Potential Therapeutic Benefits of Flaxseeds in the Treatment of Type 2 Diabetes Symptoms

by

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ABSTRACT

Background: Despite the reported improvements in glucose regulation associated with flaxseeds (*Linum usitatissimum*) few clinical trials have been conducted in diabetic participants. Objective: To evaluate the efficacy of ground flaxseed consumption at attenuating hyperglycemia, dyslipidemia, inflammation, and oxidative stress as compared to a control in adults with non-insulin dependent type 2 diabetes (T2D). Design: In a randomized parallel arm controlled efficacy trial, participants were asked to consume either 28 g/d ground flaxseed or the fiber-matched control (9 g/d ground psyllium husk) for 8 weeks. The study included 17 adults (9 male, 8 females; 46±14 y; BMI: 31.4±5.7 kg/m^2) with a diagnosis of T2D ≥ 6 months. Main outcomes measured included: glycemic control (HbA1c, fasting plasma glucose, fasting serum insulin, and HOMA-IR), lipid profile (total cholesterol, LDL-C, HDL-C, total triglycerides, and calculated VLDL-C), markers of inflammation and oxidative stress (TNF-alpha, TBARS, and NOx), and dietary intake (energy, total fat, total fiber, sodium). Absolute net change for measured variables (week 8 values minus baseline values) were compared using Mann-Whitney U non-parametric tests, significance was determined at $p \leq 0.05$. Results: There were no significant changes between groups from baseline to week 8 in any outcome measure of nutrient intake, body composition, glucose control, or lipid concentrations. There was a modest decrease in TNF-alpha in the flaxseed group as compared to the control ($p = 0.06$) as well as a mild decrease in TBARS in the flaxseed as compared to the control group ($p = 0.083$), though neither were significant. Conclusions: The current study did not detect a measurable association between 28 g/d flaxseed consumption for 8 weeks in T2D

participants and improvements in glycemic control or lipid profiles. There was a modest, albeit insignificant, decrease in markers of inflammation and oxidative stress in the flaxseed group as compared to the control, which warrants further study.

DEDICATION

I dedicate this dissertation to my family who has shown relentless support throughout the years. I am thankful to my husband Jon for his encouragement, patience, friendship and love, acceptance and for always having confidence in me. I also dedicate this dissertation to my children, Stella and Hayden who showed as much patience and support as anyone under the age of 5 can manage. You inspire me each day to believe in myself and always make me laugh. To my loving parents, Dennis and Carol, I appreciate your words of encouragement and for giving me your fullest support. To my brother Kendall, thank you for your guidance and friendship, and for believing in me.

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CHAPTER 1

INTRODUCTION

1.1 Background

According to the Center for Disease Control (CDC) national diabetes statistics report, in 2014 alone 29.1 million, or 9.4%, of the adult population in the U.S. had diabetes (CDC, 2015). In 2012 alone there were 1.7 million newly diagnosed cases of diabetes in persons over the age of 20. Additionally, the American Diabetes Association (ADA) estimated that in 2012 37% of U.S. adults aged 20 years or older were considered prediabetic, which if left unresolved can progress into type 2 diabetes (T2D). The enormous economic burden as a result of the increasing prevalence of diabetes is estimated to be $245 billion dollars in both direct and indirect costs with direct costs estimated at $176 billion and indirect costs, such as disability or missed days of work, approximated at $69 billion (ADA, 2013). Moreover, 35.7% of the U.S. population is currently obese, which is expected to contribute to a rise in individuals diagnosed with T2D. T2D is independently associated with an increased risks for cardiovascular diseases (CVD) (Taub et al. 2013), which is primarily due to the early development of advanced atherosclerotic vascular changes (Naka et al. 2012). The mechanisms underlying the association of T2D with vascular dysfunction are considered to be complex. Classical cardiovascular risk factors (hypertension, dyslipidemia and smoking) may play a role, while diabetes-related parameters such as hyperglycemia and insulin resistance interpose increased risk to impaired vascular function. Obesity, and other associated emerging risk factors such as inflammation, may also contribute to the impairment of arterial function

in T2D (Lowe et al. 2013; Paneni et al. 2014; Theuma and Fonseca, 2003). The relative importance of these risk factors in the induction of vascular dysfunction in T2D patients has not been well-studied. Prolonged hyperglycemia, elevated blood glucose, is the hallmark of diabetes and results in the glycosylation of hemoglobin molecules (HbA1c), elevations of which can be used as biomarkers of glycemic control over the prior 2-3 months. This leads to oxidative stress and inflammation, both of which contribute to the development of renal failure, blindness, and cardiovascular disease (Rolo and Palmeira, 2006). Oxidative stress may also contribute to hypertension by reducing the bioavailability of the vasodilator nitric oxide, resulting in impaired endothelium-dependent vasodilation, which can be reversed with antioxidants (Bajaj and Khan, 2012). Additionally, T2D is associated with lipid abnormalities characterized by high triglyceride concentrations, reductions in high density lipoprotein-cholesterol (HDL-C) concentrations, and increases in low density lipoprotein-cholesterol (LDL-C) concentrations. Increases in small, dense proatherogenic LDL-C particles are frequently observed in T2D (Lukic et al. 2014). Most of the lipid abnormalities in T2D can be explained by reduced action of insulin at the tissue level (Reaven, 2011). Previous research has established a link between high concentrations of LDL-C and CVD (Wing et al. 2011). Most patients with diabetes require diet and/or hypoglycemic medications to regulate their blood glucose (CDC, 2012) often resulting in little success. Moreover, costly hypoglycemic medications are often associated with complications including gastrointestinal distress, lactic acidosis and hepatotoxicity (Qaseem et al. 2012). Data has also indicated that T2D is a strong predictor of complications associated with

hypertension and other cardiovascular disease risk factors. Approximately 67% of T2D adults have an average resting blood pressure of ≥ 140 mm Hg (defined as hypertensive) (Ferrannini et al. 2012). Therefore, simple and inexpensive dietary strategies for the control of diabetes would be welcomed by patients and medical professionals and may help to reduce the economic burden related to both direct and indirect costs of this devastating illness within the United States as well as globally if widely-implemented.

Studies have shown that individuals who consume diets high in fibers such as psyllium and beta-glucan have decreased oxidative stress and inflammation along with improved lipoprotein profiles (Satija and Hu, 2012). While research has focused on the health benefits of plant-based fibers such as psyllium at reducing symptoms of T2D and cardiovascular disease, to our knowledge, no studies have examined the potential therapeutic benefits of flaxseed-derived fiber supplementation in the control of T2D in regards to endothelial function and inflammation. Moreover, a comparison of flaxseed-derived versus pysllium-based fibers on diabetic complications, including cardiovascular disease, has not been performed.

The majority of studies on flaxseeds have focused on the effects of the oil, which even at high doses (10g/day) reportedly has no impact on blood glucose, HbA1c, insulin concentrations or lipid profiles (Barre et al. 2008). In contrast, a recent study concluded that daily supplementation of ground flaxseeds (10g/day) in adults with T2D for just one month significantly reduced fasting blood glucose by 19.7%, HbA1c by 15.6%, in addition to improvements in lipid regulation (Mani et al. 2011). Moreover, recent

research has indicated that 40 g/d of flaxseed meal lead to significant improvements in lipid profiles in adult males with hypercholesterolemia (Pan et al. 2007).

1.2 Purpose of Study

The purpose of this pilot study and efficacy trial is to compare the effectiveness of eight weeks of 28 g/d ground flaxseed supplemented to an individual's habitual diet as compared to a standardized amount of psyllium fiber (9 g/d) on management of symptoms associated with T2D including glucose homeostasis, dyslipidemia, oxidative stress, inflammation, and blood pressure in subjects with non-insulin dependent T2D.

1.3 Specific Aims and Hypotheses

The hypothesis for this study is that there will be no significant difference in participants with non-insulin dependent T2D who supplement their normal diet with 28 g/d of ground flaxseed versus 9 g/d of psyllium on glycemic regulation, serum lipids, and concentrations of inflammatory as well as markers of oxidative stress. We will test this hypothesis through three specific aims.

Specific Aim 1: To compare the effectiveness of daily flaxseed (28 g/d) or psyllium (9 g/d) supplementation on improvements in acute and chronic glycemic control in adults with non-insulin dependent T2D. **Hypothesis:** Adults with T2D consuming 28g/d of ground flaxseeds for 8 weeks will have reduced fasting blood glucose, insulin, HbA1c,

and homeostatic model assessment of insulin resistance (HOMA-IR), compared to matched subjects consuming 9 g/d pysllium supplementation.

Specific Aim 2: To compare the effectiveness of daily flaxseed (28 g/d) or psyllium (9 g/d) supplementation on improvement in lipid profiles. **Hypothesis:** There will be no difference in total cholesterol, LDL-C, HDL-C, total triglycerides, VLDL-C, or HDL:LDL ratios in adults with T2D consuming 28 g/d of ground flaxseed as compared to matched subjects for 8 weeks will have a significantly lower total cholesterol, total triglycerides, and LDL-C as well as an improved LDL:HDL ratio as compared to matched subjects consuming a supplement with a similar fiber content (9 g/d psyllium husk).

Specific Aim 3: To compare the effectiveness of daily flaxseed (28 g/d) or psyllium (9 g/d) supplementation on improvements in markers of vascular reactivity through increased nitric oxide availability as well as diminished oxidative stress and inflammation in adults with non-insulin dependent T2D. **Hypothesis:** There will be no significant difference in improvements of vascular health (reduced blood pressure and increased plasma nitric oxide), reductions of markers of oxidative stress and inflammation (lipid peroxidation, and TNF-α) between the non-insulin dependent T2D participants in the flaxseed group (28 g/d) as compared to the psyllium group (9 g/d) from baseline to week 8.

1.4 Impact

The results of this study are expected to have a broad impact by contributing to the understanding of how flaxseed supplementation may improve vascular health in diabetes as well as provide a potential cost-effective nutrition-based alternative for glucose regulation that may help to alleviate the costly burden of medication to individuals suffering from diabetes.

1.5 Delimitations and Limitations

Possible limitations of this study include lack of adequate sample size to achieve power (set at 80%). To avoid these potential complications we performed a sample size power analysis based upon an extensive literature review (see section 3.6). Additionally, we modified the amount of test foods for both treatment and control groups to represent moderate intake that more accurately reflect a participant's average diet in an effort to increase compliance. Anticipated problems include contamination of samples which will be avoided by utilizing rigorous laboratory techniques and by wearing personal protective equipment at all times. There is also a potential problem in tissue availability which will be overcome by optimizing assays through proper collection and storage of tissue samples, using aliquoted samples to prevent protein degradation, and determining appropriate collected sample (i.e., plasma versus serum) to use for each parameter, and by using commercially available kits which have been previously validated for sensitivity, specificity, accuracy, precision, detection limit, range and limits of quantitation. Standard curves were also performed an analyzed during each assay,

according to the manufacturers protocol to ensure inter-assay and inter-laboratory assessment of assay and to increase the repeatability and robustness of the results.

