upon binding to the apo C-II of chylomicrons (15, 37). The enzyme is bound to heparin sulfate proteoglycans on the surface of endothelial cells and hydrolyzes most of the TG within the chylomicrons to free fatty acids (43). The liberated free fatty acids are now available to be absorbed by the tissues for energy or storage. The remaining cholesterol-enriched chylomicron remnant dissociates from the capillary endothelium and is circulated back to the liver where it undergoes further delipidation by hepatic lipase, an enzyme bound to the surface of sinusoidal capillaries anchored by heparin sulfate proteoglycans on hepatocytes (43). The remnant can also be taken up by receptor mediated endocytosis through the binding of apo E (44). In the liver, the cholesterol and remaining TG are released and the remnant is degraded in the lysosomes (15, 37). The unloaded TG can be stored in the liver or incorporated into lipoproteins such as VLDL.

3.c.ii. Very-Low-Density Lipoproteins

Excess dietary fatty acids and carbohydrates that are not needed immediately for energy are converted to TG in the liver and packaged into postprandial VLDL with many apolipoproteins including apo B-100, apo C-I, apo C-II, apo C-III and apo E (15, 37). In the fasted state, the liver can also convert fatty acid and glycogen stores into VLDL (15, 37), which are larger, more buoyant and referred to as VLDL-1 particles (45). In contrast, the postprandial VLDL particles are small and dense and referred to as VLDL-2 particles. In healthy humans, the amount of each VLDL species will depend on which energy state is occurring (45). The VLDL particles are mostly composed of TG and are exported out of the liver to travel to the capillaries (45). Like with chylomicrons, the

LPL enzyme is activated by the apo C-II of VLDL to release free fatty acids (45), which are in turn absorbed by tissues for immediate energy or for storage through re-synthesis to TG (15, 37). The removal of TG from VLDL converts this lipoprotein to a VLDL remnant, which is also referred to as an intermediate-density lipoprotein (IDL) (15, 37). The VLDL remnant can undergo a second hydrolytic process by hepatic lipase which is responsible for additional loss of TG and removal of all apolipoproteins except apo B-100, apo CII, and apo E, resulting in the conversion of IDL to LDL (45) (Figure 5).

Figure 5: The Metabolism of Chylomicrons to VLDL and LDL (44).¹

¹Abbreviations Used: VLDL=Very-low-density lipoprotein; IDL=Intermediate-density lipoprotein; LDL=Low-density lipoprotein; TG=Triglycerides; CM=Chylomicron; CR=Chylomicron remnant.

3.c.iii. Low-Density Lipoproteins

The LDL molecule is predominantly composed of cholesterol and its major apolipoprotein, specifically apo B-100 (15, 37). LDL molecules are recognized by LDL receptors which are present on cells that require cholesterol and bind to apo B-100 and apo E. It is through the LDL receptor that receptor mediated endocytosis occurs to bring the LDL molecule into the cell in an endosome. The endosome fuses with lysosome containing enzymes that hydrolyze the cholesterol esters (Figure 6). This releases cholesterol and free fatty acids to the cytosol of the cell. The apo B-100 is also degraded and the amino acids are released into the cytosol. LDL receptors that were also taken into the cell through the endocytosis process dissociate from the lysosome-endosome complex and return to the cell surface (15, 37). In clinical analysis, LDL-C is not directly measured in the blood but rather calculated according Friedewald equation (46) which, in mg/dl, is: $LDL-C = TC - (TG/5) - (HDL-C)$. For LDL-C units of mmol/L, the calculation is: $LDL-C = TC - (TG/2.22) - (HDL-C)$.

Figure 6: The Receptor Mediated Endocytosis and Degradation of LDL (47).¹

¹Abbreviations Used: LDL=Low-density lipoprotein; Apo=apolipoprotein.

3.c.iv. High-Density Lipoproteins

HDL is another major class of lipoproteins that is primarily composed of apolipoproteins thereby making it the densest of the lipoproteins (15, 37). HDL is assembled in the plasma from components obtained from other lipoproteins that have been degraded. The major apolipoproteins in HDL are apo A-I, apo C-I and apo C-II, with apo A-I being unique to HDL. Another major protein found in HDL is an enzyme called lecithin-cholesterol acyl transferase (LCAT). LCAT catalyzes the formation of cholesterol esters from phosphatidylcholine (lecithin) and cholesterol found in cell membranes, chylomicrons and VLDL remnants (15, 37). Another enzyme called cholesterol ester transfer protein (CETP) then incorporates the cholesterol esters into the

HDL particle (43). The cholesterol-rich HDL particle is transported back to the liver where the cholesterol is then unloaded. The unloading begins with receptor mediated endocytosis of the HDL molecule and which is followed by its metabolism inside the hepatic cells.

The HDL molecule is also capable of scavenging and transferring cholesterol by another transport system called reverse cholesterol transport. Reverse cholesterol transport can occur through two mechanisms. The first is when HDL binds a scavenger receptor class B, type I (SR-BI) on the plasma membrane of hepatic and steroidogenic cells which mediates partial and selective transfer of cholesterol and other lipids from the HDL molecule into the cells (15, 37). The depleted HDL is then able to retrieve more cholesterol from extra-hepatic tissues, chylomicrons and VLDL remnants, and bring it back to the liver for metabolism. The second mechanism of reverse cholesterol transport is through the apo A-I pathway in which the apo A- I of the HDL molecule interacts with ABCI active transporters found within cholesterol-rich cell membranes of extra-hepatic tissues. The newly formed HDL complex then enters the cell through endocytosis, retrieves cholesterol from the cell and is then released from the cell to transport the cholesterol back to the liver for metabolism (15, 37).

3.c.v. Summary of Serum Lipids Metabolism

The metabolism of serum lipids and the associated lipoproteins is a complex and interrelated system. Figure 7 summarizes the metabolism of serum lipids and distinguishes between the processes that occur exogenously, involving lipids consumed from the diet, and the processes that occur endogenously, involving lipids originating in the body. Measuring serum lipids is one of the most common and routine methods of

assessing CVD risk. Serum lipids are well known biomarkers of CVD such that excess imbalances in them can reflect increased risk of CVD (15, 26, 37).

Figure 7: Summary of the Metabolism of Lipoproteins (48).¹

¹Abbreviations Used: VLDL=Very-low-density lipoprotein; IDL=Intermediate-density lipoprotein; LDL=Low-density lipoprotein; HDL=High-density lipoprotein; GI=Gastrointestinal tract; C=Cholesterol; BA=Bile acids; Chylom=Chylomicron; LPL=Lipoprotein lipase; FFA=Free fatty acid; Extrahep=Extra-hepatic; LCAT=Lecithin-cholesterol acyl transferase.

3.d. Serum Lipids; Relationship to CVD Risk

The varying lipoproteins that cholesterol, TG and apolipoproteins are associated with perform different roles in the bloodstream. The balance, or imbalance, between their actions is closely linked to the risk of CVD. Lipoproteins such as chylomicrons, VLDL and LDL are referred to as atherogenic molecules because, in excess, their metabolism can have damaging effects on the cardiovascular system and increase the risk of CVD (30). HDL on the other hand, can result in cardioprotective effects with its role considered beneficial to the cardiovascular system to reduce the risk of CVD (26). Because of the differing actions of the lipoproteins, and the resulting metabolism of their serum lipids, target levels for the cholesterol, TG and apolipoprotein content of each lipoprotein have been established to decrease the risk of serum lipid imbalances that

could promote CVD. Table 2 lists the lipoprotein target levels of the cholesterol, TG, apolipoproteins as well as various ratios that have also proven useful as biomarkers of CVD risk.

Table 2: Serum Lipid and Apolipoprotein Target Levels for CVD Risk Management¹

Serum Lipid	Target Level	Reference
TC	<5.2 mmol/L	(31)
LDL-C	<3.5 mmol/L	(12)
HDL-C	>1.0 mmol/L for men >1.2 mmol/L for women	(31)
TG	<1.5 mmol/L	(12)
Apo B	<1.05 g/L	(12)
Apo A-I	>1.2 g/L	(49)
LDL-C/HDL-C	≤ 5	(50)
TC/HDL-C	<5.0	(12)
TG/HDL-C	<3.0	(51)
Apo B/Apo A-I	<0.9 for men <0.8 for women	(42)

¹Abbreviations Used: TC=Total cholesterol; HDL-C=High-density lipoprotein cholesterol; LDL-C=Low-density-lipoprotein cholesterol; TG=Triglyceride; Apo=Apolipoprotein

3.d.i. Serum Total Cholesterol and LDL-Cholesterol

TC and LDL-C are well known biomarkers of CVD risk due to their relationship to atherogenicity. TC is a measure of the complete amount of cholesterol in the bloodstream carried by all classes of lipoproteins (15, 37) whereas LDL-C represents the amount of cholesterol carried by LDL, the most atherogenic of the lipoproteins. Excessive amounts of cholesterol, particularly cholesterol within LDL, increases CVD risk because it can accumulate along the artery wall and deposit lipids and cholesterol which can develop into atherosclerosis (15, 37). Evidence of this was first noted in a large prospective study called The Framingham Heart Study, which started in 1948 to follow 1959 men and 2145 women (52). Follow-up at 36 years showed a significant positive correlation between CVD risk and serum TC and LDL-C in both men and women over the age of 49 years (53). Several other large scale studies such as, the Copenhagen Male Study (54), the Münster Heart Study (a study analyzing the data of subjects enrolled in the Prospective Cardiovascular Münster Study, (PROCAM)) (55), and the pre-screening data obtained in the Multiple Risk Factor Intervention Trial (MRFIT) (56), have all confirmed the results of the Framingham Heart Study with data showing a significant positive correlation between CVD risk and serum TC and LDL-C. The relationship between CVD risk and TC has been quantified in the Lipid Research Clinics Coronary Prevention Trial (LRC-CPPT), with results showing that a 1% reduction in TC relates to a 2% reduction in CVD risk (57).

3.d.ii. Serum HDL-Cholesterol

HDL-C is also a well known biomarker of CVD risk due to its relationship to anti-atherogenicity. HDL-C represents the amount of cholesterol carried by the HDL

molecules and is considered anti-atherogenic since it clears excess TG and cholesterol from the blood thereby reducing their accumulation in the artery wall (15, 37). This reverse cholesterol transport action of HDL make it a beneficial lipoprotein in protecting against CVD (15, 37, 43).

Studies such as the Framingham Heart Study (53, 58), the Prospective Cardiovascular Münster study (PROCAM) (59), MRFIT (60) and the LRC-CPPT (57), have all demonstrated an inverse relationship between CVD risk and circulating HDL-C. As with TC, the relationship between CVD risk and HDL-C has been quantified such that a 0.026 mmol/L increase in HDL-C relates to a 2-3% reduction in CVD risk (60).

3.d.iii. Serum Triglycerides

TG is another well known biomarker of CVD risk. The measurement of serum TG represents the TG content of all TG-rich lipoproteins (chylomicrons, VLDL and their remnants) and therefore reflects their group potential to promote vascular injury and initiate atherosclerotic lesions (33). TG-rich lipoproteins can integrate into the intima and undergo hydrolysis, releasing free fatty acids, mono-glycerol and di-acyl-glycerol, accounting for the load of TG in atherosclerotic lesions (61). Although there has been some debate regarding the use of TG as a biomarker of CVD risk, most recently, a consensus has been reached that hypertriglyceridemia is associated with increased risk of myocardial infarction and stroke (33) and that this risk is even higher when hypercholesterolemia is also present (59). For example results from the PROCAM study found that in the presence of high serum LDL-C, hypertriglyceridemia increased the CVD risk by approximately 2.5 fold (59). Hypertriglyceridemia has also been associated with insulin resistance (43) and low serum HDL-C (43, 59). TG is now recognized as

established risk factor that can help define CVD risk more precisely and aid in determining CVD prevention and treatment approaches (59).

3.d.iv. Serum Apolipoprotein B and Apolipoprotein A-I

Apolipoproteins are also considered biomarkers of CVD risk. They are incorporated into all lipoproteins to stabilize the transport of cholesterol and TG (15, 37). A single molecule of apo B is found within the atherogenic lipoproteins VLDL, IDL, and LDL (34), therefore reflecting the total number of atherogenic particles in the blood (42). Apo B is responsible for the attraction of these lipoproteins into the intima layer of the artery wall as it is the ligand for the apo B and apo E receptors that assist the uptake of cholesterol into the peripheral tissues and the liver (62). The majority (90%) of apo B measured in the blood is within LDL (62) thereby making apo B a reflection of LDL-C and therefore related to increased CVD risk. On the other hand, the amount of apo A-I measured in the blood corresponds to the amount of anti-atherogenic HDL particles in the blood since apo A-I is the main apolipoprotein in the HDL molecule (62). In addition, apo A-I plays a major role in the reverse cholesterol transport of HDL (15, 37) and it has been shown to have antioxidant and anti-inflammatory effects to provide vascular protection (42). Overall, a measurement of apo A-I is a reflection of HDL-C and related to reduced CVD risk.

There is research to suggest that apo B and apo A-I are possibly more precise biomarkers of CVD risk than the more widely used TC, LDL-C and HDL-C measurements (63) or at least, are useful complementary measurements. Part of this reasoning relates to their methodological advantages including that apo B is measured directly (as opposed to LDL-C which is measured indirectly using the Friedewald

calculation (46)) and that both apo B and apo A-I can be measured in the non-fasted state thereby increasing their practicality (62). Many studies have provided support for the idea that CVD risk is positively associated with apo B and negatively associated with apo A-I. For example, the Multinational Monitoring of Trends and Determinants in Cardiovascular Disease/Kooperative Gesundheitsforschung in der Region Augsburg Echocardiography Study (MONICA/KORA) from Augsburg Germany (64) followed 1414 men and 1436 women for 13 years and discovered a significant positive association between serum apo B and CVD risk, particularly myocardial infarction risk (64). In terms of apo A-I, a study by Schaefer et al. (65) found that low plasma apo A-I was associated with low plasma HDL-C, thereby contributing to an elevated CVD risk. Apo B and apo A-I were both examined in an epidemiological prospective study called the Apolipoprotein Related Mortality Risk Study (AMORIS) (66) in which serum apolipoproteins were related to risk of mortality from myocardial infarction (no other causes of death were considered) in 75553 men and women. After a follow up of 66.8 months, risk of mortality from myocardial infarction was significantly positively associated with apo B and negatively associated with apo A-I (66).

3.d.v. Serum Lipid Ratios

3.d.v.i Total-Cholesterol/HDL-C and LDL-C/HDL-C

The ratios of TC/HDL-C and LDL-C/HDL-C are also recognized as a predictive tool to relate to future risk of CVD (59). High TC/HDL-C or LDL-C/HDL-C have been shown to exhibit a strong correlation with increased CVD risk, specifically for deaths due to myocardial infarction (55). The ratios of serum lipids are valuable measures when analyzing CVD risk as they can provide a more relevant measure of risk compared to the

individual components of the ratio. For example, certain serum lipid or apolipoprotein ratios can reflect a balance between the atherogenic and anti-atherogenic molecules in the bloodstream such that an increase in the ratio represents an atherogenic state and elevated CVD risk whereas a decrease in the ratio indicates an anti-atherogenic state and reduced CVD risk.

The TC/HDL-C and LDL-C/HDL-C ratios are commonly used in studies as biomarkers of CVD risk. To assess the validity of these ratios, Ridker et al. (63) conducted a cohort study of 15632 initially healthy women to relate various plasma lipid measures to CVD risk. Results of the study indicated that the TC/HDL-C ratio and the LDL-C/HDL-C ratio were more related to CVD risk than any of the single components of the ratios. Overall, the TC/HDL-C ratio and non-HDL-C, were identified as the strongest predictors of future cardiovascular events (63).

3.d.v.ii. Triglycerides/HDL-C

The ratio of TG/HDL-C is another biomarker that has been studied in relation to CVD risk, but also in relation to insulin resistance. Recent research suggests that there is a link between TG and HDL metabolism (67) in that hypertriglyceridemia has been associated with low HDL-C, a combination that is typical of the dyslipidemia found in insulin resistant states (43). Insulin resistance is not only related to type 2 diabetes but also significantly increases CVD risk (1).

3.d.v.iii. Apolipoprotein B/Apolipoprotein A-I

The apo B/apo A-I ratio is another CVD biomarker and a useful way of expressing the balance of cholesterol transport. A higher ratio indicates that there are more atherogenic lipoprotein particles (LDL) than anti-atherogenic lipoprotein particles

(HDL), which means that cholesterol is likely being deposited on and integrating into the artery wall (62) thereby promoting atherosclerosis and increasing CVD risk. Several studies have shown that the apo B/apo A-I ratio is positively associated with risk of myocardial infarction (63, 64, 66, 68). In fact, many large studies, including the AMORIS study (66), the INTERHEART study (68), and the MONIC/KORA Augsburg study (64), report the apo B/apo A-I ratio as the strongest of all independent risk factors for risk of myocardial infarction, irrespective of lipid levels.

In summary, circulating levels of lipoproteins can collectively represent circulating levels of TG and cholesterol. The profile of lipoproteins (chylomicrons, VLDL, LDL or HDL) and/or associated apolipoproteins (apo B or apo A-I) that are associated with TG and cholesterol can be useful for predicting CVD risk. Measurements of serum lipids, apolipoproteins and their ratios are a very common method of determining CVD risk that can be used as both a preventative and predictive tool for the assessment of CVD risk. Individuals at high CVD risk, such as adults with type 2 diabetes, should therefore monitor their serum lipids often and keep them regulated to best protect themselves from CVD.

3.e. Serum Lipids; Alterations in Type 2 Diabetes

3.e.i. Altered Cholesterol and Serum Lipid Profiles in Type 2 Diabetes

The dyslipidemia that is often observed in type 2 diabetes begins with insulin resistance and hyperinsulinemia (69). As the body becomes resistant to the signals from insulin, its cells are not able to uptake glucose and perform glycolysis to provide energy (14). The adipose cells respond by breaking down fatty acid stores to excrete free fatty acids into the blood as a means of energy for other metabolically active cells (70). This

increase in free fatty acids causes fatty acid accumulation in the liver which leads to the abnormal hepatic metabolism of serum lipids and lipoproteins that is observed with insulin resistance and type 2 diabetes (45, 71). Excess free fatty acids also has multiple effects on several enzymes which are key to the metabolism of serum lipids and lipoproteins (71, 72). Lipoprotein lipase becomes saturated with free fatty acid which reduces the amount of time the enzyme is able to metabolize TG-rich lipoproteins thereby increasing their likelihood of damaging the artery wall to promote atherosclerosis. Excess free fatty acids also stimulate cholesterol ester transferase which causes lipid exchange between TG-rich lipoproteins and other lipoproteins of lower density (71, 72). LDL and HDL particles therefore become enriched with TG while VLDL becomes enriched with cholesterol. TG-rich LDL and HDL are substrates for hepatic lipase which will act on them and convert them to smaller, denser particles. Small HDL particles are cleared from the bloodstream more rapidly therefore reducing overall HDL levels and their positive CVD-related properties. Thus, insulin resistance and hyperinsulinemia magnify this chain reaction of lipid metabolism and the improper activation of enzymes that contribute to the dyslipidemia of type 2 diabetes (71, 72).