Delimitations of the study are that the data will be generalizable only to adults with non-insulin dependent T2D. Data will also not provide information on direct measurements of vascular dysfunction or small resistance arteries which are important in blood pressure regulation and microvascular complications associated with T2D.

Definition of Terms

Adenosine Diphosphate (ADP)- a nucleotide composed of adenine, ribose, and two phosphate groups that functions in the transfer of energy during the catabolism of glucose, formed by the removal of a phosphate group from adenosine triphosphate.

Adenosine Triphosphate (ATP)- a high energy molecule which consists of adenosine, ribose, and three phosphate groups. It is the primary source of energy in cellular metabolism due to its ability to donate a phosphate group during biochemical reactions.

Adipokines- Cytokines secreted by adipose tissue.

Adiponectin- A 244 amino acid polypeptide involved in regulating glucose concentrations as well as fatty acid breakdown.

Adipose Tissue Macrophages (ATMs)- Macrophages present in adipose tissue.

Angiotensin Converting Enzyme (ACE)- A glycoprotein (dipeptidyl carboxypeptidase) that catalyzes the conversion of angiotensin I to angiotensin II by splitting two terminal amino acids. ACE-inhibiting agents are used for controlling hypertension and for protecting the kidneys in diabetes mellitus.

Angiotensin II (AII)- An octapeptide that is a potent vasopressor and powerful stimulus for production and release of aldosterone from the adrenal cortex.

Arteriosclerosis- A condition in which an artery wall thickens as a result of the accumulation of fatty materials such as cholesterol and triglyceride. It is a syndrome

affecting arterial blood vessels, a chronic inflammatory response in the walls of arteries, caused largely by the accumulation of macrophages and promoted by low-density lipoproteins.

C-Reactive Protein (CRP)- A protein found in the blood, the levels of which rise in response to inflammation (acute phase protein).

Cytokine- Small signaling molecules used for cell signaling. Cytokines can be classified as proteins, peptides, or glycoproteins.

Diabetes- A group of metabolic diseases in which a person has high blood sugar, either because the pancreas does not produce enough insulin, or because cells do not respond to the insulin that is produced. **Dyslipidemia-** An abnormal amount of lipids (e.g. cholesterol and/or fat) in the blood.

Dyspnea- Shortness of breath.

Electron Transport Chain (ETC)- an enzymatic series of electron donors and acceptors located within the mitochondria of eukaryotic cells that transfer electrons from electron donors to electron acceptors via redox reactions, and couples this electron transfer with the transfer of protons (H+ ions) across a membrane which drives the synthesis of ATP.

Endocrine System- The collection of cells, glands, and tissues of an organism that secrete hormones directly into the bloodstream to control the physiological and behavioral activities of an organism.

Endothelin 1 (ET-1)- A protein peptide that is a potent vasoconstrictor produced by vascular endothelial cells.

Endothelium- the thin layer of endothelial cells that line the interior surface of blood vessels forming an interface between circulating blood in the lumen and the rest of the vessel wall. Endothelial cells line the entire circulatory system, from the heart to the capillaries. Functions include fluid filtration (such as in the glomeruli of the kidney), blood vessel tone, hemostasis, neutrophil recruitment, and hormone trafficking.

Fatty Acid Oxidation- (beta-oxidation) The process by which fatty acid molecules are broken down in the mitochondria to generate acetyl-coA, which enters the citric acid cycle, and NADH and FADH2, which are used by the electron transport chain.

Fibroblasts- A type of cell that synthesizes the extracellular matrix and collagen; the structural framework (stroma) for animal tissues; and plays a critical role in wound healing. Fibroblasts are the most common connective tissue in animals.

Gluconeogenesis (GNG)- A metabolic pathway that generates glucose from non-carbohydrate carbon substrates such as pyruvate, lactate, glycerol, gluconeogenic amino acids, and medium-chain fatty acids.

Glucose- A six carbon (hexose) sugar molecule that serves as the principle source of sugar in the blood and a major source of metabolic energy.

Glucose Transporters- A diverse family of membrane proteins that facilitate the transport of glucose over a plasma membrane.

Glycogenolysis- Breakdown of glycogen to glucose-1-phosphate and glucose. Glycogen branches are catabolized by the sequential removal of glucose monomers via phosphorolysis, by the enzyme glycogen phosphorylase.

Glycolysis-The catabolism of carbohydrates such as glycogen and glucose by enzymes into pyruvate or lactic acid resulting in the release of energy.

Hemoglobin A1c (HbA1C)- A form of hemoglobin that is measured primarily to identify the average plasma glucose concentration over prolonged periods of time (i.e. prior 90 days). It is formed by non-enzymatic binding of glucose to hemoglobin.

Hyperglycemia- A condition in which an excessive amount of glucose circulates in the blood plasma (fasting plasma glucose >100 mg/dl or non-fasting plasma glucose >140 mg/dl).

Hyperplasia- An increase in cell number.

Hypertrophy- An increase in cell size.

Hypoxia- A pathological condition in which the body or a region of the body is deprived of an adequate oxygen supply.

Hypoxia Inducible Factor 1 alpha (HIF-1 alpha)- a protein regulated by nuclear factor-kappa B in response to hypoxia.

Impaired Fasting Glucose (IFG)- A condition in which fasting blood glucose concentrations are consistently elevated above what is considered physiological.

Impaired Glucose Tolerance (IGT)- A sustained hyperglycemic response during a glucose challenge.

Infarction- Tissue death (necrosis) caused by a lack of oxygen, due to an obstruction of blood flow in a tissue.

Inflammation- Part of the complex biological response of vascular tissues to harmful stimuli such as pathogens, damaged cells, or irritants. Inflammation is a protective attempt by the organism to remove the injurious stimuli and to initiate the healing process.

Innate Immune System- The first line of defense of the immune system response, comprises the cells and mechanisms that defend the host from infection by other organisms in a non-specific manner (i.e. not requiring the production of antibodies).

Insulin Resistance- A physiological condition in which cells fail to respond to the normal actions of the hormone insulin.

Interleukin 1 beta (IL-1 beta)- A cytokine mediator of the inflammatory response. It is also involved in a variety of cellular activities including cell proliferation, differentiation, and apoptosis.

Interleukin 6 (IL-6)- Secreted by T cells and macrophages to stimulate immune responses. Acts in both pro-inflammatory and anti-inflammatory capacities.

Interleukin 8 (IL-8)- a chemokine secreted by any cells with toll-like receptors that are involved in the innate immune response.

Islets of Langerhans- Irregular clusters of endocrine cells scattered throughout the tissue of the pancreas that secrete insulin and glucagon.

Leptin- A 16-kDa protein hormone that plays a key role in regulating energy intake and expenditure including appetite and hunger, metabolism, and behavior.

Lipogenesis- The process by which acetyl-CoA is converted to fatty acids.

Lipolysis- The breakdown of lipids. It involves hydrolysis of triglycerides into glycerol and free fatty acids.

Macrophages- Immune cells produced by the differentiation of monocytes that are responsible for ingesting and breaking down pathogens.

Metabolic Syndrome (MetS)- A disorder of energy utilization and storage, diagnosed by a co-occurrence of three out of five of the following medical conditions: abdominal (central) obesity, elevated blood pressure, elevated fasting plasma glucose, high serum triglycerides, and low high-density cholesterol (HDL-C) levels. Metabolic syndrome increases the risk for developing cardiovascular disease.

Monocyte Chemoattractant Protein 1 (MCP1)- recruits monocytes, memory T cells, and dendritic cells to the sites of inflammation produced by either tissue injury or infection.

Nitric Oxide- A potent vasodilator.

Nitric Oxide Synthases- A family of enzymes that catalyze the production of nitric oxide (NO) from L-arginine.

Non-Alcoholic Hepatic Steatosis- Accumulation of fat in the liver; may progress to non-alcoholic fatty liver disease (NAFLD).

Normoglycemia- Blood glucose concentrations that fall within the normal range of <100 mg/dl fasting, <140 mg/dl 2 hours after a high load glucose challenge, or an A1C <6.0%.

Oxidative Stress- A situation in which there is an increase in free radicals caused by either increased production of oxidizing species or a significant decrease in the effectiveness of antioxidant defenses.

Perivascular Adipose Tissue (PVAT)- Adipose tissue surrounding blood vessels.

Polydipsia- Excessive thirst.

Polyphagia- Excessive hunger.

Polyuria- Excessive urination.

Preadipocytes- Undifferentiated fibroblasts that can be stimulated to form mature adipocytes.

Prediabetes- The state in which some but not all of the diagnostic criteria for diabetes are met.

Preproinsulin- the precursor of proinsulin which contains an additional polypeptide sequence at the N-terminal which are then removed by proteases in the endoplasmic reticulum to form proinsulin.

Reactive Oxygen Species- Chemically reactive molecules containing oxygen.

Resistin- A cysteine-rich adipokine implicated in the etiology and progression of obesity and type 2 diabetes. Resistin accelerates the accumulation of LDL in arteries, increasing the risk of heart disease.

Sleep Apnea- A sleep disorder characterized by pauses in breathing or instances of shallow or infrequent breathing during sleep.

Superoxide ($O_2^{\bullet -}$)- A free radical product of the one-electron reduction of dioxygen.

The Citric Acid Cycle (TCA or Krebs cycle)- A major metabolic pathways of cellular respiration, and involves a cyclic series of enzymatic reactions by which pyruvate converted into Acetyl Coenzyme A is completely oxidized to CO_2 and hydrogen is removed from the carbon molecules, transferring the hydrogen atoms and electrons to electron-carrier molecules (e.g. NADH and $FADH_2$) as well as the synthesis of ATP.

Toll-Like Receptors (TLRs)- A class of proteins that play a key role in the innate immune system. They are single, membrane-spanning, non-catalytic receptors usually expressed in sentinel cells such as macrophages and dendritic cells that recognize structurally conserved molecules derived from microbes.

Tumor Necrosis Factor Alpha (TNF-alpha)- An adipokine involved in systemic inflammation that stimulates the acute phase reaction.

Type 2 Diabetes (T2D)- Disease state characterized by insulin resistance, sometimes combined with an absolute insulin deficiency. This form of diabetes was previously referred to as non insulin-dependent diabetes mellitus (NIDDM) or "adult-onset diabetes".