Before insulin resistance progresses to type 2 diabetes, impaired glucose intolerance will first be experienced (69). It is at this impaired metabolic stage that dyslipidemia also begins to develop (69), which explains the similar lipid profile observed with insulin resistance, impaired glucose states and type 2 diabetes (73). In type 2 diabetes, dyslipidemia is one of the main causes for elevated CVD risk which make lipid abnormalities a more important CVD risk factor than hyperglycemia (72). The characteristic lipid profile observed with insulin resistance and type 2 diabetes

includes hypertriglyceridemia, low HDL-C and small dense LDL particles (with either normal or high LDL-C) (45, 74, 75). The mechanisms of how these individual serum lipid abnormalities occur in type 2 diabetes are not separate but rather are metabolically linked in a complex manner (45).

3.e.ii. Hypertriglyceridemia in Type 2 Diabetes

As mentioned, hypertriglyceridemia is a common lipid dysfunction in type 2 diabetes. The main TG-rich lipoproteins are the chylomicrons, derived from the intestine, and VLDL, derived from the liver (45). The TG content of the VLDL particle depends on its size in that the large buoyant VLDL-1 particles contain more TG than the smaller more dense VLDL 2 particles (45). In healthy adults, it has been found that insulin acutely inhibits the assembly and secretion of VLDL particles, specifically VLDL-1 particles (76), by increasing apo B degradation and decreasing the expression of microsomal transfer protein (MTP) in the hepatocytes (which is responsible for lipidation of apo B during VLDL production) (45). This thereby makes the smaller VLDL-2 particles the main hepatically-derived TG-rich lipoproteins in hyperinsulinemic states (77). In contrast, acute hyperinsulinemia imposed on subjects who are insulin resistant and have type 2 diabetes fails to suppress VLDL-1 production (78). This inconsistency seen in insulin resistance and type 2 diabetes is an important variation in this metabolism such that more of the larger VLDL-1 particles are present, since insulin is unable to successfully suppress their production (79). Further investigation has revealed that there is not a similar pattern seen with the VLDL-2 particles in type 2 diabetes patients in this situation as they do not increase along with the VLDL-1 particles (79). This suggests that an altered metabolism of VLDL-1 particles contributes to the increase of TG-rich

lipoproteins and TG seen in type 2 diabetes. Thus, the failure of insulin to suppress VLDL-1 production in patients with type 2 diabetes is an important contributing factor to the hypertriglyceridemia observed in type 2 diabetes (78).

3.e.iii. Altered Metabolism of Triglyceride-Rich Lipoproteins (Chylomicrons and VLDL) in Type 2 Diabetes

The metabolic alterations that accompany type 2 diabetes have a large impact on serum lipid metabolism. The metabolism of the TG-rich lipoproteins (chylomicrons and VLDL particles) is of particular interest because they both experience altered metabolism in type 2 diabetes. Both of these TG-rich lipoproteins are metabolized through a similar delipidation cascade (80) that is catalyzed by both lipoprotein lipase (LPL) and hepatic lipase. The first delipidation step that the TG-rich lipoproteins undergo is by LPL which hydrolyses TG (80). LPL is regulated by insulin and is often decreased with insulin resistance and/or type 2 diabetes (45, 72). The consequent decrease in lipolysis keeps higher numbers of TG-rich lipoprotein in the circulation for a longer duration. The second delipidation step that the TG-rich lipoproteins undergo is by hepatic lipase which converts IDL particles to LDL particles (80). Hepatic lipase is increased with insulin resistance and/or type 2 diabetes which causes an enhanced production of small LDL from IDL, but not enough to produce elevations in LDL-C (80).

3.e.iv. Decreased HDL-C in Type 2 Diabetes

A low concentration of HDL-C is a major feature of diabetic dyslipidemia (81). In an insulin resistant state, the high TG content in the blood drives the exchange of core lipids between TG-rich lipoproteins and HDL (much like it does between TG-rich lipoproteins and LDL) by the insulin resistant induction of CETP (81). When HDL molecules become enriched with TG, they become smaller and denser, making them

excellent substrates for hepatic lipase, which breaks them up into smaller particles and dissociates their constituent apo A-I, which is then cleared by the kidneys (81). Hepatic lipase is increased in type 2 diabetes which causes increased catabolism of these smaller HDL particles and subsequent abnormally low concentrations of HDL-C (81).

3.e.v. Altered LDL Metabolism in Type 2 Diabetes

It is important to note that high LDL-C in type 2 diabetes is associated with a higher CVD risk compared to individuals of similar LDL-C levels without type 2 diabetes (74). This makes it very important to target therapies to lower LDL-C in type 2 diabetes. High LDL-C however is not always seen in type 2 diabetes (71, 74, 82), rather it is the shift towards small, dense LDL that is the main alteration in type 2 diabetes (71, 74, 82). Over-stimulation of CETP that occurs with insulin resistance promotes the formation of TG-rich LDL particles which are smaller and denser than normal LDL particles (80). The smaller denser LDL can penetrate the intima of the artery and promote atherosclerosis at much higher rates than larger LDL (80). Small dense LDL are also more susceptible to glycation and oxidation compared to larger LDL molecules (74). Thus, regardless of circulating LDL-C levels, reducing LDL-C in individuals with type 2 diabetes is most important for reducing the atherogenic effects from the small dense LDL molecules.

3.e.vi. Treating Diabetic Dyslipidemia

The atherosclerotic risks of diabetic dyslipidemia require intervention to resolve the imbalance (83). One of the most common and effective methods of treatment is by pharmaceuticals which are designed to target different aspects of the dyslipidemia. Overall, pharmaceutical therapy has been beneficial at improving the lipid profile of

individuals with type 2 diabetes and decreasing their risk of CVD and myocardial infarction (34).

An alternative approach to managing dyslipidemia is through lifestyle modifications. When dyslipidemia is first diagnosed, lifestyle modifications are recommended in attempt to correct the imbalance in hopes to avoid or delay the need for pharmaceutical treatment (74). Certain lifestyle recommendations include cessation of smoking, weight loss, regular physical activity and dietary changes. Some of these dietary recommendations include reduced saturated and trans fat, reduced alcohol, increased fruit and vegetables and increased dietary fibre (20, 34, 81). In addition, there are other specific foods that have received interest for their potential to improve the serum lipid profile. An example of one of these foods is soy. Soy has been consumed in Asia for thousands of years (84) where its high intake has been correlated with the relatively worldwide low rates of diabetes, dyslipidemia and CVD of this population (85-87). Conversely, soy is consumed in substantially lower amounts in the Americas where there are high worldwide rates of diabetes, dyslipidemia and CVD (88). Thus, the soy-rich Asian diet is of great interest and deserves investigation with respect to its associated reduction in disease risks.

4. Soy Protein and Soy Isoflavones

4.a. Soy Protein

4.a.i. Structure and Composition

Soy protein represents a complete protein, providing all essential amino acids required in the human diet and has a digestibility and bioavailability comparable to animal protein (89). The protein component of the soybean is made up of two major

fractions, namely the globulin fraction and the whey fraction (90, 91). The globulin fraction composes approximately 85% of the total protein component of the soybean and contains the 2S, 7S, 11S and 15S proteins (90, 91). The remaining whey fraction, which composes approximately 15% of the total protein component of the soybean, contains the 2S and 7S proteins (92). The 2S proteins (within the globulin and whey fractions) make up 10-20% of soybean protein and consist of small proteins such as the Kunitz trypsin inhibitors and cytochrome C. The 7S proteins (within the globulin and whey fractions) make up 30-40% of soybean protein and consist mainly of β -conglycinin (7S globulin) but also smaller proteins such as hemagglutinin, lipoxygenases and amylases. The 11S proteins (within the globulin fraction) also make up 30-40% of soybean protein and consist mainly of glycinin (11S globulin) (90, 91). Finally, the 15S proteins (within the globulin fraction) make up 10-15% of soybean protein and consist of small protein aggregates of the other globulin protein fractions (93). The dominant proteins in the soybean are therefore the 7S globulin, β -conglycinin, and the 11S globulin, glycinin (94). These are both major storage proteins in the soybean and provide the amino acids and enzymes necessary for development during seed germination (95). They are also considered to be the bioactive peptides of the soybean that have the potential to confer human health benefits (94).

4.a.ii. Dietary Intake

Dietary intake of soy protein in North America is very low when compared to Asian countries (96, 97). This can historically be explained by the fact that soybeans have been cultivated in East Asia for approximately 5000 years and have been a significant component of the East Asian diet over that time (84). In Asia, the average

consumption of soy protein is approximately 40 g per day (18, 98) with the most common sources being tofu, miso and tempeh (99). In contrast, North Americans consume much less soy protein averaging at 1-3 g per day which comes from mainly processed foods such as soy drinks, breakfast cereals, energy bars and soy burgers (99).

4.b. Soy Isoflavones

4.b.i. Structure

In the natural matrix of the soybean, the proteins are associated with isoflavones. Isoflavones are naturally occurring phytochemical compounds from the subcategory of phytoestrogens called flavonoids (100). Phytoestrogens occur naturally in plants and derive their name from their structural similarity to estrogen (100). The most abundant isoflavones in soy are genistein, daidzein, and, to a lesser extent, glycitein (101) (Figure 8). These isoflavones, mentioned in their aglycone forms, are also found in their glycosylated (or glycoside) forms (genistin, daidzin, and glycitin) in which they are conjugated with a glucose molecule (101). The isoflavone precursors biochanin A and formononetin also occur, although are more present in red clover than soybeans. Following removal of their methyl group, biochanin A and formononetin can be converted to genistein and daidzein, respectively (100). Genistein and daidzein can also be metabolized further by enzymes produced by intestinal microflora into the isoflavone metabolites p-ethyl phenol, O-desmethylangolensin (O-DMA) or equol (102). Production of these isoflavone metabolites, particularly equol, varies among individuals and is thought to be influenced by both genetics and colonic microflora (102).

Figure 8: The Predominant Soy Isoflavones: Daidzein, Genistein, and Glycitein, in Their Aglycone Form (103).

4.b.ii. Dietary Intake, Food Sources and Bioavailability

Much like the dietary intake patterns of soy protein, soy isoflavone intake is higher in the Asian populations compared to the North American population (96, 97). It is not uncommon for the average person to consume 40 mg of isoflavones per day in Asia (84), which is substantially higher than the average North American person who consumes usually no more than 1 mg/day (84).

In terms of food sources of isoflavones, 1 gram of soy protein contains approximately 1-2 mg of isoflavones (101). The precise amount depends on many factors including the soybean growing conditions (99), the variety of soybean (99), food processing conditions (fermentation results in an increased amount of isoflavones in the aglycone form) (104), the food matrix (105), time of harvesting (soybeans harvested later in the season have higher isoflavone levels) (106) and soybean seed growing temperature (higher temperatures result in lower isoflavone content) (106). The

isoflavone content of soy foods can range from 0.1-3.0 mg/g fresh weight (89). The isoflavone content of some common soy foods are summarized in Table 3.

Table 3: Isoflavone Content of Some Common Soy Foods (modified from (104)).

Soy Food	Isoflavones (mg/kg fresh weight)
Soybeans	1800
Tofu	2400
Soy bean flour	1300-1650
Soy milk	450
Soy sauce	150

The bioavailability of soy isoflavones can be affected by a multiple of factors, many of which are still being researched. Some of the factors proposed to affect the bioavailability of isoflavones include the food matrix, the background diet, the isoflavone form, gender, age, and the gut microflora profile (100). In order to fully assess the bioavailability of isoflavones, there must be consideration of their absorption, metabolism, circulation and excretion.

4.b.iii. Metabolism and Absorption

The metabolism and absorption of soy isoflavones is summarized in Figure 9. When isoflavones are consumed, they are first acted upon by intestinal enzymes that are synthesized by intestinal bacteria (102). Isoflavones that are consumed in their glycoside form (genistin, daidzin, glycitin), are metabolized by intestinal β -glucosidase to their aglycone form (genistein, daidzein, glycitein) (107), which can then be absorbed by the enterocytes of the intestine. Alternatively, the aglycone isoflavones can undergo further metabolism by an array of other intestinal bacterial enzymes to produce isoflavone metabolites. Specifically, daidzein can be metabolized to equol and/or O-DMA, and

genistein can be metabolized to p-ethyl phenol (102). These isoflavone metabolites can then also be absorbed by the enterocytes of the intestine (102).

Figure 9: Metabolism of Isoflavones.¹

¹Abbreviations used: O-DMA=O-desmethylangolensin

Of important note in relation to the isoflavone metabolites is the large inter-individual variation in the ability to metabolize daidzein into equol (89). Research has found that only 30-40% of individuals have the ability to metabolize daidzein into equol and therefore excrete it through the urine (108-110). This is of particular scientific interest since equol is the most potent of isoflavones (111) and has been specifically linked to reduced risk of disease (111). Ongoing research into the reasons for the large inter-individual variation in the ability to produce equol has identified potential factors such as genetics, gut microflora, and background diet (96).

4.b.iv. Circulation

After absorption, aglycone isoflavones and metabolites travel the portal circulation to the liver where they are conjugated by UDP-glucuronosyl transferase or sulfotransferase enzymes to glucuronide and sulfate conjugates prior to entering the peripheral circulation (104). It is in this conjugated form that the isoflavones and

isoflavone metabolites primarily circulate in the blood and other bodily fluids (89), with less than 10% of circulating isoflavone remaining in their unconjugated form (112, 113). Entrance into the circulation is rapid as evident by the fact that the conjugated forms of the isoflavones can be detected in the blood within 30 minutes of isoflavone consumption (114). Peak blood concentrations occur between 4-6 hours following consumption (115, 116). The biological half-life of isoflavones ranges from 5.8 to 8.4 hours (117).

4.b.v. Excretion

The excretion of soy isoflavones has been identified in the urine, feces and bile and can vary considerably between individuals (118). The urine is the primary excretory route for isoflavones, accounting for approximately 30% of ingested isoflavones (118). Urinary excretion of isoflavones closely reflects intake in a dose dependent pattern (108, 119). As such, urinary isoflavones are frequently utilized as an indication of subject compliance in soy intervention studies (120) or as a biomarker to relate to disease risk in epidemiological studies (121).

Isoflavones can also be excreted in the feces, but at substantially lower amounts, accounting for only 1-4% of ingested isoflavones (122). The form of the isoflavone present in the feces can provide information on its metabolism. For example, if the form is an aglycone, it suggests that the isoflavone was not absorbed. Alternatively, if the form is an isoflavone conjugated with glucuronide or sulfate, it suggests that the isoflavone was absorbed, then excreted through the enterohepatic cycle back into the intestine, but then not reabsorbed.

Finally, isoflavones can also be excreted in the bile (100). Like estrogens, isoflavones undergo an enterohepatic circulation and therefore, once excreted into the

bile, can either be excreted in the feces or reabsorbed (118). If the isoflavones are to be reabsorbed, they must first be deconjugated by β -glucuronidase to yield the aglycone and allow for absorption (100, 118).

It is noteworthy that the excretion of isoflavones in the urine, feces and bile does not completely account for the amount consumed. This discrepancy could be due to unidentified isoflavone metabolites, unidentified storage of isoflavones or possibly microbial degradation of isoflavones (100).

The metabolism, absorption, circulation and excretion of isoflavones are important to consider when assessing their bioavailability and discussing their physiological impact in relation to disease prevention. Research that has been conducted with soy isoflavones, and its associated proteins, has yielded positive results on the ability of soy to reduce the risk of disease (89, 99, 104). In particular, beneficial findings have been reported on the ability of soy to reduce the risk of CVD (123-125), which has relevance for adults with type 2 diabetes who are at an elevated CVD risk (71, 126, 127).

5. Role of Soy in Reducing CVD Risk in Type 2 Diabetes

The potential for lifestyle modification to reduce risk of CVD in type 2 diabetes deserves attention as it could serve as a valuable means to improve quality and quantity of life. Diet, a significant lifestyle component, is modifiable however few studies have analyzed the effect of soy in the diet with respect to CVD risk reduction in type 2 diabetes. Some epidemiological, *in vitro* and animal studies have generated promising results, but the area of human intervention studies involving the effects of soy and CVD risk in adults with type 2 diabetes is limited.

5.a. Epidemiological Studies

The elevated risk of CVD in adults with type 2 diabetes (71, 126, 127) makes the potential ability of soy to reduce CVD risk very relevant to this population. Although there has been no epidemiological studies conducted to study the relationship between soy consumption and CVD risk in adults with type 2 diabetes, there have been studies worth mention that were conducted in adults without type 2 diabetes.

The first epidemiological study to investigate the association between soy, serum lipids and CVD risk was performed by Nagata et al. (128). This cross-sectional study involved 1242 men and 3596 women who completed food frequency questionnaires (FFQ) to quantify soy product consumption and provided blood samples for evaluation of serum TC. Results showed a significant decrease in serum TC in association with increasing soy product consumption in men with a similar trend in women but not significant (128). These results were added to by another cross-sectional study by Goodman-Gruen et al. (129) in which soy isoflavone intake and CVD risk factors were assessed using FFQ and blood samples in 208 postmenopausal women. Results showed that intakes of genistein, daidzein and total isoflavones were significantly positively associated with plasma HDL-C, but not significantly associated with plasma TC, LDL-C or TG (129).

Other epidemiological studies have investigated the association between soy consumption and actual incidence of CVD. A population-based prospective cohort study performed by Zhang et al. (130) recruited women who had participated in the Shanghai Women's Health Study to examine the relationship between soy consumption and CHD. FFQ and in-person interviews were conducted to obtain information on soy consumption

from 64915 Chinese women, without previous CVD, cancer or diabetes. After 2.5 years of follow up, there was a clear dose-response inverse relationship between soy consumption and CHD risk. The inverse relationship was even stronger between soy consumption and non fatal myocardial infarction (130).

Overall, epidemiological studies provide evidence for a beneficial role for soy consumption in the improvement of serum lipids as biomarkers of CVD risk as well as reduction of actual incidence of CVD. Although none of these studies included subjects with type 2 diabetes, the fact that they focused on CVD is highly relevant to type 2 diabetes. These studies provide valuable information however an actual cause and effect relationship cannot be established from this kind of research as it is only observational and can therefore only conclude about associations. This makes epidemiological studies important for identifying trends and generating hypotheses, but other types of studies such as *in vitro* and *in vivo* studies are needed to establish cause and effect relationships.

5.b. *In Vitro* Studies

The *in vitro* research that has explored the role of soy in modulating CVD risk in diabetes has focused on the potential antioxidant effects of soy isoflavones (131). This is relevant to type 2 diabetes as higher levels of free radicals have been observed in adults with type 2 diabetes compared to those without diabetes (132), an observation that may be caused by glucose-derived oxidants formed from hyperglycemia (133). High levels of free radicals increase CVD risk as they can oxidize LDL particles making them more susceptible to uptake by macrophages in the artery wall while promoting fatty streak and plaque formation (131). Thus, *in vitro* studies have been performed to evaluate the effect of ability of soy isoflavones to reduce LDL oxidation.