Vascular Endothelial Growth Factor (VEGF)- A signal protein produced by cells that stimulates vasculogenesis and angiogenesis.

CHAPTER 2

REVIEW OF LITERATURE

2.1 Glucose Metabolism

2.1.1 Glucose

Glucose metabolism is critical to normal physiological functioning. Glucose is the human body's key source of energy, providing about four calories per gram through aerobic respiration. Breakdown of carbohydrates (e.g. starch) yields mono- and disaccharides, most of which is glucose. Through glycolysis and later in the reactions of the tri-carboxylic acid (TCA) cycle, glucose is oxidized to eventually form CO_2 and water, yielding energy mostly in the form of adenosine tri-phosphate (ATP) which is required for nearly all types of cellular reactions. Normal plasma glucose concentration varies between about 70 and 120 mg/dL (3.9-6.7 mM) (Mantzoros et al. 2011). The brain uses about 120 grams of glucose daily which is approximately 60-70% of the total body glucose metabolism (Bergman, 2013). However, the brain has little stored glucose and no other energy stores, making glucose an essential nutrient for survival. Brain function begins to become seriously affected when glucose levels fall below 40 mg/dL; levels of glucose significantly below this can lead to permanent damage and death (Levin, 2002). The brain cannot use fatty acids for energy due to the inability of fatty acids to cross the blood-brain barrier. Ketone bodies such as acetoacetate and hydroxybutyrate, which are produced as by-products of beta-oxidation derived acetyl-CoA being further metabolized in the TCA metabolic pathway, can however be utilized

by the brain for energy in emergencies such as periods of severely restricted carbohydrate or food intake.

2.1.2 Glucose Homeostasis

Glucose entry into cells is facilitated by a family of glucose transporter proteins (GLUT). The most commonly identified glucose transporters are GLUT1-5, each of which exhibit different affinities for various monosaccharides and display tissue-specific expression (Zhao and Keating, 2007). These characteristics contribute to how the various tissues respond to glucose. Typically, glucose transporters are localized on the plasma membrane and allow for glucose to enter the cell in a passive diffusion manner (Klip and Pâquet, 1990). However, the glucose transporter isoform 4 (GLUT4), is located in intracellular membrane vesicles which must be stimulated through an insulin mediated cell signaling cascade and undergo cellular trafficking to the membrane surface (Russell et al. 1999). The GLUT4 isoform, which is specifically expressed in insulin sensitive tissues such as skeletal muscle, heart muscle, and fat, is responsible for insulin-mediated blood glucose clearance (Saltiel and Kahn, 2001).Various organs function to regulate circulating glucose levels in the plasma including the liver, brain, pancreas, skeletal muscle, adipose tissue, heart, and kidneys. There are two main endogenous metabolic pathways which function to regulate glucose levels, gluconeogenesis and glycogenolysis. Gluconeogenesis (GNG) is a metabolic pathway that results in the generation of glucose from non-carbohydrate carbon substrates such as pyruvate, lactate, glycerol (derived from odd-chain fatty acids and to a lesser extent long-chain fatty acids) in

addition to glucogenic amino acids. The liver and kidneys are the main organs which contribute to the GNG pathway. When circulating blood glucose concentrations are too low (hypoglycemia) the body acts to increase glucose levels through the breakdown of glycogen via glycogenolysis. Glycogenolysis functions to catabolize glycogen, the stored glucose energy source in liver and skeletal muscle, to glucose-1 phosphate which is then readily available to provide energy for intracellular processes.

The diet is an abundant source of circulating glucose and provides carbon and energy sources for liver gluconeogenesis. The liver is the major metabolic regulatory organ for glucose metabolism. About 90% of all circulating glucose not derived directly from the diet comes from the liver (Schwarz et al. 1995). The liver contains significant amounts of stored glycogen available for rapid release into circulation and is capable of synthesizing large quantities of glucose from substrates such as lactate, amino acids, and glycerol released by other tissues. In addition to controlling plasma glucose, the liver is responsible for synthesis and release of the lipoproteins that adipose and other tissues use as the source of cholesterol and free fatty acids (Weikert and Pfieffer, 2006). During prolonged starvation, the liver is the source of both glucose and ketone bodies that are required by the brain to maintain function. Like the liver, the kidney has the ability to release glucose into the blood. Under normal conditions gluconeogenesis in the kidneys provides only a small contribution to the total circulating glucose; however, during prolonged starvation, the contribution of the kidneys to circulating glucose may approach that of the liver (Stumvoll et al. 1998). Kidney function is critical for glucose homeostasis for another reason; plasma glucose continuously passes through the kidney and must be

efficiently reabsorbed to prevent loss. Skeletal muscle cannot release glucose into circulation; however, it has the ability to rapidly increase glucose uptake in response to sudden elevations in circulating plasma glucose as is the case after a meal or during periods of increased demand, such as during an acute bout of exercise (Rose and Richter, 2005). Adipose tissue also acts as a major site of glucose metabolism. Insulin stimulates glucose uptake into adipose tissue, and has three major actions which result in net fat deposition: 1) insulin increases the amount of lipoprotein lipase, an enzyme that mediates release of free fatty acids from circulating lipoproteins; 2) insulin stimulates synthesis of glycerol-phosphate (required for triacylglycerol synthesis) from glucose; and 3) insulin inhibits hormone-sensitive lipase, the enzyme responsible for the first step in triacylglycerol breakdown (Odegaard and Chawla, 2013). Adipose tissue is the major site of fatty acid storage. Fatty acids are stored in the form of triacylglycerol, which is synthesized from glycerolphosphate and free fatty acids. The glycerol-phosphate used must be derived from glycolysis in the adipose tissue; free glycerol cannot be phosphorylated because adipocytes lack the relevant kinase (Guo et al. 2013). In conditions when liver gluconeogenesis is necessary the adipose tissue supplies free fatty acids and glycerol into the circulation to be taken up by the liver as substrate. Finally, the pancreas is the source of insulin and glucagon, two of the most important metabolic regulatory hormones.

2.2 Regulation of Post-Prandial Glucose Metabolism

2.2.1 Endocrine regulation of glucose metabolism

Insulin is an anabolic hormone secreted by the pancreas essential for appropriate tissue development and growth. Insulin also functions to regulate circulating glucose levels in response to a meal (Pessin et al. 2000). Insulin plays a major role in the metabolism of carbohydrates and lipids (McGarry and Foster 1980). The pancreas alters its release of insulin and glucagon in response to changes in plasma glucose and other circulating nutrients such as amino acids and free fatty acids. The response to a meal varies significantly depending on the composition of nutrient intake. The release of insulin levels caused by actually eating is thought to be due to gastrointestinal peptide hormones such as cholecystokinin (CCK), glucagon-like peptide 1 (GLP-1), and ghrelin (Weir et al. 2012). These peptides are released in response to food absorption and function to mediate the glucose effect on insulin release.

When eating a meal rich in carbohydrates, insulin levels rise and glucagon levels fall (Thorens et al. 2011). The decrease of glucagon is due to inhibition of its release by insulin, and to the elevation in plasma glucose. When eating a meal rich in protein, insulin levels rise, because insulin secretion is stimulated by amino acids as well (Salehi et al. 2012). The release of glucagon in response to a meal high in protein functions to counteract the effects of insulin in order to maintain proper circulating glucose levels and avoid hypoglycemia due to the low carbohydrate content of the meal. When eating a mixed meal, insulin levels rise, and glucagon levels rise, fall, or remain unchanged as appropriate to maintain plasma glucose.

2.2.2 Mechanism of insulin

Insulin is a hormone that is exclusively produced by pancreatic beta cells. Beta cells are located in the pancreas in clusters known as the islets of Langerhans. Insulin is a small protein and is produced as part of a larger protein to ensure it folds properly. In the protein assembly of insulin, the messenger RNA transcript is translated into an inactive protein called preproinsulin (Fu et al. 2013). Preproinsulin contains an amino-terminal signal sequence that is required in order for the precursor hormone to pass through the membrane of the endoplasmic reticulum (ER) (Leem and Koh, 2011) for post-translational processing to proinsulin which is further modified to active insulin (Guo et al. 2014). Finally, insulin is packaged and stored in secretory granules, which accumulate in the cytoplasm, until release is triggered.

2.2.3 Insulin secretion

Insulin is released from beta cells, in response to changes in blood glucose concentration. Type 2 glucose transporters (GLUT2) mediate the entry of glucose into beta cells (Luni et al. 2012). Glucose ($C_6H_{12}O_6$) enters the glycolysis pathway, which is the metabolic pathway responsible for converting glucose to pyruvate ($CH_3COCOO^- + H^+$) and subsequent high energy compounds adenosine triphosphate (ATP) and reduced nicotimamide adenine dinucleotide (NADH). Glucose is phosphorylated by the rate-limiting enzyme in this pathway, glucokinase. Upon modification glucose becomes trapped within the beta cells and is further metabolized to create ATP (Richter et al. 2013). The increased in ATP to adenosine diphosphate (ADP) moleculares (ATP:ADP

ratio) causes the ATP-gated potassium channels in the cellular membrane to close, preventing potassium ions from crossing the membrane (Masini et al, 2014). The increase in potassium ions within the cell leads to an intracellular positive charge resulting in subsequent depolarization of the cell. This causes activation of voltage-gated calcium channels, which transport calcium ions into the cell. The increase in intracellular calcium concentrations results in the exportation, via exocytosis, of the insulin-storing granules from the beta cells and the diffusion of insulin into nearby blood vessels (Rorsman and Braun, 2013). There is a large and extensive vascular network which surrounds the pancreatic islets to ensure the adequate diffusion of insulin (and glucose) between beta cells and blood vessels (Dai et al. 2013).

The release of insulin occurs in two stages. The initial amount of insulin released upon increases in circulating glucose is dependent on the available concentrations stored in the beta cells. Once these initial stores are depleted a second phase of insulin release is initiated. This latter release is prolonged since insulin has to be synthesized, processed, and secreted for the duration of the increase of blood glucose. Furthermore, beta cells also have to regenerate the stores of insulin initially depleted in the fast response phase.

2.2.4 Functions of insulin and target tissues of insulin-mediated glucose disposal

Insulin molecules circulate throughout the blood stream until they bind to their receptors (IR) located on various target tissues. Insulin regulates glucose disposal at many sites including the liver, skeletal muscles, adipose tissue (AT) and cardiac muscle (Olson and Pessin, 1996). In muscle and fat cells, the clearance of circulating glucose depends on the

insulin-stimulated translocation of the glucose transporter GLUT4 isoform to the cell surface which is initiated through activation of the IR. When insulin binds to its receptor on the cell membrane surface it activates the IR tyrosine kinase, which results in tyrosine phosphorylation of members of the insulin receptor substrate (IRS) family. Activation of the IR and IRS results in phosphorylation of the phosphatidylinositol 3-kinase (PI3K) pathway resulting in activation of protein kinase B (Akt) which functions to stimulate GLUT4 vesicles to the membrane surface. Insulin also functions to reduce hepatic glucose production and output (via decreased GNG and glycogenolysis) as well as increasing lipid synthesis in liver and adipocytes which result in attenuating fatty acid release from triglycerides in AT and muscle (Pessin et al. 2000). In a healthy person, these functions allow blood glucose and insulin levels to remain in the normal range.

2.2.5 Impaired insulin signaling leading to insulin resistance

The body's regulation of glucose metabolism requires control and coordination of a series of complex mechanisms. Disruptions in normal insulin synthesis, secretion, regulation, or cell signaling lead to altered metabolism of glucose as well as lipids resulting in hyperglycemia and dyslipidemia. Impairments in normal insulin signaling are usually the result of genetic acquired traits, auto-immune disorders or environmental factors (Mlinar et al., 2007). In cases of type 1 diabetes mellitus, the body destroys insulin-producing beta cells in the pancreas, thus the individual does not produce insulin and must use an exogenous form. Other alterations in insulin signaling occur within the beta cells during synthesis and secretion, or at the post-receptor levels. At a molecular level insulin

resistance can be caused by impaired insulin signaling due to increased serine phosphorylation of the insulin receptor substrate-1 (IRS1). This change causes inhibition of IRS1 tyrosine phosphorylation, decreased binding of the downstream enzyme PI3K, and decreased phosphorylation and activation of the kinase Akt. IRS1 can be phosphorylated on serine residues by various isoforms of protein kinase C, which are activated by lipid intermediates, stress kinases, or increases in endoplasmic reticulum stress (Turban and Hujduch, 2011). The resulting hyperinsulinemia along with increases in inflammatory markers such as tumor necrosis factor alpha (TNF-alpha) and interleukin-6 (IL-6) may perpetuate the deregulation of Ser/Thr phosphorylation of IRS1 and downstream signaling intermediates including PI3K (Rains and Jain, 2011).

2.2.6 Insulin resistance and associated disorders

Impaired insulin signaling is central to development of the insulin resistance, metabolic syndrome (MetS) as well as diabetes and can promote cardiovascular disease indirectly through development of abnormal glucose and lipid metabolism, hypertension, and inflammation. Insulin resistance in the liver is the major cause of fasting hyperglycemia due to increases in hepatic glucose output which is normally suppressed by insulin. Disrupted insulin signaling in the liver also contributes to dyslipidemia associated with hyperglycemia due to increases in fatty acid synthesis and decreased clearance of LDL and very low-density (VLDL) lipoproteins (Lukic et al. 2014). Moreover, impaired insulin signaling disrupts skeletal muscle glucose disposal due to decreased GLUT4 expression through down-regulation of the insulin-mediated cell signaling cascade. In

addition to insulin resistance and hyperglycemia, dyslipidemia is a common characteristic of both MetS and T2D. Hepatic insulin resistance contributes to this dyslipidemia through increased fatty acid synthesis as well decreased clearance of LDL and very low-density lipoprotein (Lukic et al. 2014).

2.3 Insulin Resistance and Progression to T2D

2.3.1 Insulin resistance

Type 2 diabetes is a metabolic disorder that is characterized by high blood glucose in the context of insulin resistance and relative insulin deficiency (Kumar et al. 2008). Insulin resistance is a physiological condition in which cells fail to respond to the normal actions of the hormone insulin. In the context of insulin resistance, the body produces enough insulin in response to increases in glucose concentrations but its cells are too desensitized to allow adequate insulin signaling cascade response. Due to the ability to initially compensate for the lack of cell sensitivity and the need to maintain proper glucose homeostasis a person can still have glucose levels in the normal range as described below. Causes of insulin resistance may include ethnicity, certain diseases, excess weight, physical inactivity, hormones, steroid use, certain medications, age, and cigarette smoking. Mechanisms of insulin resistance as discussed in previous sections (*2.2.5*; *2.2.6)* include inhibition of IR, IRS, or insulin signaling intermediates. A person can be insulin resistant and still maintain normal blood glucose levels (normoglycemia) as long as the beta cells produce enough insulin to compensate for the decrease in cell sensitivity. Fluctuations in insulin sensitivity occur during the normal life cycle, with insulin

resistance being observed in puberty, pregnancy, and with aging (Moran et al. 1999, Buchanan et al. 1990). Conversely, lifestyle variations such as increased physical activity and increased carbohydrate intake are associated with increased insulin sensitivity (Musi and Goodyear, 2006). Thus, without lifestyle modifications (i.e., increased physical activity, improvements to diet) or medical interventions, insulin resistance will often progress to a condition known as prediabetes due to the high demand placed on the insulin-producing beta cells (McLellan et al. 2014).

2.3.2 Metabolic syndrome, insulin resistance, and progression to T2D

Insulin resistance is strongly associated with a condition known as MetS. Prevalence of MetS in 2008, according to data from the National Health and Nutrition Survey Data Set (NHANES) was estimated at 34% of adults age 20 and older (Bassin et al. 2013). MetS is thought to represent a combination of cardiometabolic risk determinants, including obesity, glucose intolerance, insulin resistance, dyslipidemia (including hypertriglyceridemia, increased free fatty acids (FFAs) and decreased HDL-C) and hypertension. The concept of a cluster of metabolic abnormalities which predispose an individual to T2D and cardiovascular disease was first proposed by Eskil Kylin in the early 1900's. Dr. Haller first used the term "metabolic syndrome" (MetS) to describe the association of obesity, diabetes mellitus (DM), hyperlipoproteinemia, hyperuricemia, and hepatic steatosis when describing the additive effects of risk factors on atherosclerosis. Dr. Gerald Phillips (1978) further developed the concept of metabolic risk factors for myocardial infarction and described a group of abnormalities including glucose

intolerance, hyperinsulinemia, hyperlipidemia, and hypertension. In 1988, Dr. Gerald Reaven proposed insulin resistance (IR) was the cause of glucose intolerance, hyperinsulinemia, increased (VLDL-C), decreased (HDL-C) and hypertension and named the constellation of abnormalities "syndrome X". Multiple Health organizations including the World Health Organization (WHO), International Diabetes Federation (IDF), the European Group for the Study of Insulin Resistance, and the National Cholesterol Education Program Adult Treatment Panel III (NCEP ATP III) all have varying definitions and diagnostic criteria for MetS (Miranda et al. 2005). However, the most common set of diagnostic criteria used to identify individuals with MetS in the U.S. by the American Heart Association and American Diabetes Association is the NCEP ATP III guideline which confirms MetS as having three or more of the following measurements present:

- Abdominal obesity (waist circumference of 40 inches or above in men, and 35 inches or above in women)
- Triglyceride level of 150 milligrams per deciliter of blood (mg/dL) or greater
- HDL cholesterol of less than 40 mg/dL in men or less than 50 mg/dL in women
- Systolic blood pressure (top number) of 130 millimeters of mercury (mm Hg) or greater, or diastolic blood pressure (bottom number) of 85 mm Hg or greater
- Fasting glucose of 100 mg/dL or greater

The associated complications of insulin resistance and MetS include atherosclerosis, coronary heart disease (CHD), non-alcoholic fatty liver disease (NAFLD), some cancers,

kidney dysfunction, pancreatic dysfunction, and polycystic ovarian syndrome (PCOS). While having insulin resistance and metabolic syndrome are risk factors for the development of prediabetes and T2D, it is not absolute. Moreover, it should be noted that while obesity appears to be a central factor in the development of both IR and MetS, normal weight individuals can develop either of these conditions or both independent of a being classified as overweight or obese (Park et al. 2003).

2.3.3 Pre-diabetes

In prediabetes, the beta cells can no longer produce enough insulin to overcome insulin resistance, causing blood glucose levels to rise above the normal range, known as impaired glucose tolerance (IGT). Prediabetes is a condition in which a person develops hyperglycemia (elevated blood glucose) or increased glycated hemoglobin (HbA1c) levels, which reflect average blood glucose levels over the prior 90 days, that are higher than normal but not high enough for a diagnosis of diabetes. A HbA1c of 5.7% to 6.4% indicates prediabetes. A fasting glucose level of 100 to 125 mg/dl indicates prediabetes and is known as impaired fasting glucose (IFG). A two hour sample of blood glucose level between 140 and 199 mg/dl for an oral glucose tolerance test, which measures blood glucose levels for up to 3 hours after a high glucose drink has been ingested indicates prediabetes. People with prediabetes are at increased risk of developing T2D and CVD, which can lead to heart attack or stroke (Ganguly et al. 2008). IR increases the risk of developing T2D and prediabetes (Cobb et al. 2013). Prediabetes usually occurs in people who already have insulin resistance. Although insulin resistance alone does not

cause T2D, it often contributes to the disease by placing a high demand on the insulin-producing beta cells which eventually leads to the loss of adequate production as stated previously.

2.3.4 Type 2 diabetes

Once a person has prediabetes, continued loss of beta cell function usually leads to T2D (Ristow et al. 2004). Among those individuals who develop T2D, the proportion of IR versus beta cell dysfunction varies, with some having primarily insulin resistance and only a minor defect in insulin secretion and others with slight insulin resistance and primarily a lack of insulin secretion (Shoback et al. 2011). Other potentially important mechanisms associated with T2D and insulin resistance include increased breakdown of lipids within adipocytes, resistance to and lack of incretin (a gastrointestinal hormone that stimulate a decrease in blood glucose by causing an increase in the amount of insulin released from the beta cells after eating, before blood glucose levels become elevated), high glucagon levels in the blood that promote hyperglycemia, increased retention of salt and water by the kidneys, and inappropriate regulation of metabolism by the central nervous system (Smyth et al. 2006). People with T2D have hyperglycemia and, due to loss of insulin regulated lipid metabolism, elevated levels of non-esterified fatty acids (NEFA) (Tolman et al. 2007). Diagnosis for T2D as defined by the WHO includes a single raised glucose reading with classic symptoms including polyuria (frequent urination), polydipsia (increased thirst), polyphagia (increased hunger), and weight loss. Other symptoms that are commonly present at diagnosis include: a history of blurred

vision, itchiness, peripheral neuropathy, recurrent vaginal infections, and fatigue (ADA, 2013). Additionally, diagnosis can be based from raised values on two occasions of either: a fasting plasma glucose ≥ 7.0 mmol/l (126 mg/dl) or plasma glucose concentrations ≥ 11.1 mmol/l (200 mg/dl) two hours after consuming a controlled dose of glucose during an oral glucose tolerance test (OGTT). A random blood sugar of greater than 11.1 mmol/l (200 mg/dl) in association with typical symptoms or HbA1c of greater than 6.5% are other diagnostic measures of diabetes. Over time, high blood glucose damages nerves and blood vessels, leading to complications such as CVD, stroke, blindness, kidney failure, neuropathies, and lower-limb amputations. IR and prediabetes often go unrecognized by patients until they have progressed to overt diabetes. People may have one or both conditions for several years without knowing they have them.