An *in vitro* study performed by Vedavanam et al. (132) used human LDL isolated from plasma to examine the effect of a soy isoflavone extract (a mixture of genistein and daidzein) on glucose-induced LDL oxidation, as measured by thiobarbituric acid-reactive substances (TBARS). Results showed that the LDL incubated with the soy isoflavone extract had significantly less glucose-induced oxidation compared to the control (132). The antioxidant effects of soy isoflavones on LDL oxidation are also supported by other *in vitro* studies. For example, Exner et al. (133), who also assessed LDL oxidation using TBARS, found that human LDL treated with genistein prevented the atherogenic modification of LDL by inhibiting the glucose autooxidation. The results of these two *in vitro* studies demonstrate the role of soy isoflavones in reducing early atherogenic events in type 2 diabetes by reducing LDL oxidation caused by hyperglycemia.

In vitro studies have also examined the potential for soy isoflavones to reduce CVD risk in type 2 diabetes through activation of the γ and α forms of peroxisome-proliferator activated receptors (PPAR), which are nuclear receptors (134). A variety of substrates are able to activate PPAR which lead to their binding to PPAR element sequences located within the promoter regions of the PPAR regulatory genes. PPAR γ controls the expression of genes involved in adipocyte differentiation and insulin sensitivity (73) while PPAR α regulates lipid catabolism by controlling the transcription of lipid catabolism genes (72, 127). When activation of PPAR γ and PPAR α occurs together it produces increased fat metabolism, improved insulin sensitivity and decreased blood and liver lipid levels (134). Since this would prove extremely beneficial in improving the dyslipidemia that occurs in type 2 diabetes, pharmaceuticals such as insulin sensitizers and fibrates have been developed for type 2 diabetes to target PPAR γ

and PPAR α , respectively (72). Soy isoflavones have also been studied for their potential to influence PPAR regulation and ultimately lipid metabolism. For example, a study by Mezei et al. (134) transfected murine macrophage-like RAW264.7 cells with a PPAR γ or PPAR α expression plasmid and incubated them in serum and treated with either conjugated isoflavone mixtures, unconjugated isoflavone mixtures or individual unconjugated isoflavones. When compared to the negative control, PPAR γ and PPAR α were not activated by the conjugated isoflavone mixture but were activated by the unconjugated isoflavone extract mixture, as well as the individual unconjugated isoflavones genistein and daidzein.

Overall, these *in vitro* studies provide evidence for mechanisms by which soy isoflavones may reduce CVD risk for those living with type 2 diabetes, including reductions in LDL oxidation caused by hyperglycemia (132, 133) and activation of PPARs to improve lipid and insulin metabolism (134). In order to progress knowledge regarding the potential for soy to benefit type 2 diabetes, it is necessary to perform *in vivo* studies, such as those using animal models.

5.c. Animal Studies

The potential for soy to benefit type 2 diabetes through reduced CVD risk has been studied using both rodent and non-human primate animal models of type 2 diabetes. In these studies, the animals have been physiologically manipulated via genetic breeding or pharmaceutical intervention so that they exhibit the type 2 diabetes condition. These specific animal models of type 2 diabetes are extremely useful for studying the disease in ways that are not feasible and/or not ethical in humans.

5.c.i. Rodents

The most common rodent model used to research the effects of soy has been the rat, with various breeds utilized. The potential for soy isoflavones to modulate lipid metabolism was examined by Mezei et al. (134) who fed male and female obese diabetic Zucker rats (a model of type 2 diabetes and obesity (135)) either a high-isoflavone soy protein, a low-isoflavone soy protein or casein. The high-isoflavone soy protein significantly reduced liver cholesterol content, liver TG content and plasma cholesterol compared to both the low-isoflavone soy protein and the casein, in both the male and female rats (134). Of note is that in the female rats, the high-isoflavone soy protein also significantly increased plasma TG compared to both the low-isoflavone soy protein and the casein (134). It is noteworthy that the favourable lipid results of this rodent study are comparable to results when humans are treated with pharmaceuticals that target PPARs to improve lipid metabolism (136).

Peluso et al. (137) also utilized obese male diabetic Zucker rats as a model of type 2 diabetes and obesity and studied the effects of 70 days of casein, low-isoflavone soy protein isolate (SPI) or high-isoflavone SPI soy on plasma and liver concentrations of cholesterol and TG and liver concentrations of cholesterol esters. Results showed that plasma concentrations of TG did not significantly change, although plasma cholesterol was significantly reduced; 21% by the low-iso SPI and 29% by the high-iso SPI both when compared to the casein. Liver concentrations of cholesterol and TG were significantly reduced by both SPIs in relation to casein (34% and 33% by the low-iso SPI, respectively and 48% and 49% by the high-iso SPI, respectively). There also was a significant reduction of 24% in liver TG concentrations in the high-iso SPI group

compared to the low-iso SPI group. No significant reductions were seen in liver cholesterol between the two SPI groups. Finally, liver concentration of cholesterol ester was also significantly reduced by both SPIs compared to the casein (46% by low-iso SPI and 77% by high-iso SPI), with the high-iso SPI effect significantly greater than both the low-iso SPI and the casein. The significance found in most of the findings in this study, supports the ability for soy protein consumption to reduce serum lipids in type 2 diabetes and encourages further research in this area.

A third study that utilized the obese Zucker rats with type 2 diabetes was conducted by Dyrskog et al. (138) to examine the individual and combined effects of the dietary supplement Abalon (soy protein, isoflavones and soy cotyledon fibre) and the nutraceutical stevioside (used for lipid lowering) on plasma lipids. A total of 40 male obese Zucker rats were randomized to receive 11 weeks of a control diet, a diet containing stevioside, a diet containing Abalon or a diet containing both Abalon and stevioside. Results revealed that plasma free fatty acids, TC and TG were significantly decreased following consumption of the Abalon-containing but not the stevioside diets, when compared to the control diet (138). This study further supports the positive effects of soy and associated isoflavones on serum lipids in diabetes.

Another rodent model that is frequently used to study type 2 diabetes is the obese SHR/N-cp rat as it is highly predisposed to developing type 2 diabetes. These rats are genetically altered to be a model for obesity, type 2 diabetes and insulin resistance (139). Bhathena et al. (139) used this rodent model to study the effects of soy consumption on plasma lipids. Rats were randomized to receive 20% of their energy intake from either soy protein or casein for 6 months after which plasma TC, LDL-C, TG and HDL-C and

TG content in the liver were measured. Compared to the casein-fed SHR/N-cp rats, the soy protein-fed SHR/N-cp rats had lower plasma TC, LDL-C and TG, however the results were not statistically significant (139). TG content in the liver was significantly reduced by the soy protein, which is considered beneficial in improving insulin resistance (139).

Ali et al. (140) also used the obese SHR/N-cp rat model of type 2 diabetes to research the effects of soy isoflavones on lipid metabolism. Rats were fed casein or casein supplemented with an isoflavone mixture (genistein, daidzein and glycitein). The duration of the study was 20 weeks and concluded with measurements of plasma TC, LDL-C, HDL-C and hormones related to lipid metabolism, specifically thyroid hormones and steroid hormones. When compared to the casein alone, the isoflavone-supplemented casein significantly decreased plasma TC, LDL-C and steroid hormones but did not significantly affect plasma HDL-C or thyroid hormones (140). Ali et al. (141) also analyzed plasma TG from the data collected in the previous study using the SHR/N-cp rat model (140) to evaluate the effect of 20 weeks of the isoflavone-supplementation on plasma TG and found no significant difference from the casein control (141).

A final rodent study worth mention is that by Lee et al. (142), who performed a study examining the effect of soy protein and genistein on serum and liver lipids in 32 male Sprague Dawley rats. The rats were divided into 4 groups including a group of non-diabetic rats fed a control diet, a group of streptozotocin (STZ)-induced diabetic rats fed a control diet, a group of STZ-induced diabetic rats fed genistein and a group of STZ-induced diabetic rats fed SPI, all for a duration of 3 weeks. At the conclusion of the study there were significant reductions in serum TC, serum HDL-C, serum TG, liver

cholesterol and liver TG in the STZ-induced diabetic rats fed SPI and in the STZ-induced diabetic rats fed genistein, both compared to the STZ-induced diabetic rats fed a control diet. When the SPI and genistein-fed STZ-induced diabetic rats were compared with the non-diabetic rats fed the control diet, the only significant result was a reduction in serum HDL-C. Results from this study demonstrate the beneficial effects of soy on serum and liver lipids and supports a role for both the protein and isoflavone components of the soy.

Overall, these rodent studies, designed to evaluate the potential for soy to improve the CVD risk of type 2 diabetes, demonstrate improvements including reduced lipid profiles (134, 137-140, 142), reduced liver cholesterol (134, 137, 142), reduced liver TG (134, 137, 139, 142) and reduced steroid hormones related to lipid metabolism (140). Although these rodent studies are beneficial and provide valuable information, their value is limited by the extent in which their results can be extrapolated to humans. In this regard, a closer relative to the human, such as non-human primates, is often studied as another animal model of diabetes.

5.c.ii. Non-Human Primates

The only study that utilized non-human primates as a model to study the effects of soy consumption on endpoints related to CVD risk in type 2 diabetes was performed by Wagner et al. (143). The study involved 23 male cynomolgus diabetic and non-diabetic monkeys who were fed an atherogenic diet along with a protein source of either SPI or casein. All results were consistent between diabetic and non-diabetic monkeys and showed that the monkeys fed SPI had significantly lower plasma TC and LDL-C as well as significant reductions in arterial-deposited LDL-C of 50% and arterial delivery of LDL-C of 40%, all compared to the monkeys fed casein (143). In addition, the monkeys

fed SPI had significantly reduced permeability of LDL in the carotid bifurcation and internal carotid arteries compared to the casein group. Overall, the results of this animal study demonstrate not only favourable effects of soy protein consumption on plasma lipids, but also more direct arterial effects thereby suggesting a more detailed mechanism by which soy consumption may reduce CVD risk in type 2 diabetes. The results derived from these animal studies are valuable as they are often extrapolated to humans. However, it is important to conduct human trials in order to directly investigate the effect of soy on serum lipids in relation to CVD risk.

5.d. Human Intervention Studies

Although studies cannot be as invasive in humans as they are in animals, human intervention studies have repeatedly demonstrated the ability of soy protein to improve circulating lipids as a key CVD risk factor. The high number of studies has prompted several meta-analyses in order to summarize their findings. The first meta-analysis was published in 1995 and summarized that consumption of soy protein resulted in overall reductions of circulating TC of 23.2 mg/dl or 9.3%, LDL-C of -21.7 mg/dl or 12.9% and TG of -13.3 mg/dl or -10.5% and an overall increase in HDL-C of 1.2 mg/dl or 2.4% (144). A second meta analyses, published in 2005, revealed similar findings by reporting that consumption of soy protein resulted in overall reductions of circulating TC of 0.22 mmol/L or 3.7%, LDL-C of 0.215 mmol/L or 5.25% and TG of 0.10 mmol/L or 7.2% and an overall increase in HDL-C of 0.04 or 3.0% (86). The most recent meta-analysis, published in 2006, reported that consumption of soy protein resulted in overall reductions in mean serum TC of 5.26 mg/dl, LDL-C of 4.25 mg/dl and TG of 6.26 mg/dl and an overall increase in HDL-C of 0.77 mg/dl (145).

The multiple studies that reported a beneficial role for soy consumption to improve circulating lipids led to the 1999 approval of a health claim for foods in the United States that relates consumption of soy protein to a reduced risk of coronary heart disease risk (146). In order to qualify for the health claim, foods must contain at least 6.25 grams of soy protein per serving such that 4 servings would provide a daily total of 25 grams of soy protein. Other criteria must be met by the foods, such as being low in fat, cholesterol and sodium. The displayed health claim on food labels states that “diets low in saturated fat and cholesterol that include 25 grams of soy protein a day may reduce the risk of heart disease” (146).

The well-documented lipid-lowering effects of soy protein consumption has prompted the need to investigate its potential for similar effects in those living with chronic diseases such as diabetes. The need for research focusing on strategies to reduce CVD risk in adults with type 2 diabetes is essential to improving the lives of those suffering from the disease. Despite the need for this research, few human studies have explored the role of soy in improving CVD risk in adults with type 2 diabetes.

Table 4 contains a summary of the 10 published human intervention studies that have examined the effects of soy protein, soy fibre or soy isoflavones on serum lipids in adults with type 2 diabetes. The first human study summarized in Table 4 investigated the effects of soy protein and soy fibre on serum lipids in adults with type 2 diabetes using a randomized crossover, double-blinded design (18). Twenty adults (14 men, 6 women) with type 2 diabetes and no diabetes complications (11 managed by diet and 9 treated with hypoglycemic medications) consumed a combination of 50 g of high-isoflavone (165 mg/day) SPI and 20 g of soy cotyledon fibre or a control combination of

50 g of casein and 20 g of cellulose for 6 weeks each separated by a 3-week washout period. Compared to the casein and cellulose control combination, the SPI and soy cotyledon fibre treatment combination significantly reduced plasma LDL-C, TG, apo B 100 as well as the LDL-C/HDL-C ratio. There were no significant effects of the soy protein and soy cotyledon fibre treatment combination on plasma TC, HDL-C, apo A-I or the apo B 100/apo A-I ratio (18). It is important to note that the use of both the soy protein and soy fibre in this study means that further study is needed to identify which is most responsible for the observed beneficial effects.

The next three studies summarized in Table 4 all investigated the effects of soy consumption on serum lipids in adults with type 2 diabetes who also had the existing complication of diabetic nephropathy (147-149). Anderson et al. (147) performed a randomized crossover study of 8 men with insulin treated type 2 diabetes with diabetic nephropathy. Subjects consumed a diet where 1 g protein/kg body weight was from either soy protein (in the form of beverages, meat analogue patties and ground meat analogues) or animal protein (in the form of ground beef and cow's milk) for 8 weeks each, separated by a 4-week washout period. The soy protein diet significantly decreased serum TC and TG but did not significantly affect serum LDL-C or HDL-C, when compared to the animal protein diet (147).

A similar study conducted by Azadbakht et al. (148) also evaluated the effect of soy consumption on serum lipids in 14 adults (10 men, 4 women) with diabetic nephropathy (subject medication use not described). Subjects consumed a diet (precise food types not indicated) containing 0.8 g protein/kg body weight/day in the profile of either a combination of 35% soy protein, 35% animal protein and 30% vegetable protein

or a combination of 70% animal and 30% vegetable protein for a period of 7 weeks each, separated by a 4-week washout period. Results revealed that the soy protein combination diet significantly decreased serum TC, LDL-C and TG but did not significantly change serum HDL-C or the LDL-C/HDL-C ratio, when compared to the animal protein combination diet (148).

The third study that evaluated the effect of soy consumption on serum lipids in adults with diabetic nephropathy was performed by Teixeira et al. (149) who included 14 men of which 8 were treated with insulin and oral hypoglycemic agents and 4 were treated with lipid-lowering medications. Following an initial 4-week run-in period to exclude all soy products from the diet, subjects replaced 0.5 g/kg body weight of their daily protein intake with either SPI (2 mg isoflavones/g protein) or casein for 7 weeks each, separated by a 4-week washout period. Results showed that the SPI diet significantly increased serum HDL-C, significantly decreased the LDL-C/HDL-C and TC/HDL-C ratios and did not significantly affect serum TC, LDL-C or TG, all when compared with the casein diet (149). Overall these three studies of adults with diabetic nephropathy have demonstrated positive effects of soy consumption on serum lipids. Although nephropathy is a common complication from type 2 diabetes, the inclusion of subjects with diabetic nephropathy introduces a potential confounding variable to these studies and rationalizes the need for further studies evaluating the effects of soy consumption in adults with type 2 diabetes.

Besides diabetic nephropathy, human intervention studies investigating the effects of soy on CVD risk in adults with type 2 diabetes have also included subjects with other diabetes-related complications. For example, subjects with microalbuminuria, a risk

factor for diabetic nephropathy, were included in a study by Wheeler et al. (150) that investigated the effect of a plant-based diet containing 62% of its protein from soy foods (protein was 17% of energy) on serum lipids. A total of 17 adults (14 men, 3 women) with type 2 diabetes were included, 16 of whom were treated with oral hypoglycemic agents and 4 of whom were treated with lipid-lowering medications. Subjects consumed the soy protein diet or a control animal protein diet for 6 weeks each, separated by a 4-week washout period in a randomized, crossover design. Although the soy protein diet significantly reduced plasma TC over the 6 weeks, it was not significantly different from the animal protein diet, nor were there any significant effects found for plasma HDL or TG (150). This study does not provide strong support for a role of soy protein in reducing circulating lipids in adults with type 2 diabetes who also have microalbuminuria.

Subjects with retinopathy, another diabetes complication, were included in a study by Oh et al. (151) that researched the effects of soy isoflavones on serum lipids in 12 postmenopausal Korean women with type 2 diabetes (subject medication use not described). The study was single-armed with no control group where the subjects consumed four isoflavone supplements daily (totaling 120 mg of genistein and 57 mg daidzein) for 12 weeks. Results showed no significant differences in serum TC, LDL-C, HDL-C, TG or HDL-C/LDL-C between baseline and week 12 (151). The lack of significance in this study could support the idea that soy isoflavones are only effective in reducing serum lipids when consumed with their associated proteins. However, a strong limitation of this study that deserves noting is the lack of a control group. Without a

control group, the results cannot be considered without questioning the potential influence of unknown confounding variables.

Other studies have included subjects without any diabetes complications. For example, in a randomized, crossover, double-blinded study conducted by Jayagopal et al. (98), 32 postmenopausal women with diet-controlled type 2 diabetes consumed 30 g of SPI (132 mg isoflavones) or 30 g of cellulose for 12 weeks each, separated by a 2-week washout period. Results showed significant decreases in serum TC, LDL-C and the TC/HDL-C ratio but no significant change in serum HDL-C or TG following consumption of SPI when compared to cellulose (98). Interpretation of this study is limited by the lack of protein in the control treatment thereby introducing the possibility that the results were due to a higher quantity of protein rather than a different quality of protein. The study is also limited by the uncertainty of whether the subjects were on a stable diet through out the course of the study.