2.4 Obesity and T2D

2.4.1 Adipose tissue as an endocrine organ

In addition to impairments in insulin signaling, accumulating AT and obesity are common factors in development of IR, T2D, and MetS. Adipose tissue (i.e., body fat) is composed mainly of adipocytes and connective tissue. In addition to adipocytes, AT contains the stromal vascular fraction (SVF) of cells including preadipocytes, fibroblasts, vascular endothelial cells and a variety of immune cells (i.e. AT macrophages (ATMs)). Adipose tissue is derived from preadipocytes. Adipose tissue is found in specific locations, which are referred to as adipose depots. In humans, major AT depots include subcutaneous (below the skin), visceral (around the organs), intrahepatic (Koska et al.

2008) and to a lesser extent bone marrow (yellow bone marrow) and breast tissue. Its main role is to store excess triglycerides which are esterified to free fatty acids and glycerol as well as function as an energy reserve, insulate the body and cushion vital organs. Additionally, AT acts as an endocrine organ (Kershaw, 2004) and has been shown to synthesize and secrete a number of hormones, called adipokines, which are involved in the regulation of energy homeostasis, insulin action, and lipid metabolism (Dyck et al. 2006). Some of the hormones considered to play key roles in energy metabolism, include leptin and adiponectin. When the body accumulates excessive AT it may lead to disruptions in endocrine signaling which can disrupt other organ systems of the body and may lead to disease (Galic et al. 2010).

Leptin is secreted by white adipose tissue (WAT) (Zhang et al. 1994). Leptin is produced in proportion to body fat mass and can act on the brain to induce satiety and regulate AT mass (Ainslie et al. 2000). It has been suggested that the primary role of leptin is in adaptation to negative energy balance (Kiem et al. 1998). As such, leptin was originally proposed to act as a signal indicating abundant adipose stores to the hypothalamus to limit energy intake and increase energy expenditure (Campfield et al. 1996). Accordingly, decreases in circulating leptin are associated with increased hunger (Kiem et al. 1998) and leptin replacement prevents the compensatory decrease in metabolic rate and thyroid function after diet-induced weight loss in humans (Faraj et al. 2003). Furthermore, there is some evidence linking leptin to a direct regulation of AT metabolism through inhibition of lipogenesis and stimulation of lipolysis (Meier et al. 2004).

Adiponectin is a protein hormone that modulates a number of metabolic processes, including glucose regulation and fatty acid oxidation (Diez et al. 2003). Adiponectin is exclusively secreted from AT and is one of the most abundant AT-specific factors (Fruhbeck et al. 2003). Data suggest that adiponectin is a mediator of insulin sensitivity and an enhancer of fatty acid oxidation (Berg et al. 2002). In contrast to leptin, plasma levels of adiponectin are lower in obese subjects, and the low levels are associated with increased risk factors for insulin resistance (Hotta et al. 2000). Low levels of adiponectin are also associated with the reduced ability of insulin to phosphorylate insulin receptor tyrosine residues and are therefore predictive of the development of insulin resistance in humans since tyrosine phosphorylation is essential to activation of the insulin signaling pathway (Faraj et al. 2003). Administration of adiponectin to rodents increases insulin sensitivity, an action that appears to result from lowered hepatic glucose production and increased muscle fatty acid oxidation. Moreover, adiponectin knockout mice exhibit insulin resistance (Kubota et al. 2002) and circulating adiponectin concentrations have been reported to increase after weight loss (Yang et al. 2001).

2.4.2 Differentiating overweight and obesity

Obesity or being overweight in humans and most animals does not depend on body weight alone, but rather on body composition, specifically the amount of AT that accumulates. A poor diet combined with physical inactivity is known to cause overweight and obesity. Obesity is an excessive accumulation of fat within the body, this accumulation results in an imbalance between energy intake and energy expenditure

(Cummings and Shwartz, 2003). In this case, the nutrient supply, particularly in the form of high fat foods, exceeds energy expenditure of the individual thus increasing body fat stores (Trembly et al. 1989). To determine whether accumulating adiposity results in an individual becoming overweight or obese is usually determined through measurements such as the body mass index (BMI), waist circumference, or waist-to-hip ratio (American Diabetes Association). The risk of secondary diseases increases with the severity of overweight. BMI is calculated from the size and weight and is only considered clinically relevant for persons 18 years and older. Categories of BMI include: 1) normal weight is a BMI between 19 to 24.9 kg / m2 for women and between 20 to 24.9 for men; 2) overweight is a BMI between 25 and 29.9 kg / m2; 3) obesity is considered a BMI above 30 kg / m2 (CDC, 2004).

2.4.3 Causes of increased adiposity

Causes of increased adiposity can range from physical, psychological, and environmental factors such as: high-fat diet and sedentary lifestyle, depression, emotional eating, familial predisposition, lack of access to healthy food or safe places to walk/play, cultural ideologies regarding food, secondary to disease states (hypothyroidism, abnormal cortisol metabolism), as well as undetermined factors (CDC, 2004) .

2.4.4 Pathophysiological consequences of increased adiposity

Increased adiposity may lead to both adverse physical and/or metabolic consequences such as respiratory distress; dyspnea or respiratory arrest attacks during the night (sleep

apnea); overbearing joint and articular problems such as back or knee pain; cardiac disorders ranging up to infarction and heart failure after fat accumulation in the heart; arteriosclerosis; insulin resistance, pre-diabetes, type 2 diabetes; increased levels of blood lipids (cholesterol and triglycerides) (Poirie et al. 2006r); non-alcoholic hepatic steatosis; calcifications of the gallbladder and coronary artery (Aoqui et al. 2013); leg vein thrombosis (partial or complete obstruction of leg vein); damage to the joints (osteoarthritis); and complications during pregnancy .

2.5 Progression from Increased Adiposity to T2D

2.4.1 Pathogenesis of increased adiposity

As stated earlier, obesity is characterized by an excess of body fat and is associated with a state of chronic, low-grade inflammation. The link between obesity and inflammation was first established by Hotamisligil et al, (1993) who showed a positive correlation between adipose mass and expression of the pro-inflammatory gene tumor necrosis factor-alpha (TNF-alpha) (Hotamisligil et al. 1993). The link between obesity and inflammation has been further illustrated by increased plasma concentrations of several pro-inflammatory markers including cytokines and acute phase proteins like C-reactive protein (CRP) in obese individuals (Trayhurn et al. 2005). Adipocytes are associated with the production of pro and anti-inflammatory adipokines.

As a secretory organ, AT displays several unusual characteristics. First, the different fat depots (visceral vs. subcutaneous) are heterogeneous not only in terms of metabolic capacities, but also of adipokine secretion patterns (Guerre-Millo, 2004). Differences in where adipose is distributed may have a direct impact on secretion thus variations in local repercussions on the AT by autocrine or paracrine mechanisms. Second, AT is composed of distinct cell types: mature adipocytes and stromal-vascular cells (SVC, non-fat cells; including macrophages), all of which may contribute to the secretory functions and effects of AT (Guerre-Millo, 2004).

Mature adipocytes represent 50–85% of the total cellular components of AT. The cellular composition of AT can vary according to anatomical location and body weight. In human obesity, AT is characterized by adipocyte hypertrophy (increased size) and hyperplasia (increased number), macrophage infiltration, endothelial cell activation and fibrosis (Faust et al. 1978, Henegar et al. 2008 and Maury et al. 2007). Increased fat storage in fully differentiated adipocytes, resulting in hypertrophy is thought to be the most important determinant whereby fat depots increase in adults (Spalding et al. 2008). With increasing AT accumulation, such as in the case of obesity, adipocytes undergo metabolic changes. Additionally, adipocyte size is related to dysregulated adipokine expression and secretion in humans, wherein the hypertrophic adipocytes have altered intracellular signaling that shifts the balance towards the production of pro-inflammatory molecules (Jernas et al. 2006 and Skurk et al. 2007). These pro-inflammatory mediators include TNF-alpha, interleukin (IL)-6, IL-1beta, adiponectin, leptin, and resistin. WAT is also known to produce chemokines such as IL-8 and monocyte chemoattractant protein

(MCP)-1. Obese AT is characterized by macrophage infiltration and these macrophages are an important source of inflammation in this tissue as well as an elevated production of adipose-derived pro-inflammatory mediators (Suganami et al. 2005).

TNF-alpha appears to be one of the most central pro-inflammatory mediators released by AT that contributes to and promotes obesity-associated inflammation. Adipose tissue and circulating levels of TNF-alpha are elevated in obese subjects and fall after weight loss (Dandona et al. 1998; Maury et al. 2009). TNF-alpha is also higher in visceral than subcutaneous fat, and more abundantly produced by macrophages that have infiltrated AT than adipocytes themselves (Fain et al. 2004; Maury et al. 2009). Most effects of TNF-alpha on AT are mediated by the TNF-alpha receptor 1 subtype (TNFR1) and subsequent activation of various transduction pathways (Cawthorn and Sethi, 2008). Two transcription factor-signaling pathways have been linked to the pro-inflammatory effects of obesity: the nuclear factor-kappa B (NF-kappa B) and c-Jun NH2-terminal kinase (JNK) pathways (Shoelson et al. 2007). Cell culture studies have supported this regulatory effect of TNF-alpha in obesity as demonstrated by an increase in constitutive NF-kappa B activity (Berg et al. 2004).

Adipocytes in obese human subjects have additionally been shown to be hyper-responsive to TNF-alpha which in turn functions to propagate inflammation by up-regulating pro-inflammatory mediators such as IL-6 and MCP-1, both of which have been shown to be elevated in obesity (Bastard et al. 2006). In fact, adipose tissue may contribute up to 15–35% of the systemic IL-6 in humans (Mohamed-Ali et al. 1997).

Additionally, adipose-derived TNF-alpha acts to down-regulate anti-inflammatory adipokines such as adiponectin (Bruun et al. 2003, Maury et al. 2009).

Two other biological pathways have been suggested in the relationship between obesity and activation of the immune system. The first pathway which could link obesity to altered production of adipokines is AT hypoxia (Trayhurn et al. 2008 and Ye, 2009). The causal link between hypoxia and changes in adipokine production has been confirmed in vitro and has been demonstrated in obese mice by several techniques (Hosogai et al. 2007 and Ye et al. 2007). In several models of cultured adipocytes, hypoxia decreased mRNA levels of adiponectin, while increasing those of pro-inflammatory genes (plasminogen activator 1 (PAI-1), TNF-alpha, IL-1, IL-6, and MCP-1,) together with those of hypoxia response genes (hypoxia inducible factor-1 alpha (HIF-1 alpha), glucose transporter 1, vascular endothelial growth factor (VEGF)) (Hosogai et al. 2007, Wang et al. 2007 and Ye et al. 2007). The expression of inflammatory genes was also induced by hypoxia in cultured macrophages (Ye et al. 2007). The molecular mechanisms of dysregulated gene expression are related to activation of the transcription factors NF-kappa B and HIF-1 alpha, to endothelial stress and to post-transcriptional alterations (Hosogai et al. 2007 and Ye, 2009). Thus, hypoxia may underlie the development of the inflammatory response in AT, leading to obesity-associated diseases.

Second, it has been suggested that a paracrine loop between adipocytes and macrophages establishes a vicious circle that aggravates the inflammatory changes in AT (Suganami & Ogawa, 2010). This paracrine loop involves free fatty acids (FFAs) and TNF-alpha. Enlarged adipocytes release excess saturated FAs that activate macrophages

via toll-like receptor 4 (TLR4) signaling (Suganami & Ogawa, 2010). TLRs play a critical role in the innate immune system by activating pro-inflammatory signaling pathways in response to microbial pathogens. The activation of TLR4 upregulates intracellular inflammatory pathways related to the induction of insulin resistance, such as JNK or NF-kappa B. TLR4 has been proposed to be a molecular link between lipids, inflammation and insulin resistance (Shi et al. 2006). Thus, TLR4-knockout mice are protected against lipid-induced inflammatory changes, insulin resistance and obesity (Shi et al. 2006 and Tsukumo et al. 2007). Additionally, TLR4 expression was increased in AT of obese mice (Shi et al. 2006). TLRs are also expressed in human AT (Vitseva et al. 2008). As a result, macrophages secrete TNF-alpha, which in turn acts on TNFR1 to induce inflammatory changes in hypertrophic adipocytes through activation of the NF-kappa B pathway and enhanced FFA release (Suganami & Ogawa, 2010). FFAs may also act on adipocytes in an autocrine fashion to generate inflammation and chemokine/adipokine overproduction at least in part via TLR4 (Jiao et al., 2008).

Obesity is often associated with the pathogenesis of insulin resistance, MetS, and T2D. This link between obesity-induced dysfunction in adipocytes and its related co-morbities appears to be increased in adipose tissue-mediated adipokines. In states of increasing adiposity the majority of adipokines with pro-inflammatory properties are overproduced while those adipokines with anti-inflammatory or insulin-sensitizing properties, like adiponectin are diminished. This dysregulation of adipokine production may promote obesity-linked metabolic disorders and cardiovascular disease.

2.6 Impaired Insulin-Mediated Vasodilation

2.6.1 Insulin-mediated vasodilation and vascular health

Insulin has important vascular actions to stimulate production of nitric oxide from the endothelium. This leads to capillary recruitment, vasodilation, increased blood flow, and subsequent augmentation of glucose disposal in classical insulin target tissues (e.g., skeletal muscle) (Muniyappa et al. 2007). The PI3K insulin-signaling pathway also regulates endothelial production of nitric oxide (NO). Insulin binding to its membrane-bound receptor (IR) subsequently stimulates IRS-1/IRS-2 which activates PI3K leading to phosphorylation of protein kinase B (PKB/Akt) and phosphatidylinositol-dependent kinase 1(PDK-1) therefore stimulating endothelium nitric oxide synthase (eNOS) (Assunta Potenza et al. 2009). eNOS is an SH-dependent enzyme with a cysteine residue at its active site that is important in regulating vascular tone by catalyzing the production of NO from L-arginine (Figure 1; Chen et al. 1994, Vasdev et al. 2006). These and other cardiovascular actions of insulin contribute to the coupling of metabolic and hemodynamic homeostasis under healthy conditions. Cardiovascular diseases are the leading cause of morbidity and mortality in insulin-resistant individuals (ADA). Therefore, examination of markers of inflammation and oxidative stress that directly impact the vascular insulin signaling pathway may be relevant in evaluating the efficacy of specific therapeutic interventions aimed at ameliorating endothelial dysfunction.

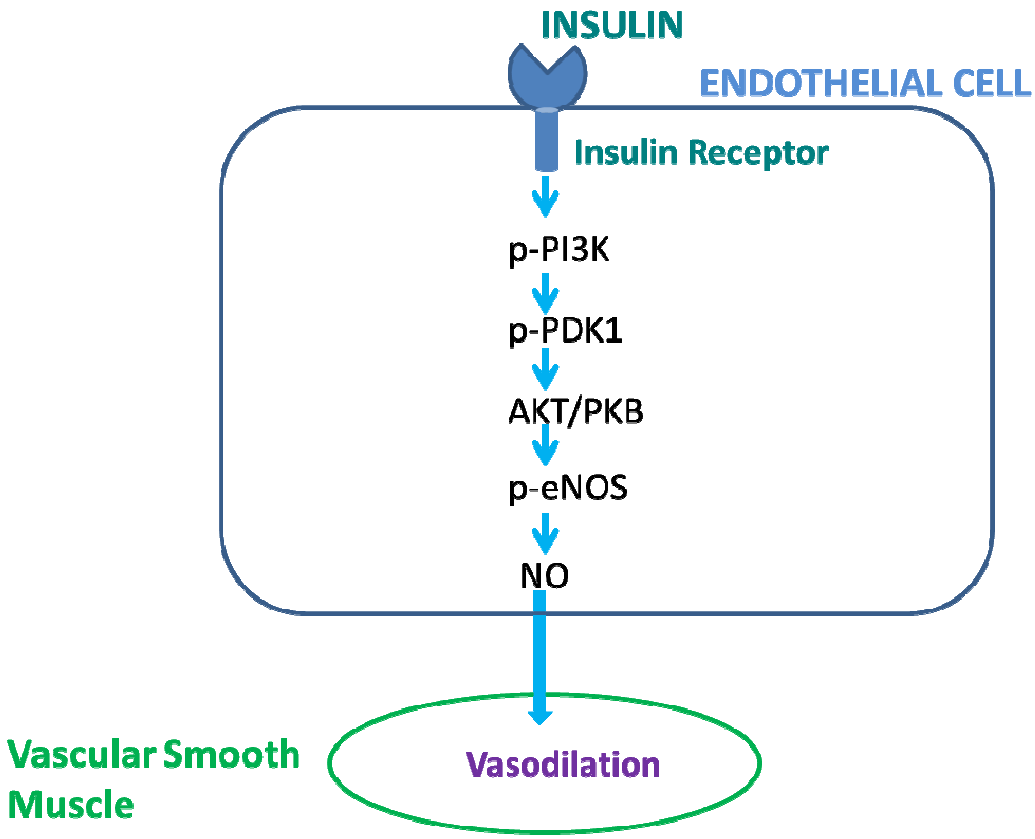

Figure 1. Insulin-Mediated Vasodilation Pathway.

2.6.2 Pathogenesis of Impaired Insulin-Mediated Vasodilation

Increased adiposity is associated with the development of diabetes, hypertension, and cardiovascular disease. Oxidative stress is a complication of diets high in fat and increased adiposity and can directly induce hypertension through $O2^{\cdot-}$-mediated scavenging of the endogenous vasodilator NO (Dobrian et al. 2001, Taniyama et al. 2003).

Oxidative stress can occur as a result of decreased activity or expression of antioxidant enzymes with an increase in enzymatic generation of $O2^{\cdot-}$. Superoxide generation serves as a precursor for a number of reactive oxygen and nitrogen species.

Therefore oxidative stress results from an imbalance of reactive oxygen species (ROS) generation. Some major sources of ROS include uncoupling of the electron transport chain resulting in increases in NOS as well as by NADPH oxidases. Hyperglycemia and dyslipidemia also contribute to increased ROS production in addition to initiating insulin resistance through down-regulation of insulin signaling pathway intermediates (Newsholme et al. 2007). In addition to increases in oxidative stress, HFDs inhibit the vascular insulin signaling pathway. Since normal tyrosine-autophosphorylation of the insulin receptor (IR) is down-regulated in favor of serine/threonine phosphorylation, stimulation of intracellular signaling intermediates is impaired leading to downregulation of IRS-1, PI3K, PKB/Akt, and PDK-1 phosphorylation leading to further decreases in eNOS and NO production (Figure 2; Montagnani et al. 2002). Alterations to the cysteine residue of eNOS also results in loss of catalytic activity. Moreover, oxidation of the eNOS cofactor tetrahydrobiopterin causes the uncoupling of eNOS, resulting in decreased formation of NO and an increase in $O_2^{\cdot -}$ production (Thum et al. 2007, Xia et al, 1998). NO is not only a potent vasodilator, it also inhibits platelet aggregation, vascular smooth muscle cell (VSMC) migration and proliferation, monocyte adhesion, and adhesion molecule expression (Taddei et al. 2004). Decreasing NO bioavailability while at the same time producing excess ROS, therefore, leads to the endothelial dysfunction (Tian et al. 2012). Endothelial dysfunction, or impaired endothelium-dependent vasodilation, is observed with insulin resistance, diabetes, obesity, and dyslipidemia is therefore a prominent component of hypertension, coronary heart disease, and atherosclerosis.

2.6.3 Oxidative Stress

Proteins, lipids and DNA are cellular targets for oxidation, which lead to alterations in their structure and function. ROS can cause these alterations through oxidation of critical amino acid residues, such as cysteine residues as previously described for eNOS, which may alter enzyme activity or may affect transcriptional activities if they are within the binding site of transcription factors. Intra- or intermolecular conformational changes, such as the formation of disulfide bridges between or within proteins, may result in an alteration of protein activity or function. Metal-catalyzed oxidative reactions may cause modifications to membrane proteins and lipids, leading to degradation whereas peroxidation reactions may lead to degradation of membrane lipids with loss of membrane integrity, and release of aldehydes, possibly altering ion channel or receptor function. Finally, overstimulation of ROS-mediated signaling pathways may result in over-expression of inflammatory factors, increased vascular cell proliferation and apoptosis (Giordano et al. 2005, Vasdev et al. 2006).