A more recent study involving subjects with type 2 diabetes who were free from diabetic complications, but did exhibit lipid imbalances, was completed by Ristic Medic et al. (152). This 12-week single-armed study had no control group and included 47 adults with type 2 diabetes and hyperlipidemia (23 men and 24 postmenopausal women), 22 of whom were treated with oral hypoglycemic agents. The subjects were stratified into two groups according to their serum lipids status, including a hypertriglyceridemic group of 25 subjects ($TG \geq 1.7$ mmol/L and $LDL-C < 3.5$ mmol/L) and a combined hyperlipidemic group of 22 subjects ($TG \geq 1.7$ mmol/L and $LDL-C \geq 3.5$ mmol/L). All subjects consumed a dietary soy supplement powder composed of 35% soy protein, 24% carbohydrates, 12% lecithin, 19.2% high polyunsaturated phospholipid fat mix, 7.6% ash

and 0.3% aspartame (isoflavone content was not specified) for 12 weeks. Results showed significant changes at week 12 compared to baseline in many serum lipids including increased HDL-C and decreased TC, LDL-C, TG, TC/HDL-C and LDL-C/HDL-C, both in subjects overall as well as in the stratified subject groups (except LDL-C which was not reduced in the hypertriglyceridemic group). These findings support the role of soy in improving lipid profiles however it is important to note that this study did not include a control group. Without a control group, determination of how much the soy consumption specifically contributed to the changes cannot be determined.

Another human intervention study worth mention is a study by Li et al. (153) that included subjects with type 2 diabetes who also were obese, which is highly relevant since obesity often accompanies type 2 diabetes (34). This weight loss study examined the effects of a soy-based meal replacement on serum lipids in 82 obese adults with type 2 diabetes (51 men and 31 women) who were all treated with oral hypoglycemic agents. Subjects were randomly assigned to supplement their diet with a SPI meal replacement (SMR) shake (15 g protein, isoflavone amount not specified) or follow the American Diabetic Association food exchange system, for a total duration of 12 months. The subjects who were randomized to the SMR group were instructed to replace all three main meals with the SMR for the first 5 days and after that replace two meals a day with the SMR for the next 3 months. Finally, after 3 months they were instructed to replace 1-2 meals a day (based on subject preference) with the SMR for the remaining 9 months. Body weight and serum lipids were analyzed at baseline and at 3, 6 and 12 months. Although the SMR group experienced a significant reduction in weight loss compared to the control group, there were no significant improvements in serum lipids. There was,

however, significant improvements in serum lipids within the SMR group alone, including reduced TC, TG and LDL-C at 3 months, reduced serum TC and TG at 6 months, and reduced serum TG at 12 months (153). These reductions are encouraging but can be debated whether the effects are due to the soy consumption or the weight loss. The inconsistent consumption of the SMR between subjects in the SMR group after 3 months, when they had the choice to replace 1 or 2 meals a day with the SMR may also have confounded the results.

A final human intervention study of adults with type 2 diabetes worthy of mention is one that investigated the effects of isoflavones from red clover in an extracted form. Howes et al. (154) investigated the effects of isoflavones extracted from red clover (57 mg/day) on CVD risk factors in 19 postmenopausal women with type 2 diabetes of which 15 were treated with oral hypoglycemic agents. The randomized crossover, double-blinded, placebo controlled trial was 16 weeks long, composed of a 4-week run in period where subjects adhered to a low-isoflavone diet followed by the two 4-week treatment periods separated by a 4-week washout period. When compared with a placebo, the red clover isoflavone supplement did not significantly affect serum lipids but there was a trend toward reductions in TC ($p=0.092$) and LDL-C ($p=0.061$) (154). Even though this study did not use soy-derived isoflavones, the similarity between soy and red clover isoflavones justifies the importance of including this study in reviewing the literature of the role of soy on CVD risk in adults with type 2 diabetes.

Overall, there is limited research investigating the effects of isoflavone-rich soy protein on the effects of serum lipids in adults with diet controlled type 2 diabetes who have no existing diabetes complications. It would be very valuable to conduct more of

these studies as it would provide more data that can be utilized to promote prevention and/or improving the quality and prolonging the lives of those living with type 2 diabetes who have not yet had to introduce medications to reduce their CVD risk. Studies that have included adults with type 2 diabetes have primarily included subjects with diabetic nephropathy (147-149), microalbuminuria (150), retinopathy (151), hyperlipidemia (152), obesity (153) and/or subjects on medications related to management of their glycemic and/or lipid control (18, 147, 149, 150, 152-154). Study treatments have ranged and included soy fibre (18), SPI (98, 147-149, 152, 153) and isoflavones extracted from soy (151) or red clover (154). Since the ability of soy protein to reduce CVD risk through improved serum lipids has been well documented in adults without type 2 diabetes (86, 144, 145), there is rationale to continue investigation in adults with type 2 diabetes. The progress of this research is of particular importance to adults who are managing their type 2 diabetes without glycemic or lipid-lowering medications as this scenario represents a prevention opportunity. Any findings that aid this population in preventing the advancement of their diabetes into a more serious and advanced condition is valuable and prudent to the maintenance of the quality and quantity of their life.

Table 4: Summary of Human Intervention Studies Evaluating the Effects of Soy Protein and/or Isoflavones on CVD Risk Factors in Adults with Type 2 Diabetes¹

Reference	Study Design	Subject Characteristics	Study Treatments	Treatment Length	Study Endpoints	Significant Results
Hermansen et al. 2001 (18)	Randomized Crossover Double-blinded	n=20 adults with type 2 diabetes (14 men, 6 women; 11 subjects treated with diet, 9 subjects treated with hypoglycemic medications). None with diabetic complications.	50 g of SPI (165 mg/day isoflavones) and 20 g of soy cotyledon fibre 50 g of casein and 20 g of cellulose	6 weeks each separated by a 3-week washout period	Plasma TC, LDL-C, HDL-C, TG, apo B100, apo A-I, LDL-C/HDL-C, apo B100/apo A-I	↓LDL-C ↓TG ↓apo B100 ↓LDL-C/HDL-C (all by SPI and soy cotyledon fibre treatment)
Anderson et al. 1998 (147)	Randomized Crossover Not blinded	n=8 men with type 2 diabetes (all insulin treated) with diabetic nephropathy	1 g of soy protein/kg body weight (amount of isoflavones not reported (beverages meat analogues) 1 g of animal protein/kg body weight (beef and cows milk)	8 weeks each separated by a 4-week washout period	Serum TC, LDL-C, HDL-C, TG	↓TC ↓TG (all by the soy protein diet)

¹Abbreviations used: SPI=Soy protein isolate; TC=total cholesterol; LDL-C=Low-density lipoprotein cholesterol; HDL-C=High-density lipoprotein cholesterol; TG=triglyceride, apo B100=apolipoprotein B100; apo A-I=apolipoprotein A-I.

Table 4: Summary of Human Intervention Studies Evaluating the Effects of Soy Protein and/or Isoflavones on CVD Risk Factors in Adults with Type 2 Diabetes (Continued)¹

	Study Design	Subject Characteristics	Study Treatments	Treatment Length	Study Endpoints	Significant Results
Azadbakht et al. 2003 (148)	Randomized Crossover Not blinded	n=14 adults with type 2 diabetes (10 men, 4 women) with diabetic nephropathy (subject medication not described)	0.8 g protein/kg body weight composed of 35% soy protein, 30% vegetable protein and 35% animal protein (isoflavone amount not reported) 0.8 g protein/kg body weight composed of 70% animal protein and 30% vegetable protein (diet composition not published)	7 weeks each separated by a 4-week washout period	Serum TC, LDL-C, HDL-C, TG LDL-C/HDL-C	↓TC ↓LDL-C ↓TG (all by the soy protein combination treatment)
Teixeira et al. 2004 (149)	Randomized Crossover Not blinded	n=14 men with type 2 diabetes with diabetic nephropathy (8 subjects treated with insulin along with hypoglycemic agents, 4 subjects treated with lipid-lowering drugs)	Replaced 0.5 g/kg body weight of total dietary protein with SPI (2 mg isoflavones/g protein) Replaced 0.5 g/kg body weight of total dietary protein with casein	4-week run-in period followed by 7 weeks each separated by a 4-week washout period	Serum TC, LDL-C, HDL-C, TG LDL-C/HDL-C TC/HDL-C	↑HDL-C ↓LDL-C/HDL-C ↓TC/HDL-C (all by SPI)

¹Abbreviations used: SPI=Soy protein isolate; TC=total cholesterol; LDL-C=Low-density lipoprotein cholesterol; HDL-C=High-density lipoprotein cholesterol; TG=triglyceride, apo B100=apolipoprotein B100; apo A-I =apolipoprotein A-I.

Table 4: Summary of Human Intervention Studies Evaluating the Effects of Soy Protein and/or Isoflavones on CVD Risk Factors in Adults with Type 2 Diabetes (Continued)¹

Reference	Study Design	Subject Characteristics	Study Treatments	Treatment Length	Study Endpoints	Significant Results
Oh et al. 2005 (151)	Single-arm No control group	n=12 postmenopausal women with type 2 diabetes and retinopathy (subject medications not described)	Extracted isoflavone supplement (120 mg of genistein and 57 mg of daidzein)	12 weeks	Serum TC LDL-C HDL-C TG LDL-C/HDL-C	None
Jayagopal et al. 2002 (98)	Randomized Crossover Double blinded	n=32 postmenopausal women with diet-controlled type 2 diabetes	30 g of SPI (132 mg isoflavones) 30 g of cellulose	12 weeks each separated by a 2-week washout period	Serum TC, LDL-C, HDL-C, TG, TC/HDL-C	↓TC ↓LDL-C ↓TC/HDL-C (all by SPI)
Ristic Medic et al. 2006 (152)	Single-arm No control group	n=47 adults with type 2 diabetes and hyperlipidemia (23 men and 24 postmenopausal women) stratified into groups: 25 with hypertriglyceridemia and 22 with combined hyperlipidemia (22 treated with oral hypoglycemic agents)	dietary soy protein powder supplement: 35% soy protein (isoflavone content not specified)	12 weeks	Serum TC LDL-C HDL-C TG TC/HDL-C LDL-C/HDL-C	↓TC ↓LDL-C ↑HDL-C ↓TG ↓TC/HDL-C ↓LDL-C/HDL-C Observed in all subjects as well as both stratified subject groups

¹ Abbreviations used: SPI=Soy protein isolate; TC=total cholesterol; LDL-C=Low-density lipoprotein cholesterol; HDL-C=High-density lipoprotein cholesterol; TG=triglyceride, apo B100=apolipoprotein B100; apo A-I=apolipoprotein A-I.

Table 4: Summary of Human Intervention Studies Evaluating the Effects of Soy Protein and/or Isoflavones on CVD Risk Factors in Adults with Type 2 Diabetes (Continued)¹

Reference	Study Design	Subject Characteristics	Study Treatments	Treatment Length	Study Endpoints	Significant Results
Li et al. 2005 (153)	Randomized Parallel-arm Not blinded	n=82 obese adults with type 2 diabetes (51 men and 31 women) treated with oral hypoglycemic agents	SPI meal replacement shake (15 g SPI; amount of isoflavones not reported); replaced 3 meals per day for first 5 days; 2 meals per day for next 3 months and 1-2 meals per day for final 9 months) Control: followed the American Diabetic Association food exchange diet	12 months	Serum TC, LDL-C, TG HDL-C, TG	At 3 months: ↓TC, ↓TG and ↓LDL (all within SPI treatment relative to baseline) At 6 months: ↓TC and ↓TG (all within SPI treatment relative to baseline) At 12 months: ↓TG (within SPI treatment relative to baseline)
Howes et al. 2003 (154)	Randomized Crossover Double blinded	n=19 postmenopausal women with type 2 diabetes (15 subjects treated with oral hypoglycemic agents)	Red clover isoflavone extract (57 mg/day) Isoflavone-free placebo	4-week run-in period followed by 4 weeks each separated by a 4-week washout period	Serum TC, LDL-C, HDL-C, TG	None Non-significant trend toward ↓TC (p=0.092) and ↓LDL-C (p=0.061) (both by the red clover isoflavone extract treatment)

¹ Abbreviations used: SPI=Soy protein isolate; TC=total cholesterol; LDL-C=Low-density lipoprotein cholesterol; HDL-C=High-density lipoprotein cholesterol; TG=triglyceride, apo B100=apolipoprotein B100; apo A-I=apolipoprotein A-I.

B. STUDY RATIONALE

It is clear that numerous studies have revealed the positive effect of soy protein and its associated isoflavones on circulating lipids and reducing CVD risk in the general population (86, 144, 145). Since this effect would be of specific value in adults with type 2 diabetes, studies at the epidemiological, *in vitro*, animal and human intervention levels have begun to investigate the impact of soy on circulating lipids in this population. However, the human intervention studies that have been conducted in this area of research, have often included subjects on anti-diabetic and/or lipid lowering medications (18, 147, 149, 150, 152-154), which could confound the effects of a soy intervention. Many of these human intervention studies have also included subjects who are obese (153) or have advanced secondary diabetes-related complications or conditions such as nephropathy (147-149), microalbuminuria (150), retinopathy (151) and hyperlipidemia (152). Other human intervention studies have examined isoflavones extracted from soy protein (151) or red clover, (151, 154) removing the natural matrix in which the isoflavones are associated. Finally, other human intervention studies have used treatments of SPI but used controls such as fibre (18) or cellulose (98) which make the treatment and control imbalanced and incomparable. Overall, there are several human intervention studies that have examined the effect of soy on circulating lipids in adults with type 2 diabetes but none have focused on the effects of SPI in adults with diet-controlled type 2 diabetes in comparison to a protein-matched control. Thus, studying the effect of SPI (with its naturally occurring isoflavones intact) in comparison to a protein-matched control on serum lipids in adults with type 2 diabetes (who are not on

anti-diabetic or lipid-lowering medications and do not have any diabetes complications) would be of value and add to the existing literature.

C. STUDY PURPOSE, OBJECTIVES AND HYPOTHESES

The purpose of this study was to determine the effect of isoflavone-rich soy protein consumption on serum lipids in adults with diet-controlled type 2 diabetes when compared to a protein-matched control. The specific objectives of the study were to:

1. determine the effect of isoflavone-rich soy protein consumption on serum concentrations of TC, HDL-C, LDL-C, TG in adults with diet-controlled type 2 diabetes;
2. determine the effect of isoflavone-rich soy protein consumption on serum concentrations of apo B and apo A-I, apo B in adults with diet-controlled type 2 diabetes;
3. determine the effect of isoflavone-rich soy protein consumption on specific ratios of serum lipids (TC/HDL-C, LDL-C/HDL-C, TG/HDL-C, non-HDL-C/HDL-C) and apolipoproteins (apo B/apo A-I) in adults with diet-controlled type 2 diabetes.

The hypotheses of the study in relation to each study objective were:

1. the consumption of isoflavone-rich soy protein will decrease serum TC, LDL-C and TG, and increase HDL-C, when compared to milk protein isolate, in adults with diet-controlled type 2 diabetes;
2. the consumption of isoflavone-rich soy protein consumption will decrease serum apo B and increase apo A-I when compared to milk protein isolate, in adults with diet-controlled type 2 diabetes;

3. the consumption of isoflavone-rich soy protein consumption will decrease specific ratios of serum lipids (TC/HDL-C, LDL-C/HDL-C, TG/HDL-C, non-HDL-C/HDL-C) and apos (apo B/apo A-I), when compared to milk protein isolate, in adults with diet-controlled type 2 diabetes.

D. MATERIALS AND METHODS

1. Subjects

1.a. Recruitment and Screening

Adults with diet controlled type 2 diabetes were recruited from Guelph, ON and the surrounding area. Recruitment methods included newspaper advertisements, group emails, recruitment posters (Appendix 1), letters to local family physicians, and interaction with the local Diabetes Education Centres (DEC) to make announcements to clients.

Potential subjects were screened for study eligibility with a preliminary phone questionnaire (Appendix 2). An in-person meeting between the potential subject and the study coordinators occurred soon after the phone questionnaire. At this meeting, potential subjects completed a more in-depth questionnaire (Appendix 3), had their body weight measured, provided their most recent glycosylated hemoglobin (HbA1c) value, and sampled the study treatment protein powders. In addition, they were provided with oral and written information about the study design, expectations and requirements and encouraged to ask questions. After reviewing the phone and in-depth study questionnaires, and body weight to confirm eligibility, the potential subject was contacted to determine if they were still interested in participating in the study. If they were, then an official study

welcome letter was mailed to them which included a schedule of their study visit dates (Appendix 4).

1.b. Inclusionary and Exclusionary Criteria

Subject inclusion criteria included:

- adult (> 19 years old) males and postmenopausal females (no menstrual bleeding for at least 12 months or >50 years old with a hysterectomy)
- diagnoses of type 2 diabetes (fasting plasma glucose ≥ 7.0 mmol/L) (155)
- good glycemic control (blood HbA1c $\leq 8\%$) (155)
- on stable medications for at least 3 months before study entry
- on a stable diet (those who had undergone dietary training at their local Diabetes Education Centre and were stable in these diet modifications).

Subject exclusionary criteria included:

- premenopausal females (to avoid influences of the menstrual cycle on study endpoints)
- body mass index (BMI) > 35 kg/m² (to exclude individuals who were more likely to lose body weight during the study)
- treatment with oral hypoglycemic agents or insulin therapy
- treatment with lipid-lowering medications
- hormone replacement therapy or use of hormonal birth control (may interfere with isoflavone-related effects)
- renal or liver disease
- frequent alcohol consumption (> 2 drinks/day or 7 drinks/week)
- antibiotic use within 3 months (can influence the metabolism of soy isoflavones)
- allergy to soybeans or milk
- frequent soy consumption (> 3 servings/week)

- vegan diet.

1.c. Study Orientation

Following recruitment screening and receipt of the study welcome letter, subjects were invited to a study orientation designed to explain all aspects of the study. A comprehensive study handbook was provided to the subjects and included all the information they would need to know to participate in the study. The study handbook included contact information for the study coordinators, explanation of the significance of the research, a calendar of events outlining what will occur at each study visit, instructions on how to consume the study treatment powders including numerous recipes, information on the study dietary and medication restrictions, instructions on all sample collections and explanations of all study measurements. At the conclusion of the study orientation, subjects provided written consent of their participation in the study by signing a study consent form (Appendix 5).

2. Study Design

The study was approved by the Human Research Ethics Board of the University of Guelph (Appendix 6) and received funding from the Heart and Stroke Foundation of Ontario. The study utilized a randomized crossover design in which both the study researchers and subjects were blinded to the order of the treatments. The study consisted of two 8-week treatment periods separated by a 4-week washout period for a total duration of 20 weeks.

3. Study Treatment Powders

3.a. Randomization of Study Treatments

The study included two treatments of which were assigned the letters A and B to facilitate blinding. Subjects were randomly assigned to one of the treatments for the first treatment period and the other treatment for the second treatment period. Fifty three percent of subjects (17/29) started treatment period I with the milk protein isolate (MPI) treatment and 47% of subjects (15/29) started treatment period I with the SPI treatment. The complete study treatment randomization table is provided in Appendix 7.

3.b. Study Treatment Powders

The treatment protein powders included a MPI and an isoflavone-rich SPI, both provided by The Solae Company, St. Louis, MO. Study treatment powders were provided to subjects in a 14-day supply on study days 1, 15, 29 and 43 of each treatment period. Subjects received two 29 g packets of treatment powders for each day, which provided a total of 40 grams of milk or soy protein and 0 or 80 mg of aglycone isoflavones (for the MPI and SPI, respectively). Both protein powders were unsweetened and similar in energy and nutrient composition. The nutritional and isoflavone composition of one 29 g packet of the treatment powders is presented in Table 5.