Numerous enzymes are involved in cellular regulatory functions, and oxidative modification of these enzymes may result in adverse effects contributing to the development and progression of hypertension and atherosclerosis. Antioxidants, which normally attenuate ROS-mediated damage, are often down-regulated in disease states. An important antioxidant pathway is the breakdown of $O_2^{\cdot -}$ by superoxide dismutase (SOD) to form hydrogen peroxide (H2O2), which is further converted by catalase or glutathione peroxidase to water and oxygen. Increases in the vascular levels of $O_2^{\cdot -}$ lead to

endothelial dysfunction through decreasing the bioavailability of NO. Animal and cell culture studies have demonstrated glutathione peroxidaxe (GPx) and glutathione reductase (GRed) are two additional key antioxidant enzymes that protect cells from oxidative damage. The enzymes themselves have been shown to be susceptible to modifications resulting from oxidation with loss of activity, resulting in perpetuation of oxidative stress (Cullen, 2010, Jones, 2006, Vasdev 2006). In both *in vitro* and *in vivo* studies of spontaneously hypertensive rats (SHRs) (models of human essential hypertension) there are increased ROS levels in vascular tissue, with a decrease in glutathionine (GSH) levels and antioxidant enzyme activity (Elmarakby and Imig, 2010, Dobrian et al. 2001).

ROS act on yet another enzyme, angiotensin-converting enzyme (ACE), to increase catalytic activity, resulting in an increase in angiotensin II (AII) production (Takemori et al. 2007). AII is a potent vasoconstrictor implicated in the pathogenesis of hypertension and atherosclerosis. AII binds to its type 1 receptor, which results in an increase in contraction, hypertrophy, proliferation and apoptosis. AII stimulation of its type 1 receptor also increases the production of $O_2^{\cdot -}$ by the enzyme NADPH oxidase (Roberts et al. 2000). This enzyme is implicated as a major source of oxidative stress in cardiovascular disease. AII-induced oxidative stress (via NADPH oxidase) may be responsible, at least in part, for the decrease in NO bioavailability and the subsequent endothelial dysfunction found in hypertension and atherosclerosis.

2.6.4 T2D, Obesity, and Vascular Dysfunction

A final link between adipokines, vascular dysfunction and cardiovascular disease involves the influence of perivascular adipokines, adipokines released from the immediate adipose tissue adjacent to the vasculature, on the vascular effects of insulin. Insulin normally induces vasodilation in muscle tissue to promote high glucose uptake and vasoconstriction in muscle tissue with low glucose uptake (Clark et al. 2003). This involves a delicate balance of the vasodilatory effects of NO and the vasoconstrictor effects of endothelin-1 (Blendea et al. 2003). This insulin-mediated blood flow is a major determinant of whole-body insulin resistance. In obesity, NO production is reduced whereas endothelin-1 production is increased along with reduced capillary recruitment (Sacks et al. 2007). Adipokines secreted by perivascular AT are believed to contribute to these changes that would work together to impair blood flow. For example, TNF-alpha has been shown to impair insulin-induced vasodilation (Enringa et al. 2007) possibly through reduced expression of IRS1. Adiponectin also influences insulin signaling, but in contrast to TNF-alpha and IL-6, is associated with increased sensitivity to insulin and increased glucose uptake (Hopkins et al. 2007).

2.7 Diabetes and increased risk of cardiovascular disease

Cardiovascular disease (CVD) in the US is the leading cause of mortality and accounts for 36% of all deaths. Furthermore, the economic burden of CVD was estimated as $475 billion dollars in 2009 in both direct and indirect costs (AHA, 2010). Risk factors for CVD include both non-controllable factors such as age, gender, ethnicity, and genetics, as well as controllable factors such as diet, physical activity levels, weight, and smoking.

Other reversible risk factors include high blood pressure and unhealthy lipid profiles (low HDL, high LDL, high total cholesterol, and dyslipidemia). Additionally, other emerging independent factors such as hyperglycemia, postprandial lipidemia, hyperinsulinemia, oxidative stress, endothelial dysfunction, increased concentrations of inflammatory markers, and accumulating adipose tissue including perivascular adipose tissue have been suggested as contributing factors to the onset and progression of CVD (Cullen, 2000; Hackam and Anand, 2003; Pearson et al. 2003; Willerson and Ridker, 2004). With the US population currently experiencing a high rate of events including a large aging population, as well as an increased prevalence of obesity, metabolic syndrome, and T2D there is potential for a dramatic increase in the incidence of CVD. In fact, the AHA (2010) has estimated that in 2030 40.5% of the population within the US will develop CVD as compared to 37% of the population in 2010 resulting in an increase in the economic burden of CVD to $818 billion in direct costs and indirect costs of $276 billion. The risk of deaths of patients with type 2 diabetes from CVD-related events is 2 to 4 times higher as compared to persons without diabetes (Buse, 2007). Among white, non-hispanic Americans, the age-adjusted prevalence of coronary heart disease (CHD) is twice as high among those with type 2 diabetes as among those without diabetes (Isomaa et al, 2001). These cardiovascular events associated with T2D as well as the high incidence of other macrovascular complications, such as strokes and amputations, further complicate the treatments and outcomes of illness and add to the already enormous economic burden of T2D. Multiple modifiable risk factors for late complications in patients with T2D, including hyperglycemia, hypertension, and dyslipidemia, increase the

risk of a poor outcome (Nandish et al, 2011). Recent guidelines from the ADA and other national organizations such as the AHA recommend a multifactorial approach to the management and treatment of diabetes and diabetes-related CVD risk factors.

The NCEP ATP III and the AHA consider diet modification as a primary approach to prevent and treat hyperlipemia and hypertension. In 2010, the USDA released the 2010 "American Dietary Guidelines" (DGA) which emphasized healthy eating behaviors and physical activity aimed at reducing the prevalence of overweight and obesity and diet-related chronic disorders. AHA set forth nutritional guidelines aimed at preventing CVD and stroke or reducing associated risk factors. This guideline targeted 5 major dietary intake factors including increasing daily fruit and vegetable intake, increasing consumption of fatty fish, reducing sodium as well as sugar-sweetened beverages, and increasing daily fiber intake. In a two-year follow-up, it was demonstrated that participants who followed the AHA nutritional guidelines did see significant improvements in measured lipid and cardiometabolic parameters (Redaelli et al. 2012) However, according to NHANES data, the prevalence of meeting all seven AHA health metrics at ideal levels was 1.2% in 2005-2010 (Shay et al, 2013). Moreover, only 20% of adults followed at least two dietary recommendations. Interestingly, in a recent study it was demonstrated that clinical trials that focused on only 1 of the 5 healthy eating factor goals, specifically fiber, resulted in a significantly greater adherence rate. Furthermore, those nutrition interventions that focused on daily fiber consumption had improvements in CVD risks (Post et al. 2012).

While it is agreed upon that meeting a greater number of healthy eating parameters in accordance with the AHA guidelines results in decreases in CVD mortality as evidenced by a 49% risk reduction in all-cause mortality in individuals who met all healthy eating factors (Ford et al.), only a minor percent of the population fits into this quintile as described previously. Therefore, finding simple dietary strategies, which have the potential to attenuate cardiometabolic risk factors, which are easy to adhere to and incorporate into a normal diet may prove to be tremendously beneficially to populations such as patients with T2D who are at increased risk.

2.8 Nutrition interventions and dietary fibers

2.8.1 Potential Use of Dietary Fibers in the Treatment of T2D

Dietary fiber is defined as the portion of plants that is composed of complex carbohydrate polymers of simple sugars. Fibers can be classified as either soluble fiber or insoluble fiber. Soluble fibers, commonly referred to as viscous fiber, consist of gel-forming substances, such as pectin, gum, and mucilage that tend to be efficiently degraded by bacteria in the colon. Insoluble fibers, known as bulk fiber, consist of structural or matrix fiber such as, lignin, cellulose, and some hemicellulose that passes through the body unchanged (Gray, 1995; Hunt et al. 1993). The fibers used in this study, flaxseed (*Linum usitatissimum*) and psyllium (*Plantago ovato*) are both water-soluble fibers. Soluble fibers can function in nutrient metabolism in the body by delaying gastric emptying as

well as adsorbing or binding bile acids and products of fat digestion such as bile acids during passage through the intestines (Anderson et al. 1988; Hunt et al. 1993; Levin et al. 1990). Epidemiological data suggests that dietary fibers may function to attenuate hyperglycemia (Anderson et al. 2009). Clinical trials have supported this association between dietary fiber intake and regulation of glucose homeostasis by demonstrating dietary fiber decreases post-prandial glucose and insulin responses in T2D patients (Anderson et al. 1999; Chandalia et al. 2000; Pastors et al. 1991; Rodríguez-Morán et al. 1998). Additionally, clinical research has shown that fibers improved long-term glucose control in T2D (Giacco et al. 2000; Wolk et al. 1999). The benefits of a high-fiber diet on reducing glucose levels are still controversial (Nuttall et al. 1993) and have not been fully studied or appropriately tested in T2D as there are few controlled studies. Furthermore, the considerable amount of fiber used in these studies is controversial due to lack of adherence in a normal diet and adverse effects that are associated with sudden increases in fiber or long term very high fiber consumption such as gastrointestinal distress, disruption of the gut flora, and impaired mineral absorption (Beattie et al. 1988; Scott et al. 1988; Tattersal et al. 1990). Alternatively, the ADA recommends a fiber consumption of 25 g/d for women and 38 g/d for men, however, it is estimated that the average U.S. adult consumes only 15 g/d which is well below the recommended intake. There is general consensus that moderate amounts of fiber from a variety of plant-based food sources are a vital component of a healthy diet, thus most controversy regarding the effects of fiber in the regulation of glucose is not due to the effectiveness of the dietary approach but whether or not it is feasible.

2.8.2 Reported Benefits of Psyllium and glucose control

Psyllium that is used for the majority of the world's consumption is comprised of the mucilage portion of the psyllium plant obtained from the outer layer of the seed known as the seed coat. Mucilage yield amounts to about 25% (by weight) of the total seed yield. The mucilage is often referred to as husk, or ground psyllium husk. The ground husk mucilage is a white fibrous material that is hydrophilic, meaning that its molecular structure causes it to attract and bind to water. Upon absorbing water the mucilage can increase its volume up to ten-fold. Previous studies have demonstrated a beneficial effect of dietary psyllium-derived fiber for reducing cholesterol levels in patients with hypercholesterolemia (Anderson et al. 2000; Olson et al, 1997; Sprecher et al. 1993) whereas the effect on glucose serum levels of a diet supplemented with soluble fibers varies according the dose consumed (Chandalia et al. 2000; Weickert et al. 2008). In studies examining the dosage amount of psyllium required to elicit a change in glucose-related outcomes has demonstrated that there is little change in measured variables using lower quantities of Psyllium (7 g/d) (Rodríguez-Morán et al. 1998). However, when the dose is increased up to 20 g/d there was a significant reduction in both basal and postprandial hyperglycemia (Abraham et al. 1988; Anderson et al. 2000; Jenkins, 2002; Pereira et al. 2001).