Table 5: Nutritional and Isoflavone Composition of One 29 g Packet of Treatment Powder.^{1, 2, 3}

	Amount
Energy (kilocalories)	100
Protein (g)	20
Isoflavones ⁴ (mg aglycone)	40
Total Carbohydrate (g)	4 – 5
Sugars (g)	2
Dietary Fibre (g)	0
Total Fat (g)	0.5 – 1
Cholesterol (mg)	0 – 5
Calcium (mg)	700
Magnesium (mg)	40 – 100
Sodium (mg)	120 - 190
Potassium (mg)	5 - 150
Iron (mg)	0.9 – 3.6
Phosphorus (mg)	500
Vitamin A (IU)	500
Riboflavin (mg)	0.4
Folate (µg)	30-60
Vitamin B ₁₂ (µg)	0.9
Vitamin C (mg)	0
Vitamin D (IU)	100

¹Data provided by The Solae Company, St. Louis, MO.

²All data applies to both the MPI and SPI treatment powders, with the exception of isoflavone content, which only applies to the SPI treatment powder.

³Subjects consumed two 29 g treatment powder packets per day.

⁴Isoflavone content of SPI treatment powder only.

Subjects supplemented their habitual diets with the treatment protein powders and were provided with multiple methods of consuming them. To avoid consumption of excess protein, subjects were counseled to replace certain foods (e.g. milk, cheese, lunch deli meat) with their treatment protein powders. Subjects were encouraged to simply mix their treatment powders with water and had the option of using Nestle™ flavour packets which were available in multiple flavours (chocolate, vanilla, orange, cappuccino and

Irish cream). In attempt to encourage and maximize compliance, subjects were asked to record how and when they consumed their treatment powders each day in a daily study diary. To create a more consistent exposure to the treatment powder, subjects were asked to consume their two treatment powder packets at different times each day, preferably one in the morning and one in the afternoon.

3.c. Study Dietary Restrictions

Subjects were instructed to consume their habitual diet and maintain their lifestyle habits throughout the study with a few specific modifications and restrictions. Subjects were provided with various dietary restrictions to minimize their background phytoestrogen consumption. These restrictions included the avoidance of soy products (soy lecithin and soy oil were permitted), flaxseed, beans and legumes (green beans were permitted) and whole grains. Further, since the study treatment powders were fortified with calcium, subjects were instructed to avoid fluid milk and calcium-fortified beverages. Subjects were also instructed to avoid all dietary supplements, protein bars, green tea and limit alcohol consumption to 7 drinks per week and 2 drinks at one sitting (with 1 drink defined as 350 mL of beer, 5 oz of wine or 1.5 oz of liquor).

4. Data Collection

All subject study visits occurred at the Human Nutraceutical Research Unit (HNRU) in the J.T. Powell building at the University of Guelph. Subjects came to the HNRU on days 1, 15, 29, 43 and 57 of each treatment period for various study measurements. The study coordinators used a binder for each subject to keep individual subject information organized and referred to this binder to record data obtained from each study visit. Study flow sheets (documents which outlined what should be

accomplished on each study day) were included in the subject binders and fully informed the study coordinators with what needed to be done on each study day. Figure 10 illustrates the data collection schedule for each 57-day treatment period.

Figure 10: Data Collection Schedule for Each 57-Day Treatment Period.

¹Food Records were recorded for three consecutive days on study days 2-4; study days 26-28; and study days 50-52. In addition, there was one pre-study 3-day food record completed during the week before the first treatment period.

²Height, blood pressure, heart rate and hemoglobin A1c were measured only on day 1 to characterize the subjects.

³BIA = Bioelectrical Impedance Analysis for measurement of body composition.

⁴Urine was collected for three consecutive 24 hour days on study days 54-56.

4.a. Anthropometric and Other Body Measurements

Fasted body weight was measured on all study days (days 1, 15, 29, 43 and 57) in each treatment period. A digital scale (OHAUS® ES200L) was used and measured body weight to the nearest 0.1 kg. Subjects were instructed to wear light clothing and remove their shoes for the body weight measurement.

Height was measured on study day 1 of the first treatment period. Subjects were instructed to stand tall, facing forward with their feet flat on the ground and their back against the measuring stick attached to a manual weigh scale. Height was measured in inches to the nearest 0.25 inch and then converted to meters.

Body composition was measured on study days 1 and 57 of each treatment period using bioelectrical impedance analysis (BIA) (BodyStat 1500™, Tampa, FL) at which time subjects were fasted for 12 hours. This machine measures the resistance of an electric current through the body to determine body composition of fat, lean mass and water. Since the measurement is most accurate when the subject is well hydrated, subjects were instructed to drink two glasses of water the night prior to their BIA measurement and another two glasses of water the morning of their BIA measurement. To perform the BIA procedure, subjects were instructed to lie down on a medical bed and remove their right sock, and any metal jewelry or accessories. Rubbing alcohol was applied to the back of their right hand and foot. Two electrodes were then placed on the back of their hand (one on the wrist bone and one beneath the middle knuckle) and two electrodes were placed on the back of their foot (one on the ankle bone and one below the middle toe knuckle). Subject gender, age, height, and body weight were entered into the

BIA machine and body composition was determined and digitally available within seconds and recorded.

Waist and hip circumference were measured to the nearest 0.1 cm on days 1 and 57 of each treatment period using a metric measuring tape. Waist circumference was measured on bare skin at the midpoint between the bottom rib and the top of the hip of the subjects. Subjects aided in identifying those points on their body and the midpoint was determined. A coloured crayon was applied to the subject's skin to mark the waist point on both the left and right side. A measuring tape was then wrapped around the subject and aligned with the marked points to obtain waist circumference. Hip circumference was measured, clothed, around the widest point of the subject's lower body with the same measuring tape. Subjects were asked to remove any wallets or other items from back pockets which could add to the measurement.

Systolic and diastolic blood pressure was analyzed using a digital blood pressure machine with an automatic inflatable upper arm cuff on study day 1 of the first treatment period to provided baseline subject characteristics. Heart rate was determined at the same time by the same blood pressure machine. This measurement was performed after the subjects had been seated for 5 minutes.

4.b. Food Records

To monitor energy and nutrient intakes throughout the treatment periods, 3-day food records were completed at periodic time points during each treatment period including study days 2-4, 26-28 and 50-52. In addition, one 3-day food record was completed one week before the first treatment period. Subjects were provided with pre-dated food record sheets that included spaces to provide the time of consumption, amount

of food and or beverage consumed and a description of the food or beverage consumed. Subjects were fully trained on how to complete accurate food records with an emphasis on detail including the provision of cooking method, brand names and attachment of food labels. For complex homemade dishes, subjects were asked to provide the recipe and indicate the fraction of the recipe they consumed. For meals consumed at restaurants, subjects were asked to indicate the restaurant they dined at and offer as much detail as they could about the preparation and size of their meal. If subjects consumed packaged foods the food packaging or label was also requested to be handed in. Food records were reviewed by study coordinators throughout the study and subjects were consulted as needed to confirm completeness and accuracy.

4.c. Urine Collections

Twenty-four hour urine collections were completed on study days 54, 55 and 56 during each treatment period. On study day 43 of each treatment period, subjects were provided with an opaque 3-liter container (VWR International) containing 3 g of ascorbic acid (as a preservative) for each urine collection day. Urine containers displayed a label with space to record the start and end times of the collection and volume of any spills. In addition, subjects were provided with a 1-liter bottle (Nalgene™) and an insulated lunch bag with 2 ice packs to provide temporary storage for urine during travel. Subjects were instructed to transfer the urine from the 1-liter container into the 3-liter container as soon as possible and to keep the 3-liter container refrigerated. Female subjects were provided with a urine collection “hat” to assist in urine collection.

Subjects were provided with comprehensive individual instruction on how to complete their 24-hour urine collections. For their first 24-hour urine collection, subjects

were instructed that their first urination of the day was not to be collected but rather discarded and that the time of this first urination was to be recorded as the start time for the 24-hour urine collection. All subsequent urinations would then be collected with the final urination of the 24-hour collection as the first urination in the morning of the following day. This time was to be recorded as the end time for the first 24-hour urine collection but also as the start time for the second 24-hour urine collection. This pattern was to then be followed for each of the three 24-hour urine collections. Subjects were instructed to wake up at the same time each day during their urine collection period in order to have a consistent start and end time for each of their 24-hour urine collections.

On study day 57, subjects dropped off their three 24-hour urine collections after which study coordinators recorded start and end times, total volume and spill volumes for each 24-hour collection. The entire urine collection was then gently mixed and aliquoted into 15 ml conical tip polypropylene tubes (Sarstedt™) and stored at -20°C until analysis.

4.d. Blood Collections

Blood samples were obtained on study days 1 and 57 of each treatment period at a consistent time for each subject. Subjects received specific instructions prior to their blood sample including no food or beverages for 12 hours but were encouraged to drink water. They were also instructed to avoid alcohol, strenuous activity and over-the-counter medications for 72 hours prior to their blood sample. Blood collection occurred by venipuncture and was performed by a trained phlebotomist. Blood was collected into red-top vacutainers with no anti-coagulant. Vacutainers were then left to sit at room temperature for 30 minutes prior to centrifugation at 5°C for 15 minutes at 2500 rpm. Serum was then aliquoted from the centrifuged sample by a disposable pipette into 1 mL

cryovials and stored at -80°C until analysis. Samples of whole blood were also collected and analyzed for hemoglobin A1c for baseline subject characteristics purposes.

4.e. Study Diaries

Subjects were required to complete a daily study diary during each treatment period. Dated study diary sheets for the entire treatment period were provided at the beginning of each treatment period and were handed in by subjects every 14 days at each study visit. The study diary included space for subjects to record information such as: how and when they consumed their treatment powders, medications consumed, physical activity, illnesses and any other information they thought was relevant.

5. Subject Compliance

5.a. Communication with Subjects

Communication with subjects was initiated and maintained on a consistent basis from the very beginning of the study, specifically starting at the study orientation. At the study orientation, the study coordinators went through the entire study handbook with the subjects, deliberately emphasizing how important communication was to the study and how much it would be valued. During the study, study day reminder calls and emails were sent to every subject providing ample opportunity for subjects to ask questions and express any concerns. Communication with subjects was always documented and recorded. A subject comment box was also present for all study visits and subjects were encouraged to offer comments, concerns or questions by putting a written note in the comment box which could be either anonymous or identified.

Communication with subjects was also promoted through periodic study newsletters. These newsletters were distributed throughout the study and informed

subjects on key study-related issues, reminders and instructions. Study newsletters also included information on several study contests with prizes; examples of contests included ‘naming of the newsletter’ and raffles for diabetes related cookbooks.

Communication with subjects was also achieved through the study diaries (see section 4.e.). Study diary sheets were handed in at every study visit (except study day 1) and represented the previous 14 days of the study. These sheets were reviewed by the study coordinators thereby allowing for prompt identification of any study-related issues, such as missed powders, illness or medication changes, that could then be discussed with the subject.

5.b. Urinary Creatinine and Urinary Isoflavones

Subject compliance with the 24-hour urine collections was assessed through the measurement of creatinine in every 24-hour urine collection. Creatinine is excreted in the urine as a waste product from muscle metabolism (156). Since the amount of creatinine that is excreted per kg of muscle (due to non-enzymatic breakdown reactions including hydrolysis and dehydration) remains fairly consistent, urinary creatinine is commonly utilized as a marker of completeness for a 24-hour urine collection (156). To determine the variability of urinary creatinine among the subject’s three 24-hour urine collections, a coefficient of variation (CV) was calculated for each treatment period. Information reported by the subject about their urine collection, such as the start and end time or if there were any spills was taken into consideration when evaluating their CV. If the subject’s CV was greater than 15% in their first treatment period, the subject was counseled regarding proper urine collection for their second treatment period.

5.c. Treatment Period Reviews

At the completion of each treatment period, all available study data for each subject (which was compiled in the subject's study binder) was reviewed in a treatment period review document (Appendix 8). The review consisted of a summary of information about the treatment period. Details noted about the treatments themselves included what treatment the subject consumed, how and when they consumed their treatment powder, and any feedback they provided about the treatment powders. The treatment period review also included a summary of the subject's study days in terms of any problems or schedule deviations. Subject medications were also reviewed and any changes were noted. Subject's body weight during the treatment period was summarized in a table to identify any changes that required attention. Completeness and detail of both food records and study diaries were noted and any issues for follow-up were identified. Details about the urine collection including CV, start and end times, spills or problems were summarized. Finally, any overall comments about the subject were noted and any action items that required follow-up were listed.

6. Data and Laboratory Analysis

6.a. Food Records

Information from all 3-day food records was entered into a computer program (Food Processor SQL Edition ESHA Research, Salem, Oregon © 2006 Version 8.9.1) which analyzed for energy, nutrient and dietary fibre intakes. Average intakes of energy, carbohydrates, total fat, saturated fatty acids, monounsaturated fatty acids, polyunsaturated fatty acids, protein, cholesterol, dietary fibre and calcium were calculated for each 3-day food record.

6.b. Serum Lipids

All serum samples were analyzed for TC, HDL-C and TG at the Guelph General Hospital using an auto-analyzer (Synchron CX Systems, Beckman Coulter, Inc. Mississauga, ON). All samples were analyzed on the same day by the same individual and a control sample was included at the start and end of the analysis for calculation of intra-assay variability. Intra-assay variability was 0.97% for TC, 0.61% for HDL-C and 8.90 % for TG.

Specific reagents for TC, HDL-C and TG were used to prepare samples for each analysis and the quantification of TC, HDL-C and TG in the samples was determined by the system measuring the change in absorbance at 520nm, 560nm and 520nm, respectively. The change in absorbance is directly proportional to the concentration of TC, HDL-C or TG in the blood and is used by the auto-analyzer to calculate and express the lipid concentrations. LDL-C was calculated using the Friedewald equation (46).

6.c. Serum Apolipoproteins

All serum samples were analyzed for apo B and apo A-I at the Lipid Research Lab at St. Michael's Hospital, Toronto ON. All samples were analyzed on the same day by the same individual and a control sample was included at the start and end of the analysis for calculation of intra-assay variability. Intra-assay variability was 2.53% for apo B and 1.44% for apo A-I.

Serum apo B and apo A-I were analyzed by nephelometry (157) using a Behring Nephelometer ProSpec® System Assay Loader (Dade Behring Inc., Mississauga, ON). This system takes an aliquot of serum and mixes it with an apolipoprotein specific antibody reagent. The mix of serum sample and reagent then has a light source (an

infrared high performance light emitting diode) directed through it. The scattering of light that occurs on the other side of the sample is measured and used to determine the apolipoprotein content in the sample. When the antibodies present in the reagent attach to their respective apolipoproteins, they form insoluble complexes that scatter light in solutions. Therefore the more apolipoproteins present the more scattering of light that will be detected.

6.d. Urinary Creatinine

Aliquots from every 24 hour urine collection were analyzed for creatinine by the Animal Health Laboratory of Laboratory Services Division at the University of Guelph. The analysis utilized an enzymatic ultraviolet method (Randox Laboratories Canada Ltd., Mississauga, ON) on a Roche Hitachi 911 auto analyzer. The Animal Health Laboratory included control samples in every analysis, indicating an inter-assay variability of 2.85%.

6.e. Urinary Isoflavones

Aliquots from each 24-hour urine collection of each 3-day urine collection were thawed and combined proportionally to create a pooled sample to represent the entire 3-day urine collection. The pooled samples were analyzed for isoflavones (genistein and daidzein) and isoflavone metabolites (equol and O-DMA) using high performance liquid chromatography at the Fred Hutchinson Cancer Research Center, Seattle, WA in the laboratory of Dr. Johanna Lampe. Control samples were included in every assay, revealing an inter-assay variability of 3.11% for genistein, 4.20% for daidzein, 4.87% for O-DMA and 3.97% for equol.

7. Statistical Analysis

Endpoints were independently examined using box plots, stem leaf diagrams and normal probability plots to assess their distribution. Examination revealed that all endpoints were normally distributed and did not require any transformation to comply with assumptions of the statistical analyses. Residual plots were also examined for all endpoints in order to assess their variance distribution. This examination revealed random patterns in all residual plots, confirming that no major unaccounted confounding variables were contributing to the variations in the endpoints.

To ensure the washout period was sufficient, day 1 values for serum TC, LDL-C, HDL-C, TG, apo B and apo A-I from the both treatment periods were compared using repeated measures analysis of variance (ANOVA), controlling for subject, treatment period and treatment.

The effect of treatment on serum lipids, apolipoproteins and their ratios at study day 57 was determined using repeated measures ANOVA controlling for subject, treatment period, and treatment and including study day 1 values as a covariate.

The effect of treatment on anthropometric, food record and urinary isoflavone data was determined using repeated measures ANOVA controlling for subject, treatment period and treatment.

The Statistical Analysis System, version 9.1 (SAS Institute, Inc., Cary, NC), was used for all statistical analyses with results considered statistically significant if $p \leq 0.05$.

E. RESULTS

1. Subject Dropouts and Exclusions

As summarized in Table 6, there were a total of 5 subjects who dropped out of the study, or were excluded from the study. One subject dropped out due to personal family issues. One subject dropped out due to dislike of the study treatment powders. One subject dropped out due to a cancer diagnosis. One subject was excluded due to their lack of commitment to the study schedule. One subject was excluded from the study after the laboratory analysis when it was discovered that his study entry HbA1c of 11.5% did not actually meet the study inclusionary cutoff criteria of less than 8%. Similarly, his serum lipid data was much higher than the remainder of the subjects, specifically his baseline (treatment period one, study day one) total-cholesterol was 5.30 mmol/L in comparison to the study average of 4.36 mmol/L. A total of 29 subjects were included in the final statistical analysis.

Table 6 Summary of Subject Dropouts and Exclusions¹

Subject Number	Removal Time	Dropped out or Excluded	Reason
22	TP I, Study Day 13	Dropped out	Personal family issue
28	TP I, Study Day 15	Dropped out	Disliked study treatment powders
34	Last week of Washout Period	Dropped out	Cancer diagnosis
16	TP II, Study Day 1	Excluded	Lack of commitment to study schedule
28 (re-assigned)²	Post study	Excluded	Data outlier

¹Abbreviations used: TP=Treatment period.

²Following the drop-out of the original subject 28, the number 28 was re-assigned to another incoming subject.

2. Subject Characteristics

Table 7 presents baseline data for anthropometrics, glycemic control (HbA1c), serum lipids and medication use for the 29 subjects (13 women, 16 men) whose data were included in the final analysis. The average BMI value at baseline was below the study inclusionary cut-off of 35 kg/m². Baseline serum lipid concentrations were classified as normal according to the Heart and Stroke Foundation of Canada classification system (31), indicating the subjects were not hyperlipidemic. Subjects had good glycemic control (as indicated by an average HbA1c of 5.98%) and were taking an average of 1.80 medications which did not include any glycemic, lipid-lowering or hormonal medications.

Table 8 presents the body weight, BMI and percent body fat data for each treatment and indicate no significant differences.

3. Energy, Macronutrient, Dietary Fibre, Cholesterol and Calcium Intakes

Results of the food record analysis are presented in Table 9. There were no significant differences in energy, protein, carbohydrate, total fat, monounsaturated fat, polyunsaturated fat, dietary fibre, cholesterol or calcium intakes between the MPI and SPI treatments periods. However, saturated fat intake was significantly ($p=0.04$) lower during the SPI treatment period compared to the MPI treatment period.

When the 3-day food record completed before the study was compared to the 3-day food records completed during the study, results indicated that both protein ($p<0.0001$) and calcium ($p<0.0001$) were significantly higher during the study, whereas there were no significant differences in energy, carbohydrate, fat, or dietary fibre.

Table 7: Subject Characteristics at Baseline^{1,2}

Age (years)	60.1 ± 9.6
Gender (n)	Female (13), Male (16)
Body Weight (kg)	83.4 ± 10.9
Height (m)	1.69 ± 0.10
BMI (kg/m ²)	29.5 ± 3.38
Body Fat (%)	38.5 ± 10.1
Waist/Hip Ratio	0.92 ± 0.07
Systolic Blood Pressure (mg Hg)	134.7 ± 16.1
Diastolic Blood Pressure (mg Hg)	83.7 ± 8.51
Heart Rate (beats per minute)	65.9 ± 8.49
Serum Total cholesterol (mmol/L)	4.58 ± 0.87
Serum LDL-cholesterol (mmol/L)	2.92 ± 0.69
Serum HDL-cholesterol (mmol/L)	1.16 ± 0.26
Serum Triglycerides (mmol/L)	1.10 ± 0.44
Serum non-HDL-C (mmol/L)	3.41 ± 0.76
Apolipoprotein A-I (g/L)	1.42 ± 0.23
Apolipoprotein B (g/L)	0.93 ± 0.20
HbA1c (%)	5.98 ± 0.62
Number of Medications (n)	1.80 ± 1.0

¹Values are means ± SD unless otherwise indicated.

²Abbreviations used: BMI=Body mass index; Hg=Mercury; LDL=Low-density lipoprotein; HDL=High-density lipoprotein; HbA1c=Hemoglobin A1c.

Table 8: Subject Characteristics During the Study^{1,2}

	MPI (n=29)	SPI (n=29)	P Value
Body Weight (kg)	83.3 ± 10.5	83.1 ± 10.3	0.3127
BMI (kg/m ²)	29.4 ± 3.71	29.2 ± 3.59	0.2605
Body Fat (%) ³	37.5 ± 12.4	36.2 ± 10.6	0.1180

¹Values are means ± SD.

²Abbreviations used: MPI=Milk protein isolate; SPI=Soy protein isolate; BMI=Body mass index.

³Percent body fat was determined using bioelectrical impedance analysis (BIA).

Table 9: Energy, Macronutrient, Dietary Fibre, Cholesterol and Calcium Intakes^{1,2}

	Pre-Study ³ (n=29)	MPI ⁴ (n=29)	SPI ⁴ (n=29)
Energy (kcal)	1988 ± 69.0	2139 ± 41.9	2055 ± 41.9
Protein (g)	87.3 ± 3.41 ^a	120.1 ± 2.43 ^b	118.6 ± 2.43 ^b
Carbohydrate (g)	239.7 ± 8.92	237.4 ± 5.75	230.2 ± 5.75
Fat (g)	75.6 ± 3.86	78.8 ± 2.09	73.4 ± 2.09
SFA (g)	22.0 ± 1.57 ^{ab}	24.4 ± 0.73 ^a	22.2 ± 0.73 ^b
MUFA (g)	22.3 ± 1.45	21.2 ± 0.77	20.7 ± 0.77
PUFA (g)	10.7 ± 0.74	10.3 ± 0.60	10.1 ± 0.60
Dietary Fibre (g)	24.6 ± 1.33	24.0 ± 1.01	22.8 ± 1.01
Cholesterol (mg)	253.0 ± 22.8	276.1 ± 16.9	261.1 ± 16.9
Calcium (mg)	920.7 ± 59.5 ^a	1959 ± 35.9 ^b	2002 ± 35.9 ^b

¹Values are least squares means ± SE. Values with different letter subscripts within a row are significantly different ($p \leq 0.05$).

²Abbreviations used: MPI=Milk protein isolate; SPI=Soy protein isolate; kcal=Kilocalories; SFA=Saturated fatty acids; MUFA=Monounsaturated fatty acids; PUFA=Polyunsaturated fatty acids.

³Values are based on the results of one 3-day food record completed before the study.

⁴Values are based on the average of three 3-day food records completed on treatment period days 2-4, 26-28, 50-52; these data include the contributions from the study treatment powder proteins.

4. Urinary Isoflavone Excretion

Results of the urinary isoflavone analysis are presented in Table 10. A significant increase in excretion of isoflavones (genistein, daidzein) and isoflavone metabolites (equol, O-DMA) occurred following consumption of the SPI treatment, when compared with the MPI treatment ($p < 0.02$ for all comparisons). The variability in urinary equol excretion within the SPI treatment was further analyzed and revealed that 6 subjects

could be classified as equol excretors (urinary equol>1000 nmol/24hours (158)) and the remaining 23 subjects could be classified as equol non-excretors.

Table 10: Urinary Isoflavone Excretion^{1, 2, 3}

	MPI (n=29)	SPI (n=29)	P Value
Genistein (nmol/24 hour)	92 ^a (55, 154)	11735 ^b (7034, 19578)	<0.0001
Daidzein (nmol/24 hour)	134 ^a (78, 228)	13723 ^b (8033, 23444)	<0.0001
O-DMA (nmol/24 hour)	32 ^a (17, 59)	7761 ^b (4149, 14517)	<0.0001
Equol (nmol/24 hour)	21 ^a (10, 43)	77 ^b (38, 158)	0.0149

¹All data were transformed on the natural log scale prior to statistical analysis. Values are presented as geometric means (95% confidence interval).

²Values with different letter superscripts within a row are significantly different (p<0.02).

³Abbreviations used: MPI=Milk protein isolate; SPI=Soy protein isolate; O-DMA=O-desmethylangolensin.

5. Serum Lipids and Apolipoproteins

Serum lipid and apolipoprotein data are presented as averages in Table 11 and as percent changes from baseline in Figure 11. Day 1 concentrations for TC, LDL-C, HDL-C, non-HDL-C, TG, apo B and apo A-I were not significantly different between treatment periods, providing evidence that the washout period between the two treatment periods was sufficient. Serum LDL-C was significantly decreased following consumption of the SPI when compared to the MPI (p=0.0443). There were no

significant effects of SPI consumption on any other serum lipids or apolipoproteins including TC, HDL-C, non-HDL-C, TG, apo B and apo A-I.

Ratios of serum lipids and apolipoproteins are presented as averages in Table 12 and percent changes from baseline in Figure 12. The ratios of LDL-C/HDL-C and apo B/apo A-I were significantly decreased following consumption of the SPI when compared to the MPI ($p=0.0227$ and $p=0.0533$, respectively). There were no significant effects of SPI consumption on any other ratios including TC/HDL-C, TG/HDL-C and non-HDL-C/HDL-C.

TABLE 11: Serum Lipids and Apolipoproteins^{1,2,5}

	MPI (n=29)	SPI (n=29)	P Value
Total cholesterol (mmol/L)			
Day 1	4.67 ± 0.07	4.63 ± 0.07	
Day 57	4.53 ± 0.06	4.43 ± 0.06	0.2103
LDL-cholesterol (mmol/L) ³			
Day 1	2.98 ± 0.05	2.94 ± 0.05	
Day 57	2.90 ± 0.04 ^a	2.78 ± 0.04 ^b	0.0443
HDL-cholesterol (mmol/L)			
Day 1	1.16 ± 0.02	1.19 ± 0.02	
Day 57	1.12 ± 0.02	1.14 ± 0.02	0.3071
Non-HDL-cholesterol (mmol/L) ⁴			
Day 1	3.52 ± 0.06	3.45 ± 0.06	
Day 57	3.41 ± 0.05	3.29 ± 0.05	0.1122
Triglycerides (mmol/L)			
Day 1	1.18 ± 0.05	1.10 ± 0.05	
Day 57	1.13 ± 0.05	1.14 ± 0.05	0.8560
Apolipoprotein B (g/L)			
Day 1	0.95 ± 0.01	0.95 ± 0.01	
Day 57	0.93 ± 0.01	0.91 ± 0.01	0.2161
Apolipoprotein A-I (g/L)			
Day 1	1.44 ± 0.02	1.45 ± 0.02	
Day 57	1.40 ± 0.02	1.43 ± 0.02	0.3431

¹Values are least square means ± SE.²Values with different letter superscripts within a row are significantly different ($p \leq 0.05$).³LDL-cholesterol was calculated using the Friedewald equation (46).⁴Non-HDL cholesterol was calculated by subtracting HDL-cholesterol from Total cholesterol.⁵Abbreviations used: LDL=Low-density lipoprotein; HDL=High-density lipoprotein.

Figure 11: Percent Change in Serum Lipids and Apolipoproteins^{1, 2, 3}

¹Values are least square means + SE.

²Endpoints marked with ** indicate a significant difference between the SPI and MPI treatments ($p \leq 0.05$).

³Abbreviations Used: MPI=Milk protein isolate; SPI=Soy protein isolate; TC=Total cholesterol; LDL-C=Low-density-lipoprotein cholesterol; HDL-C=High-density lipoprotein cholesterol; TG=Triglyceride; Apo=Apolipoprotein.

Table 12: Serum Lipid and Apolipoprotein Ratios^{1,2,5}

	MPI (n=29)	SPI (n=29)	P Value
Total cholesterol/HDL-cholesterol			
Day 1	4.15 ± 0.06	3.97 ± 0.06	0.1200
Day 57	4.13 ± 0.06	3.99 ± 0.06	
LDL-cholesterol ³ /HDL-cholesterol			
Day 1	2.66 ± 0.04	2.53 ± 0.04	0.0227
Day 57	2.66 ± 0.04 ^a	2.50 ± 0.04 ^b	
Triglycerides/HDL-cholesterol			
Day 1	1.10 ± 0.05	0.97 ± 0.05	0.7065
Day 57	1.05 ± 0.05	1.08 ± 0.05	
Non-HDL-cholesterol ⁴ /HDL-cholesterol			
Day 1	3.15 ± 0.06	2.99 ± 0.06	0.1200
Day 57	3.13 ± 0.06	2.99 ± 0.06	
Apolipoprotein B/Apolipoprotein A-I			
Day 1	0.67 ± 0.01	0.67 ± 0.01	0.0533
Day 57	0.67 ± 0.01 ^a	0.64 ± 0.01 ^b	

¹Values are least square means ± SE.

²Values with different letter superscripts within a row are significantly different ($p \leq 0.05$).

³LDL-cholesterol was calculated using the Friedewald equation (46)

⁴Non-HDL cholesterol was calculated by subtracting HDL-cholesterol from Total cholesterol.

⁵Abbreviations used: LDL=Low-density lipoprotein; HDL=High-density lipoprotein.

Figure 12: Percent Change in Serum Lipid and Apolipoprotein Ratios^{1, 2, 3}

¹Values are least square means + SE.

²Endpoints marked with ** indicate a significant difference between the SPI and MPI treatments ($p \leq 0.05$).

³Abbreviations Used: MPI=Milk protein isolate; SPI=Soy protein isolate; TC=Total cholesterol; LDL-C=Low-density-lipoprotein cholesterol; HDL-C=High-density lipoprotein cholesterol; TG=Triglyceride; Apo=Apolipoprotein.

6. Subject Compliance

6.a. Subject Communication

A high level of communication occurred between subjects and study coordinators for the duration of the study. Subjects frequently discussed study protocol with the study coordinators and shared concerns or comments they had with respect to the study. The subjects appeared to be comfortable in addressing issues and asking questions which helped build a good rapport between the subjects and study coordinators. There was

great appreciation and high participation in the many study contests and newsletters that were part of the study and helped promote communication and interaction. Although no data are available to objectively quantify subject communication, it was an important aspect of the study that was taken seriously and ultimately contributed to the success of the study.

Review of the subjects' study diaries showed compliance to study procedures including consumption of study treatment powders everyday, few urinary spills during the 24-hour urine collections, avoidance of restricted medications and consistent activity levels throughout the study. In the event that a study diary indicated an issue of concern, follow-up with the subject improved the situation and resulted in improved subject compliance in subsequent study diaries.

6.b. Urinary Creatinine and Urinary Isoflavones

If a subject had a $CV > 30\%$ for urinary creatinine from each of their 3 days of urine collection, it was most often due to a missed collection throughout the day or improper start and end times for the collection. In the case of improper start and/or end times for treatment period one, the subject was counseled and an improvement was noted in treatment period two. Evaluation of the urinary creatinine data revealed that there was a slight improvement in urine collection from the first to the second treatment period. Specifically, in treatment period one a total of 26 subjects (90%) had urinary creatinine $CVs < 30\%$ compared to a total of 28 subjects (97%) in treatment period two. A more detailed summary of the distribution of the urinary creatinine CVs within both treatment periods is presented in Table 13 and reveals the trend toward reduced CVs in treatment period two.

Table 13: Treatment Period Summaries of Subject Urinary Creatinine Coefficient of Variances^{1,2}

CV	Number of Subjects	
	Treatment Period I	Treatment Period II
0 to 4.99%	4	6
5 to 9.99%	10	11
10 to 14.99%	6	7
15 to 24.99%	5	2
25 to 29.99%	1	2
30 and above	3	1
Total	29	29

¹Abbreviations used: CV=Coefficient of variation.

²CV percents values were calculated from the standard deviation and mean values of the subject urinary creatinine excretion from each of the three days (standard deviation/mean x 100).

The significantly higher level of urinary isoflavone excretion observed during the SPI treatment period compared to the MPI treatment period (Table 10) provides evidence that subjects complied with consuming the SPI study treatment powders during the SPI treatment period and complied with refraining from dietary phytoestrogen consumption during the MPI treatment period.

6.c. Treatment Period Reviews

Treatment period reviews resulted in summaries of any issues that occurred with the subjects throughout each treatment period. Any major issues were followed up on with the subjects by the study coordinators and clarified. Some of these issues included discussing body weight fluctuations, medication changes, urine collection start and end times and study schedule changes.

F. DISCUSSION

1. Study Summary

The purpose of this study was to determine the effect of isoflavone-rich soy protein on serum lipids in adults with diet-controlled type 2 diabetes. Each of the 29 subjects involved in the study underwent two 57-day treatment periods, during which they consumed 40 g of SPI or MPI each day. Data collected throughout the study included anthropometric measures (height, body weight, waist and hip circumference, body composition), dietary information (3-day food records), lifestyle information (provided in daily study diaries) and 24 hr urine collections. Blood samples were also collected and analyzed for a full range of serum lipids (TC, LDL-C, HDL-C, non-HDL-C, TG, apo A-I and apo B) and serum lipid ratios (TC/HDL-C, LDL-C/HDL-C, TG/HDL-C, non-HDL-C/HDL-C and apo B/apo A-I), all of which have been associated with risk of CVD (43, 64, 66, 159). Few previous studies have focused on the effects of soy protein on serum lipids in adults with type 2 diabetes, especially those who are not on glycemic or lipid medications and/or have no diabetes-related complications. This study is therefore a valuable addition to the expanding literature examining the potential for soy consumption to reduce risk of CVD risk in adults with type 2 diabetes.

2. Anthropometrics and Nutrient Intakes

There were no significant differences in anthropometric data (body weight, percent body fat, waist and hip circumference) within or between treatments in the current study. These results are consistent with the research goal of maintaining these variables, since changes in body composition have been shown to affect lipid metabolism (158). Thus, the variations in the serum lipid data between treatments are unlikely due to

variations in anthropometric measurements since they did not significantly differ during the study.

Food records were completed over three consecutive days once before the study and three times throughout each treatment period, for a total of seven 3-day food records. Comparison of the 3-day food record average intakes from the pre-study food records to those completed throughout the study treatment periods revealed a significant increase in protein and calcium intake. This can be explained by the high protein and calcium content of the SPI and MPI that subjects consumed each day. Dietary intake during the study, however, remained consistent as confirmed by the comparison of the 3-day food record average intakes between the SPI and MPI treatment periods that revealed no significant differences in energy, macronutrients, cholesterol or calcium. One exception to this is saturated fat which was significantly lower during the SPI treatment period compared to the MPI treatment period. Although this difference could contribute to the significant changes observed in serum lipids between the treatments, the magnitude of its contribution is likely to be low, given that the absolute difference in saturated fat was relatively low at 2.2 g (10%). Research has indicated that changes in certain aspects of diet, such as total energy, total fat, type of fat and amount of dietary fibre can influence blood lipid levels and their metabolism (160, 161). Thus, with the possible exception of saturated fat, the lack of any significant change in these dietary variables between treatments suggests that the variations observed in the serum lipid data are not due to variations in dietary intake.

3. Urinary Isoflavones

Statistical analysis of urinary isoflavone excretion by the subjects in this study showed significantly greater excretion during the SPI treatment compared to the MPI treatment. This data provides evidence of subject compliance, both with consuming the SPI treatment and implementing the background phytoestrogen dietary restriction during the MPI treatment.

Further analysis of the urinary isoflavone data during the SPI treatment allowed for the identification of subjects who excreted substantial quantities of equol. Examining the ability to excrete equol is relevant since equol is not excreted by everyone (102), has been shown to be the most potent isoflavone in terms of its ability to bind to the estrogen receptor (115) and has been postulated to mediate the health effects of isoflavones (89). In the current study, 6 out of the 29 subjects (21%) were classified as equol excretors. Although no other studies of adults with type 2 diabetes have reported equol excretor status, this observation is inconsistent with previous studies of healthy individuals that have reported equol excretor rates of 30-50% (97, 105, 109, 111, 158). The lower rate of equol excretors observed in the current study is therefore an interesting addition to the equol literature and may suggest that disease states, such as type 2 diabetes, influence the ability to excrete equol. This idea and its implication regarding the health effects of soy require further investigation.

4. Serum Lipids

The current study found that serum LDL-C was significantly reduced by the SPI treatment compared with the MPI treatment, however there were no significant effects for serum TC, HDL-C or TG. These results are both consistent and inconsistent with

previous studies that have examined effects of various forms of soy on serum lipids in adults with type 2 diabetes.

The significant reduction in serum LDL-C in the current study is consistent with the majority of previous intervention studies that have examined the effect of SPI (18, 98, 148, 152, 153) for time periods ranging from 6 weeks to 12 months in adults with type 2 diabetes and observed a significant reduction in LDL-C, including one study that found a trend toward reduced LDL-C following 4 weeks of red clover isoflavone extract (154). In contrast, the significant reduction in LDL-C in the current study is inconsistent with three other previous studies that also examined the effect of some form of soy in adults with type 2 diabetes but did not see any significant effect on LDL-C (147, 149, 151). Subjects in these studies were different from the current study and included men with diabetic nephropathy (147, 149) or postmenopausal women with retinopathy (151) who were also using medications including insulin (147), oral hypoglycemic agents (149) or lipid-lowering medications (149). Although duration of these studies (7 (149), 8 (147) and 12 (151) weeks) was comparable to the current study's duration of 8 weeks, the treatments differed in that the studies that evaluated SPI did so on an individualized body weight basis (147, 149) while the third study evaluated extracted isoflavones (151). These differences in subject characteristics and study treatments limit the ability to explain the inconsistent results between these studies and the current study.

The lack of significant effect of SPI consumption on serum TC in the current study is similar to the findings of six previous soy intervention studies that have studied the lipid-altering effects of soy protein (18, 149, 150, 153) and extracted isoflavones (151, 154) for durations ranging from 6 weeks to 12 months in adults with type 2

diabetes. In contrast, four other studies of adults with type 2 diabetes did demonstrate a significant reduction in TC following consumption of 7-12 weeks of soy protein (98, 147, 148, 152). Subjects in these studies differed from the current study in that they included postmenopausal women with diet-controlled diabetes (98) as well as groups of men (147, 148, 152) and postmenopausal women (152) or premenopausal women (148), who were treated with oral hypoglycemic agents (152), insulin (147) or living with diabetic nephropathy (147, 148). The fact that these studies did demonstrate a reduction in circulating TC warrants further study into the effect of soy on circulating total cholesterol to help clear existing inconsistencies in the literature.

Similar to serum TC, the lack of significant effect of SPI consumption on serum HDL-C in the current study is consistent with eight other studies investigating the role of SPI (18, 98, 153), soy protein consumed through soy based foods (147, 148, 150) and extracted isoflavones (151, 154) on circulating lipids in adults with type 2 diabetes. In contrast, two other studies did observe a significant increase in serum HDL-C, one of which included 47 hyperlipidemic adults with type 2 diabetes using lipid lowering drugs who consumed a dietary soy supplement for 12 weeks (152) and the other which included 14 men with diabetic nephropathy (treated with hypoglycemic agents and lipid lowering drugs) who consumed SPI for 7 weeks (149). The inconsistency between these two studies and the current study could be due to a difference in the study samples or use of medications as these studies involved subjects who have diabetic complications, indicated by hyperlipidemia and nephropathy and were using glycemic and lipid-lowering medications, compared to the subjects in the current study who were free from

any secondary diabetic complications and did not use any glycemic or lipid-lowering medications.

The current study found no significant effect of SPI consumption on serum TG which is consistent with five previous studies that investigated the effects of soy protein (98, 149, 150) and extracted isoflavones (151, 154) on circulating lipids in adults with type 2 diabetes. In contrast, five other studies found significant reductions in TG which is inconsistent with the current study. The inconsistent studies included; a study of 47 adults with type 2 diabetes and hyperlipidemia (treated with oral hypoglycemic agents) who consumed a soy protein dietary supplement for 12 weeks (152); a weight loss study in which 82 obese adults with type 2 diabetes treated with oral hypoglycemic agents consumed a SPI meal replacement shake for 12 weeks (153); a study of 20 adults with type 2 diabetes treated with oral hypoglycemic agents who consumed SPI for 6 weeks (18); and two studies of adults with type 2 diabetes and nephropathy who consumed dietary soy protein distributed on a body weight basis for 7 (148) and 8 (147) weeks. Possible explanations for the differences in TG findings between these studies and the present study are the use of subjects samples with diabetic complications, indicated by hyperlipidemia (152), nephropathy (147, 148) and obesity (153), compared to the subjects in the current study who did not have any diabetes-related complications. Other reasons for inconsistencies could be due to the use of glycemic and/or lipid-lowering medications and/or differences in type and duration of study treatments. The conflicting results among studies provides rationale that serum TG deserves future investigation on how it is influenced by soy consumption

The current study found no significant effect of SPI consumption on serum non-HDL-C. Although no previous soy intervention study of adults with type 2 diabetes has included non-HDL-C as an endpoint, previous studies of adults without type 2 diabetes have included non-HDL-C as an endpoint (162-166). Results of these studies are inconsistent with the current study in that they have all observed a significant decrease in non-HDL-C following consumption of SPI. While all of these studies examined effects of SPI, their durations varied from 5 weeks (162), 6 weeks (166), 12 weeks (164), 24 weeks (163) to 6 months (165). Since the results of the current study are inconsistent with all the previous studies of adults without type 2 diabetes, there is a need for more research in adults with type 2 diabetes to understand if soy consumption can impact circulating non-HDL-C in this population.

Overall the results of the current study with regards to LDL-C and TC are consistent with the majority of the previous studies of adults with type 2 diabetes. There is more variation in comparison of the current study's serum HDL-C and TG results to previous studies thereby warranting further research to examine the effects of soy on serum lipids in adults with type 2 diabetes. When comparing the results of these studies it is important to consider the vast differences in methodologies that limit comparability including variations in study design, soy treatment, study duration, number of subjects and subject medication use. Despite these variations in methodologies and results, the current study along with some previous studies (18, 98, 147-149, 152, 153) have demonstrated beneficial effects of soy protein on lipid profiles in adults with type 2 diabetes. The current study also advances the literature by introducing the measurement

of non-HDL-C in adults with type 2 diabetes as no other studies focusing on the type 2 diabetes population have measured non-HDL.

5. Serum Apolipoproteins

The current study found no significant effects of the SPI treatment on serum concentrations of apo B or apo A-I. Research investigating the effects of soy on apolipoproteins in adults with type 2 diabetes is very limited in that only one previous study of adults with type 2 diabetes has included an examination of apolipoproteins. This study, conducted by Hermansen et al. (18), found that 6 weeks of SPI and soy cotyledon fibre consumption resulted in a significant reduction in plasma apo B but had no effect on apo A-I, when compared with a cellulose control, in 20 adults with type 2 diabetes. It is possible that the inclusion of the soy fibre in the Hermansen et al. (18) study contributed to its ability to demonstrate a significant effect on plasma apo B (which is inconsistent with the current study), but this does not explain its lack of significant effect on apo A-I (which is consistent with the current study).

Although not directly comparable to the current study, there are some previous soy intervention studies of adults without type 2 diabetes that have included an examination of circulating apolipoproteins. For example, serum apo B was significantly reduced (apo A-I was not significantly changed) in a study by Teixeira et al. (166) in which 81 hypercholesterolemic men consumed varying ratios of SPI and MPI for 6 weeks. Plasma apo B was also significantly reduced in two other studies that evaluated the effects of SPI for a duration of 6 weeks in 20 hypercholesterolemic men (167) and a duration of 4 weeks in 26 hypercholesterolemic men (168). The 6-week study (167) also measured apo A-I but found no significant effect of SPI consumption.

Further, another study observed significantly decreased serum apo B (apo A-I was not measured) when 117 hypercholesterolemic men and women consumed SPI for 12 weeks (169). Plasma apo A-I (apo B was not measured) was significantly increased in one study following 2 weeks of consumption of high-isoflavone SPI (56 mg of isoflavones/day) compared to low-isoflavone SPI (2 mg isoflavones/day) by 22 healthy men and women (170). Finally, neither serum apo B or apo A-I were significantly affected in two other previous studies that investigated the effect of SPI of varying isoflavone content in a sample of 35 healthy males for 8 weeks (171) and a sample of 41 hyperlipidemic men for 4 weeks (85).

Overall the limited inclusion of measures of circulating apolipoproteins in the soy intervention studies conducted to date, in adults both with and without diabetes, suggests a need for future studies to include apolipoproteins in their study endpoints. As such, the current study's inclusion of both serum apo B and apo A-I is a valuable addition to this overall research area.

6. Serum Lipid and Apolipoprotein Ratios

When the ratios of the serum lipids and apolipoproteins were calculated, the current study found that SPI consumption significantly reduced LDL-C/HDL-C and apo B/apo A-I, whereas there were no significant changes in TC/HDL-C, TG/HDL-C or non-HDL-C/HDL-C.

The significant reduction in LDL-C/HDL-C following SPI consumption in the current study is consistent with three previous studies in which adults with type 2 diabetes consumed SPI for 4 weeks (subjects also had diabetic nephropathy) (149), 6 weeks (18) or a dietary soy protein supplement for 12 weeks (subjects also had

hyperlipidemia) (152), but inconsistent with two other studies in which men with diabetic nephropathy consumed a soy protein diet for 7 weeks (148) or in which postmenopausal women with type 2 diabetes and retinopathy consumed an extracted isoflavone supplement for 12 weeks (151).

The significant reduction in apo B/apo A-I following SPI consumption in the current study has not been previously observed in adults with type 2 diabetes. The only previous soy intervention study of adults with type 2 diabetes that included apolipoproteins as endpoints did not find any significant effect of 6 weeks of SPI on apo B/apo A-I, despite a significant reduction in plasma apo B (18). The differences between this and the current study can be attributed to multiple study design differences including number of subjects, subject medication use, duration of treatment and type of treatment controls used. Previous research that has been conducted with subjects samples without type 2 diabetes have shown results consistent with the current study. Significant reductions apo B/apo A-I (despite no significant changes in either apo B or apo A-I) was demonstrated in two studies that investigated the effect of SPI of varying isoflavone content in a sample of 35 healthy males (171) and a sample of 41 hyperlipidemic men (85).

The lack of significant effect of SPI consumption on the TC/HDL-C ratio in the current study is in contrast to the three previous studies of adults with type 2 diabetes that have evaluated this lipid ratio (98, 149, 152). These three studies included a group of 32 postmenopausal women with diet-controlled diabetes who consumed SPI for 12 weeks (98), a group of 47 hyperlipidemic men and postmenopausal women who consumed a dietary soy supplement for 12 weeks (152) and a group of 14 men with diabetic

nephropathy who consumed 0.5 g/kg body weight SPI for 7 weeks (149). The significant reduction observed in TC/HDL-C within each of these studies is logical given that each of them observed a significant reduction in TC (98, 152) and/or a significant increase in HDL-C (149, 152), neither of which occurred in the current study.

Consumption of SPI did not significantly affect either the TG/HDL-C or the non-HDL-C/HDL-C ratio. No previous soy intervention studies of adults with type 2 diabetes have included either of these lipid ratios. Similarly, no previous soy intervention studies of adults without type 2 diabetes have included TG/HDL-C, but one study did include non-HDL-C/HDL-C. This study was conducted by Sagara et al. (162) and involved 5 weeks of SPI supplementation to the diets of 156 hyperlipidemic men which resulted in significantly reduced non-HDL-C/HDL-C. The current study therefore advances the literature by expanding the examination of lipid ratios in the population of adults with type 2 diabetes and establishes a foundation for future research to investigate these endpoints further. The TG/HDL-C and non-HDL-C/HDL-C ratios are worthy of investigation as they have been shown to be valuable markers of CVD risk (43, 63, 67) and serve as diagnostic tools for assessing cardiovascular health.

In summary, the current study advances the literature by demonstrating that consumption of SPI can significantly reduce the LDL-C/HDL-C and apo B/apo A-I ratios, which have both been associated with a reduction in CVD risk (62-64, 66, 172). The significant effect of SPI consumption on the LDL-C/HDL-C ratio is consistent with previous studies (18, 149, 152) and the lack of significant effect of SPI consumption on TG/HDL-C and non-HDL-C/HDL-C has not been previously studied. Overall, the serum lipid and apolipoprotein ratio results of the current study adds information to the

literature since many previous soy intervention studies in adults with type 2 diabetes did not include lipid ratios (98, 147, 150, 153, 154).

7. Study Strengths

There are many strengths of this study in relation to its subject sample, study design, study treatments, data collection and study endpoints. One of the strengths of the current study was the fact that the subjects were not treated with either glycemic or lipid-lowering medications. This aspect is a definite strong point to the study as many of the previous soy intervention studies of adults with type 2 diabetes (18, 147, 149, 150, 152-154) have involved subjects on oral hypoglycemic agents or lipid-lowering medications. The inclusion of only subjects with diet-controlled type 2 diabetes allowed for a focus on the potential for soy to act as a prevention strategy, to possibly prevent or delay the need for pharmaceutical treatment.

Another strength of the current study is its randomized, crossover, double-blinded, placebo-controlled design in which each treatment period lasted 57 days. The randomized crossover aspect of the study design allowed for each subject to act as their own control, minimizing the inter-subject variation that would have been introduced with a parallel arm study design. The double-blinded aspect of the study design minimized bias on the part of both the subject and the study coordinators. The 57-day treatment periods (separated by a 4-week washout period) makes this study one of the longer interventions that has investigated the effects of soy on serum lipids in adults with type 2 diabetes (18, 148-150, 154).

The treatments used in this study added strength for specific reasons. Firstly, the SPI and MPI were closely matched in terms of macro- and micronutrient composition,

and only differed in type of protein and amount of isoflavones. Secondly, the SPI and MPI were similar in texture, taste and appearance which allowed for the study to be double-blinded. Finally, the SPI was naturally high in isoflavones thereby allowing for an evaluation of the isoflavones as naturally present in their soy protein matrix.

Another strength of this study relates to the numerous quality control techniques that were employed throughout the data collection. For example, all blood samples were collected at a consistent time within 30 minutes for each subject and subjects were always fasted for 12 hours, and had avoided strenuous activity, alcohol, and over-the-counter medications for 72 hours prior to the blood samples. In addition, timing of the blood sample processing along with the accuracy and techniques of the processing were kept consistent.

Also contributing to the strength of the data collection was the effort put towards the development of rapport between the subjects and study coordinators. The study orientation, study handbook, regular communication through reminder calls, newsletters, contests and feedback boxes were all utilized to improve subject understanding of the study and offer interaction and enjoyment in the study beyond the regular requirements of the study. Employing these techniques helped develop an open rapport with subjects and aided compliance to certain aspects of the study such as completion of study diaries and foods records, consistency of treatment consumption, consistency of physical activity and avoidance of dietary restrictions. The attention given to subject rapport could have served as a method of subject retention.

A final strength worth mention is the endpoints included in the current study. Although previous soy intervention studies of adults with type 2 diabetes have included

select serum lipids (18, 98, 147-154), serum apolipoproteins (18) and serum lipid ratios (18, 98, 148, 149, 151, 152), this is the first study to include a full profile of all three types of endpoints. Analyzing for all three of these CVD-related biomarkers provides a more comprehensive evaluation of the effect of soy on lipids in adults with type 2 diabetes.

In summary, there are many strengths to the present study that advance previous research and expand analysis to provide a more complete investigation into soy's effects on serum lipids in adults with type 2 diabetes. Overall, this study advances the research in this area of study and is a valuable contribution to the literature.

8. Study Limitations

Although there are many strengths of this study, there are also some limitations that are worth noting. These limitations relate to the subject sample as well as the study treatments, including the dose of treatment and the type of treatments.

The first limitation relates to the subjects' management of their type 2 diabetes. Since these subjects were not on glycemic or lipid-lowering medications, it meant that they were managing their type 2 diabetes very well. This could have made it harder to detect an effect of the SPI treatment on serum lipids (as opposed to a study of subjects with less controlled glycemia and/or circulating lipids). Although the detection of an effect may have been more difficult to identify in a sample of well-managed, diet-controlled adults with type 2 diabetes, inclusion of these subjects avoided any confounding effects of diabetic-related medications.

Another potential limitation of the study could have been the consistent dose of SPI provided. It is arguable that the provision of the treatment in a more individualized

fashion, such as g/kg of body weight, as has been done in other studies (147-149), is superior since it standardizes the treatment dose relative to body size. Nonetheless, the use of a common dose has been utilized in many previous studies (18, 98, 152, 153) and it is noteworthy that its use in the current study produced a significant treatment effect.

The use of MPI as a control could be considered another limitation of this study. Since there is evidence that bioactive compounds present in milk protein can improve CVD risk (173), the use of MPI as a control may have masked some SPI effects. Despite the limitations of MPI as a control it served as a very appropriate control as it has been used in previous studies including adults with type 2 diabetes (18, 149), thereby allowing for comparison to the literature. The MPI was also nutritionally matched to the SPI for both macro- and micronutrient content, thereby only differing in type of protein and presence of isoflavones. These design aspects allowed for a focus on both type of protein and quantity of isoflavones as factors contributing to the study results.

Overall, although there are limitations to the present study there are aspects of them that proved beneficial as well. It is possible that future research could help address the limitations from this study and advance this area of research

9. Future Research

There are many potential areas of future research that could add to the understanding of how soy can affect serum lipids in adults with type 2 diabetes. These areas could include expanding aspects of the subject sample that is studied, expanding the study treatments that are tested, accounting for inter-individual differences in isoflavone metabolism and expanding the study endpoints that are measured.

One quality that has made many studies difficult to interpret is the variations in the medication use of their subjects. Future studies would benefit from including more homogeneous subject samples such that subjects are either all on no medications or all on the same types of medications. Since length of time since diagnosis is another factor that varies within studies, it would be interesting to design future studies to focus on the potential for soy to affect serum lipids in adults at specific time lengths since diagnosis for their type 2 diabetes. It would also be informative to study homogenous samples of insulin-resistant subjects to examine if soy has a role in delaying the onset of type 2 diabetes or if it could delay the need for pharmaceutical use to treat type 2 diabetes.

Another area of future research could relate to an expansion of the study treatments. For example, there could be a focus on the constituents of soy protein that may be responsible for its CVD-risk reducing effects. Studies could focus on either the isoflavones or the specific soy protein peptides in terms of their lipid-lowering effects and potential mechanisms behind those effects. Some potential mechanisms worth investigation include LDL-receptor activity, estrogen receptor activity, lipid transfer between lipoproteins, LDL and apolipoprotein mRNA levels and thyroid hormone levels.

Also related to an expansion of study treatments could be research into the effects of soy in food forms. For example, expanding soy treatments to include whole soy foods such as whole soybeans or tofu, would extend an understanding of the CVD risk-reducing potential of soy protein to foods that adults with type 2 diabetes are more likely to purchase in the grocery store.

Future research could also focus on the inter-individual differences in isoflavone metabolism, specifically the ability to produce the isoflavone metabolite equol, and how

this might influence the effects of soy on CVD risk in adults with type 2 diabetes. The observation that there was a lower prevalence of equol excretors in the current study relative to previous studies of adults without type 2 diabetes (97, 105, 109, 111, 158) deserves follow up to determine if there is a specific aspect of type 2 diabetes that may influence the ability to produce equol.

Finally, another area that deserves research into the effects of soy in type 2 diabetes is an expansion of endpoints related to CVD risk. There is a need for more studies to include apolipoproteins and serum lipid ratios, as was done in the current study. Further, the inclusion of other CVD biomarkers such as C-reactive protein, oxidized LDL, blood pressure, endothelial activity and vasoreactivity is also worth pursuit in subjects with type 2 diabetes. Of particular relevance would be the inclusion of varying lipoprotein particle sizes, since changes in these sizes, particularly LDL, occur in type 2 diabetes (45) and these changes are associated with increased atherogenicity (45) and risk of CVD (33, 45, 67).

In summary, there are many opportunities for future research in the area of soy and CVD risk in type 2 diabetes. Advancing with this research would be helpful in understanding the effects of soy in this population.

G. SUMMARY AND CONCLUSION

In summary, this study evaluated the effects of isoflavone-rich SPI in comparison to MPI on serum lipids in adults with type 2 diabetes. The study utilized a randomized, crossover, double-blinded design that included two 8-week treatment periods separated by a 4-week washout period. Blood samples were obtained on days 1 and 57 of each treatment period and analyzed for a full profile of serum lipids including apo B and apo

A-I as well as ratios of serum lipids and apolipoproteins. Study results showed that the SPI treatment significantly reduced LDL-C as well as the ratios of LDL-C/HDL-C and apo B/apo A-I, in comparison with the MPI treatment. These results demonstrate a beneficial effect of SPI consumption that relates to a reduction in CVD risk in adults with type 2 diabetes. This study therefore provides evidence for soy as a nutritional strategy for adults with type 2 diabetes to help improve the quality of their life through a reduction in CVD risk and possibly extension of life.

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Adults with Type 2 Diabetes Needed for a University of Guelph SOY Nutrition Study

Individuals with type 2 diabetes, who are not on medications for blood sugar or cholesterol, are needed for a study that will evaluate the effectiveness of soybeans to improve diabetes

Participants will be required to:

- Attend a study orientation
- Consume a soy or milk protein shake every day for a period of 8 weeks each
- Give blood samples and collect urine samples
- Complete food diaries

Financial compensation provided.

This study is run out of the Department of Human Health & Nutritional Sciences at the University of Guelph and has received approval from the University of Guelph Human Research Ethics Board.

If you are interested, please contact Colleen at

519-824-4120 x58081 or

gobertc@uoguelph.ca

Thank you ☺

Appendix 2: Study Subject Phone Eligibility Questionnaire

Effect of Soy Protein on Glycemic Control and Serum Lipids in Adults with Type 2 Diabetes

Name of caller: _____ Date: _____ Interviewer Name: _____

Phone: _____ Email: _____ Best way to get in touch: _____

1. How did you hear about the study? _____
2. How old are you? _____
3. How tall are you? _____ How much do you weigh? _____ BMI _____
4. For Females: Are you postmenopausal? YES NO
 - a. If YES, have you had a hysterectomy? YES NO
 - b. If YES, have you had any bleeding within the last 12 months? YES NO
 - c. If YES, are you taking hormone replacement therapy? YES NO
5. Do you smoke? YES NO
6. When were you diagnosed with type 2 diabetes? _____
7. Are you taking any medications to lower your blood sugar? YES NO
8. Are you taking insulin? YES NO
9. Are you taking medications for your blood cholesterol? YES NO
10. Are you allergic to soy? YES NO
11. Are you taking any dietary supplements? YES NO
 - a. If YES, would you be willing to discontinue your supplements during this study? YES NO
12. Are you a vegan? YES NO

If caller meets the study eligibility requirements thus far, describe the study to them and answer any questions they have. If they are still interested, inform them of our study timeline and explain that the next step will involve us getting in touch with them to set up an in-person meeting where they will fill out a more detailed questionnaire and learn more about the study. THANK them and also ask them if they have any friends who might be interested in the study.

In-person Study Meeting: **Date:** _____ **Time:** _____

Appendix 3: Study Subject In-Person Eligibility Questionnaire

Effect of Soy Protein on Glycemic Control and Serum Lipids in Adults with Type 2 Diabetes

Name: _____ Date: _____

Address: _____

Phone: work: _____ home: _____ Email: _____

Best way to communicate: _____

Date of Birth: _____ Age: _____ Height: _____ Weight: _____

1. How did you hear about the study? _____
2. When were you diagnosed with type 2 diabetes? _____
3. Are you taking any oral hypoglycemic agents? YES NO
4. Are you taking any insulin? YES NO
5. Are you taking any medications for your blood cholesterol? YES NO
6. Are you currently taking any other medications? YES NO
 - a. If yes, please list _____

 - b. Would it be possible for your medications to remain stable during the study? YES NO
7. Do you use any over the counter drugs, including pain relievers? YES NO
 - a. If yes, please list _____

8. Do you use recreational drugs? YES NO
9. Do you take vitamin or mineral supplements? YES NO
 - a. If yes, please list type and amount _____
 - b. Would you be willing to discontinue your supplement during the study? YES NO
10. Do you take dietary supplements, including herbs? YES NO
 - a. If so, please list type and amount _____
 - b. Would you be willing to discontinue these for the duration of the study? YES NO

11. Do you currently smoke? YES NO
 a. If no, have you ever smoked? YES NO
 b. If yes, how long ago did you quit? _____
12. Do you have any allergies (drug, food, environmental)? YES NO
 a. If yes, what are they _____
13. Are you aware that you have any impaired kidney function? YES NO
 a. If yes, please expand _____

14. Do you have any medical conditions other than your diabetes? YES NO
 a. If yes, please list (be as specific as you can) _____

15. Are there any other health issues we should be aware of? YES NO
 a. If yes, please list (be as specific as you can) _____

16. Do you exercise regularly? YES NO
 a. What do you do? _____
 b. How often and how intense? _____
17. Are you satisfied with your current weight? YES NO
18. Has your weight changed in the past 2 months? _____ past year? _____
 a. If yes, how much? _____
 b. If necessary, please explain: _____
- It will be very important to maintain your body weight throughout this study. Will you be OK with this? YES NO
19. Are you on a special diet now? YES NO
 a. If yes, can you explain it? _____

20. Are you a vegetarian? YES NO
 a. If yes, do you eat dairy products? YES NO
 b. If yes, do you eat eggs? YES NO
 c. If yes, do you eat fish? YES NO
21. Do you eat beans and/or legumes? YES NO
 a. If yes, how often? _____

22. Do you eat soy products? YES NO
a. If yes, how often? _____

23. Do you consume flax products? YES NO
a. If yes, how often? _____

24. Do you consume caffeine (coffee, tea, pop)? YES NO
a. If so, how much per day? _____

25. Do you consume alcohol? YES NO
a. If so, how many drinks per week? _____ (1 drink = 12oz beer, 5oz wine, 1.5oz hard liquor)

26. Do you drink milk? YES NO
a. If yes, how often? _____

The study product contains enough calcium to meet your daily needs. Due to this, would you be OK

with not drinking milk during the study? (note that milk on cereal, in coffee and cooking would be OK) YES NO

27. 24-hour diet recall (study coordinator will go over this with you)

28. Are you currently working? YES NO
a. If so, what hours so you normally work? _____

29. Are you comfortable with having your blood taken? YES NO

30. Are you currently involved in any other research study? YES NO

31. Have you ever been involved in a research study before? YES NO
a. If so, please expand briefly _____

32. Why do you want to be in this study?

Appendix 4: Study Subject Welcome Letter

Date Sent

Subject Name

Subject Address

Dear Subject,

We are writing to officially welcome you to our research study entitled "Effect of soy protein on glycemic control and serum lipids in adults with type 2 diabetes". We are very excited about this research and are so pleased that you have volunteered to be a participant in the study. You are making a valuable contribution to research into the improvement of the management of type 2 diabetes.

As discussed with Beth/Colleen, you will have your study orientation on _____ in room 212 Powell Building at the University of Guelph. Please feel welcome to have your spouse or partner come along with you. You can park in the lot beside the White Covered Athletic Dome (parking lot #6; entrance off East Ring Road). The lot is enforced from 7:30am to 5:30pm Monday to Friday, so you will not have to pay to park there during your orientation (since it is from 7-9pm). After you park, walk back to East Ring Road, turn right and you will notice some double doors that enter the Powell Building immediately on your right. You can enter the building through these doors; the doors will be locked, so someone will be there to let you in.

At the study orientation, you will learn everything you need to know about being a subject in this study. You will receive a detailed study handbook that you can refer to throughout the study. As you know, you are making a large commitment to be a subject in this study and we really appreciate your dedication to our study. We will be with you throughout the whole study and can answer any questions and address any concerns along the way.

We have enclosed a summary of all your study dates and visits for your information. Please mark these dates in your calendar and let us know if you have any concerns or questions.

As well, please remember to do the following one month before your study start date:

- discontinue consumption of soy & flax products
- discontinue consumption of beans and legumes
- discontinue consumption of vitamin supplements

Please let us know if you have any questions or concerns about this.

Once again, welcome to this study and we look forward to working with you! Please don't hesitate to call or email any of us at any time if you have any questions.

Sincerely,

Alison M. Duncan, Ph.D., R.D.
Assistant Professor
Ph: 824-4120 ext. 53416
amduncan@uoguelph.ca

Colleen Gobert, M.Sc., R.D.
Graduate Student
824-4120 ext. 58081
gobertc@uoguelph.ca

Beth Phillips, B.Sc.
Graduate Student
824-4120 ext. 58081
ephill01@uoguelph.ca

Your Schedule of Study Visits

Please record these in your personal day planner and be sure to consult your dates before making any plans. Contact us right away if you have any concerns:

Colleen Gobert, M.Sc., R.D.

Phone: 519-824-4120 x58081

Email: gobertc@uoguelph.ca

Elizabeth Phillips, B.Sc.

Phone: 519-824-4120 x58081

Email: ephill01@uoguelph.ca

For every study visit, you will need to come, at your scheduled appointment time, to the **Human Nutraceutical Research Unit (HNRU)** located in room 212 of the **J.T. Powell Building** at the **University of Guelph**. The following summarizes your study visits:

Your Study Visit Appointments Room 212 J.T. Powell Building, University of Guelph, 824-4120 x53925	
Treatment Period I	
Day 1	Date: _____ Time: _____
Day 15	Date: _____ Time: _____
Day 29	Date: _____ Time: _____
Day 43	Date: _____ Time: _____
Day 57	Date: _____ Time: _____
Study Washout Period	
Treatment Period II	
Day 1	Date: _____ Time: _____
Day 15	Date: _____ Time: _____
Day 29	Date: _____ Time: _____
Day 43	Date: _____ Time: _____
Day 57	Date: _____ Time: _____

Please remember to do the following for 12 hours before every study visit:

- Don't eat or drink anything (except water is OK and is encouraged)

Please remember to also do the following for 72 hours before each Day 1 and Day 57 study visit:

- Refrain from drinking alcohol, consuming extra medications (above and beyond your regular medications) or doing strenuous exercise
- Don't eat or drink anything (except water is OK and is encouraged) for 12 hours before.
- Drink at least 2 glasses of water in the evening before and at least 2 glasses the morning of to hydrate for the body composition measurement.
 - Be consistent with the amount of water you drink prior to every study visit that has a body composition measurement.

Appendix 5: Study Subject Consent Form

Effect of Soy Protein on Glycemic Control and Serum Lipids in People with Type 2 Diabetes. Study Consent Form

Names of Research Institute and Researchers: This project has been reviewed and received approval from the University of Guelph Human Subjects Committee. The researchers conducting this study are listed below. If you have any questions, feel free to contact them.

- Alison M. Duncan, Ph.D., R.D., Assistant Professor, Department of Human Health and Nutritional Sciences, University of Guelph, 519-824-4120 ext. 53416, amduncan@uoguelph.ca
- Colleen Gobert, M.Sc., R.D., Graduate Student, Department of Human Health and Nutritional Sciences, University of Guelph, 519-824-4120 ext. 53416, gobertc@uoguelph.ca
- Beth Phillips, B.Sc., Graduate Student, Department of Human Health and Nutritional Sciences, University of Guelph, 519-824-4120 ext. 53416, ephill01@uoguelph.ca
- Sarah E. Capes, M.Sc., M.D., Assistant Professor, Department of Medicine, McMaster University
- Gerarda A. Darlington, Ph.D., Assistant Professor, Department of Mathematics and Statistics, University of Guelph

Introduction: You are being invited to take part in a 20-week research study directed by Dr. Alison Duncan of the Department of Human Health and Nutritional Sciences at the University of Guelph. Before you agree to take part in this study, it is important that you understand the purpose of the study, how it may affect you, the risks and benefits, and what you will be asked to do. This information is explained in this consent form to help you decide if you would like to participate in this study.

Study Purpose: The purpose of the study is to evaluate the effect of consuming isoflavone-rich soy on glucose control and blood lipids in people with type 2 diabetes. Heart Disease is a leading cause of death in people with type 2 diabetes, making it important to explore strategies for its prevention. Soy protein and its constituent isoflavones show beneficial effects related to type 2 diabetes in animal and preliminary human studies. This research will determine if consuming isoflavone-rich soy protein can improve glucose control and reduce heart disease risk in people with type 2 diabetes. Results will provide information about a nutritional strategy that could reduce heart disease risk as well as improve the quality and prolong the lives of people living with type 2 diabetes.

Procedures: You will take part in a 20-week research study, which will include two 8-week treatment periods, separated by a 4-week break. You will attend a study orientation that will involve training in all aspects of the study. Before the study starts, you will have your height, body weight and body composition measured. The study will then involve two 8-week treatments periods that will include 5 morning visits to the Human Nutraceutical Research Unit (HNRU) in the Powell building at the University of Guelph. The following describes what you will need to do during EACH of the two treatment periods:

- Consume a soy or milk protein powder EVERY DAY. This powder can be mixed with water and flavour packets that will be provided.
- Complete 3-day food records at weeks 1, 4 and 8 for a total of THREE times.
- Have your body weight measured every at weeks 1, 2, 4, 6 and 8 for a total of FIVE times.

- Have your body composition measured at weeks 1 and 8 for a total of TWO times.
- Give a fasted blood sample at weeks 1 and 8 for a total of TWO times.
- Have an oral glucose tolerance test at weeks 1 and 8 for a total of TWO times (see below).
- Keep a daily study diary to record exercise, illnesses, and any other relevant details.

Body Composition: Your body composition will be measured using bioelectrical impedance analysis, a very common procedure for the measurement of body composition. It involves placing 2 electrodes on your hand and 2 electrodes on your foot; a machine will then be analyze your body composition in less than a minute. There is no pain involved and you will be lying down the entire time.

Oral Glucose Tolerance Tests (OGTT): As mentioned, the study will involve four OGTTs; one at the beginning and end of each of the two treatments periods. An OGTT is a common procedure used to explore glucose control. It will involve you arriving at the HNRU in the morning after not having any food or drink (except water) for 12 hours. A trained technician will place a catheter in your arm, which will remain for 3 hours. During this time, you will be kept comfortable, can walk around and can watch movies or read. You will consume an orange-flavoured drink and the technician will take blood samples from your catheter periodically throughout the 3 hours. OGTTs are very commonly done and the technician at the HNRU has been involved in numerous studies that use OGTTs.

During the study, you will be asked to: maintain your usual exercise and lifestyle habits, maintain your body weight, avoid other sources of soy, avoid beans and legumes and not participate in any other research studies. If you decide to participate, you are free to withdraw at any time without any penalty.

Possible Risks and Benefits: Potential risks from this study include slight discomfort and small bruises from the blood draws. Potential benefits include knowledge of your dietary data, body fatness and overall knowledge of the study results.

Financial Compensation: To cover your expenses and inconveniences in this study, you will receive \$300 after the completion of each treatment period and \$100 after the completion of the entire study, for a total of \$700.

Confidentiality: Every effort will be made to maintain confidentiality in all aspects of this research. All subjects will be assigned a number and any data reported in a publication or presentation will be overall group data. Your name will not appear in any report, publication or presentation resulting from this study. All documents or computer files related to the study will be stored in a locked office and/or on a password-protected computer. Data collected from this study will be used as part of a graduate student thesis and may be published in a journal article. The data will be retained for a period of 5 years after publication.

Declaration of Consent: I, _____ have read the contents of this consent form, have had any questions answered, and am therefore providing informed consent, as indicated by my signature below. I understand that I am free to withdraw from the study at any time and that every effort will be made to maintain my confidentiality.

Subject Signature: _____ Date: _____

Investigator Signature: _____ Date: _____

**Appendix 6: University of Guelph Human Research Ethics Board Approval
Certificate**

UNIVERSITY OF GUELPH	
OFFICE OF THE VICE-PRESIDENT RESEARCH	
<i>Certification of Ethical Acceptability for Research Utilizing Human Subjects</i>	
DATE:	January 14, 2004
APPLICANT:	Dr. Alison Duncan Human Biology & Nutritional Sciences
SPONSOR:	N/A
TITLE OF PROJECT:	<i>"Effect of Soy Protein on Glycemic Control and Serum Lipids in People with Type 2 Diabetes"</i> REB# DE009
<p>The members of the University of Guelph Research Ethics Committee have examined the experimental protocol which describes the participation of the human subjects in the above-named research project and they consider the experimental procedures, as described by the applicant, to conform to the University's ethical standards.</p>	
Approved:	Date: JAN 14 2004
C. McKenna Associate Vice-President (Research)	
Expedited Review ☒	

Appendix 7: Summary of Study Treatment Randomization

HSFO Study Study Treatment Randomization Table¹

There are two possible orders: treatment A (146) then treatment B (933) OR treatment B (933) then treatment A (146). If we have 30-35 subjects, we will want half of them with one order and the other half with the other order. Let's start with putting 15 slips of paper with B-A and 15 slips of paper with A-B in a hat (we will add 3 more of each later). Each time a subject starts the study, draw from the hat (without replacement) and record what their treatment order is on this table. (Note that treatment A=study product 146; B=study product 933).

Subject #	Treatment Period I	Treatment Period II
1	A	B
2	A	B
3	A	B
4	B	A
5	B	A
6	B	A
7	B	A
8	A	B
9	B	A
10	A	B
11	B	A
12	A	B
13	B	A
14	B	A
15	A	B
16	B	A
17	A	B
18	A	B
19	B	A
20	A	B
21	no subject	no subject
22	A	B
23	B	A
24	A	B
25	B	A
26	B	A
27	B	A
28	A	B
29	A	B
30	no subject	no subject
31	A	B
32	B	A
33	A	B
34	A	B

A=study product code 146
B=study product code 933

Total Number Subjects; A-B: 17
Total Number Subjects; B-A: 15

¹Subjects 16, 22, 34 dropped out of the study. Subject 28 was not included in the study analysis.

Appendix 8: Study Subject Treatment Period Review

HSFO Study

Treatment Period Review I II

Subject Name: _____

Subject #: _____

Date of Review: _____

Treatment Powder (how and when consumed, any feedback)

Study Day Visits (on time, any deviations?)

Study Medications (what are they, any changes?)

Body Weight

Day 1	Day 15	Day 29	Day 43	Day 57

Food Records (all handed in? good level of detail?)

Urine Collections (creatinine value, start end times)

General/Overall

Action Items