2.8.3 Nutrient Composition of Flaxseeds

Flaxseed is a food that is commonly consumed as whole seed, ground seed (powder or meal), or in its oil form. The nutritional composition varies between the three forms of flaxseed (refer to Table 1) as flaxseed oil is devoid of any fiber and lignan components but is unique as it contains high percentage by weight of both monounsaturated fatty acids (18%) as well as polyunsaturated fatty acids (73%) and is the richest food source of alpha lineoleic acid (ALA, 55%). Studies conducted on the bioavailability of ALA in flaxseed after consumption indicates that both flax oil and ground flaxseed provide significant levels of ALA after digestion, however, whole flaxseed did not (Katare et al. 2012). The whole seed and ground meal forms contain both insoluble and soluble fibers. The soluble fiber content of flaxseeds is derived from the mucilage portion of the seed and contributes to approximately 25% of the total dietary fiber of flaxseeds. Previous research in both animal models as well as clinical trials has demonstrated that this soluble fiber component may explain the cardioprotective effects attributed to flaxseeds (Carter et al. 1993; Kritchevsky et al. 1995; Wolever et al. 1995). In addition to high concentrations of the omega-3 fatty acid ALA and soluble fibers, flaxseeds are also a good source of multiple micronutrients associated with vascular health including vitamin E as gamma-tocepherol and the B vitamins (thiamin (B_1), riboflavin (B_2), niacin (B_3), and pyridoxine (B_6)). Flaxseeds also contain high concentrations of folate, potassium, phosphorous, magnesium, and zinc, however, due to the presence of phytic acid and oxalate which act as chelators and bind magnesium and zinc to form insoluble complexes in the intestine (Robson, 2009), the bioavailable amounts of these minerals is relatively

low. It is worth noting that some studies of animal models indicate that phytic acid independently lowers blood glucose levels (Lee et al. 2006).

Table 1. Comparison of the Nutrient Composition of Flaxseed Forms

Flaxseed Form	Weight (g)	Energy (Kcals)	Total fat (g)	Total ALA (g)	Total fiber (g)	SDG content (mg)[a]
Whole	41.2	220	17.37	11.84	11.2	33.4
Ground	28	150	11.8	8.04	7.6	24.32
Oil	54.4	481	50.4	39.1	0.0	0.0

Source: NDSR, 2012

[a] secoisolariciresinol diglucoside

2.8.4 Flaxseed-derived lignans and potential antioxidant properties

Flaxseeds also contain a large concentration of phytoestrogen lignans. Plant lignans are phenolic compounds which are a class of chemical compounds consisting of a hydroxyl group ($^{-}$OH) bonded directly to an aromatic hydrocarbon group. Plant lignans are found in a variety of commonly consumed foods and are considered naturally occurring phenolics. After ingestion they are metabolized via the xenobiotic pathway which is also the pathway responsible for drug metabolism. The carbohydrate portion is removed by intestinal bacteria to form the bioactive mammalian lignans: enterodiol and enterolactone (Bloedon et al. 2004). These mammalian lignans are then absorbed in the small intestine and conjugated in the liver. Conjugated lignans are then excreted from the body through urine and bile in addition to undergoing enterohepatic circulation which promotes reabsorption. Flaxseeds are the richest source of the lignan precursor secoisolariciresinol diglucoside (SDG) in addition to also containing minor lignan components isolariciresinol, pinoresinol, and matairesinol (Hutchchins et al. 2001; Meagher et al. 1999). Flaxseed consumption has demonstrated a linear, dose-dependent

relationship with urinary excretion of enterodiol, enterolactone, and total lignans in clinical trials (Morton et al. 1995). Prasad (1999) demonstrated that SDG, isolated from flaxseeds, lowered serum total cholesterol and LDL cholesterol and reduces hypercholesterolemic atherosclerosis in rabbits.

Recent clinical trials have demonstrated flaxseed-derived lignans have serum cholesterol and glucose lowering effects in hypercholesterolemic subjects (Bloedon et al. 2008; Pan et al. 2007; Zhang et al. 2008). Additionally, lignans, such as SDG, and its mammalian metabolites secoisolariciresinol, enterodiol, and enterolactone have demonstrated antioxidant activities (Prasad, 2000; Prasad, 1997). Studies exploring the effects of flaxseed-derived lignans have established antioxidant activity *in vivo* and *in vitro* resulting in increased total antioxidant capacity as well as decreased lipid peroxidation (Hu et al. 2007; Kitts et al. 1999; Niemeyer and Metzler, 2003). However, whole flaxseed had no significant effect on markers of lipid peroxidation in humans (Cunnane et al. 1995; Cunnane et al. 1993), but partially defatted flaxseed lowered serum protein thiol groups, indicating increased oxidation (Jenkins et al. 1999). The antioxidant activity of secoisolariciresinol and enterodiol found in flaxseeds is higher than that of vitamin E or the parent glucoside present in flaxseed (Prasad, 2000). As described earlier, oxidative stress has been shown to be a major contributor to the onset and progression of insulin resistance and T2D. Interestingly, SDG also reduces the incidence of diabetes in streptozotocin-induced diabetic rats (Prasad et al. 2000) and Zucker rats, a model for type 2 diabetes (Prasad, 2001).

2.8.5 Flaxseeds-derived omega 3, T2D and CVD risks

As previously stated (see Table 1), flaxseeds are a rich source of ALA. ALA is an essential omega-3 fatty acid which cannot be synthesized in the human body though can be converted (in variable amount) into the longer chain omega 3 fatty acids eicosapentaenoic acis (EPA) and docosahexanoic acid (DHA) (Burdge, 2004). The majority of research regarding potential benefits of flaxseed consumption is focused on markers of cardiovascular disease. While results are still inconclusive, recent data suggests that consumption of flaxseeds in all forms is associated with attenuated CVD risks. Potential mechanism by which flaxseeds may exert this protective effect include reduction of inflammation and oxidative stress as well as reducing total serum cholesterol, improving serum lipids, decreasing platelet aggregation, and attenuating vasoconstriction. These effects are primarily attributed to the large concentration of ALA as well as phytoestrogen lignans and the soluble fiber content of flaxseeds as previously discussed. Research regarding flaxseed consumption in animal models has primarily focused on the effects of flaxseed lignans in improvement of serum lipids (as discussed in*2.7.2*). However, when weanling female Wistar rats were fed diets supplemented with 200 g/kg flaxseed for 4 weeks as compared to rats supplemented with ALA or diets high in saturated fatty acids, the flaxseed group demonstrated significantly decreased oxidative stress levels in response to the administration of a oxidative stress inducer, CCL_4 (MacDonald-Wicks and Garg, 2002).

Epidemiological data on flaxseed consumption and risk of clinical studies observing the use of flaxseed oil have demonstrated that ground flaxseed intake can result

in modestly reduced total cholesterol and LDL-C concentrations without significantly decreasing HDL-C (Albert et al., 2005; Hu et al. 2004). Eleven major studies (Mantzioris et al, 1994; Allman et al. 1995; Caughey et al. 1996; Gibson et al. 2000; James et al. 2000; Nordstrom et al. 1995; Nestel et al. 1997; Cunnane et al. 1993; Loria et al. 1993; Guan et al, 1998; Paschos et al. 2007; Ueshima et al. 2007) have reported that flaxseed-derived ALA levels are inversely correlated with primary cardiovascular events. The results are from large sample populations or collected over multiple years. More evidence that dietary ALA has significant cardioprotective benefits has been demonstrated in secondary prevention trials. Studies such as the Nurses' Health Study (Hu et al. 1994) found that the intake of ALA in the diet protected against fatal ischemic heart disease and that this protection probably resulted from an antiarrhythmic effect of ALA. However, the protective effect of ALA did not extend to nonfatal myocardial infarction, for which there was a nonsignificant trend for an effect. Although there may be a direct effect on cardiac arrhythmias from dietary ALA, it is likely that its effect was mediated, in part, through the syntheses of EPA and DHA. In the Lyon Diet Heart Study (Axelson et al. 1982), ALA was associated with a decreased risk of recurrent fatal and nonfatal myocardial infarction, and a 73% reduction in risk of primary end points (cardiac mortality and morbidity) between the experimental and control groups. In a double-blinded, placebo-controlled study (Shakir et al. 2007) conducted in India, 120 patients with suspected acute myocardial infarction were followed and supplemented with 2.9 g/day of ALA (enriched oil). After one year of follow-up, both cardiac death and nonfatal myocardial infarction were significantly lower in this group of patients compared with

those on placebo. Animal models exploring the relationship of flaxseed and CVD have demonstrated that flaxseed (0.4 g/day) effectively inhibited the expression of inflammatory markers such as interleukin (IL)-6, vascular cell adhesion molecule (VCAM)-1 in aortic atherosclerotic tissue from LDL receptor-deficient mice (Cunnane et al. 1995). It was concluded that an important antiatherogenic role of ALA may involve a potent anti-inflammatory action. These results have further been demonstrated in clinical trials. Two clinical trials with healthy subject populations have observed significant reductions of TNF-α, IL-1-beta, thromboxane B5 and prostaglandin E5 after administration of an ALA-rich diet (13.7 g/day of ALA from flaxseed) (Austria et al. 2008). Additionally, in response to 2 g/d of flaxseed-derived ALA, expression of VCAM-1 and E-selectin were significantly decreased (Bloeden et al, 2008). ALA intake (8 g/day) from a flaxseed source decreases serum concentrations of serum amyloid A and IL-6 (Alonso et al. 1995). Additionally, Nestel et al (1997) reported that in obese human subjects, 20 g/day of ALA from flaxseed oil significantly increased arterial compliance and decreased LDL-C oxidation when it was compared with an oleic acid and saturated fat intervention.

Flaxseed consumption may improve glucose regulation. Studies in animal models of diabetes mellitus have shown that SDG from flaxseed can prevent the development of type 1 diabetes by approximately 71% (Prasad, 2000) and T2D by 80% (Prasad, 2001). Pan et al (2007) reported more modest but statistically significant improvements in glycemic control in type 2 diabetic patients treated for 12 weeks with 360 mg/day of flaxseed-derived lignan supplement. Ingestion of flaxseed or ALA may help in

preventing or treating a variety of diabetic complications. Bloedon et al (2008) demonstrated that 40 g/d of ground flaxseed significantly improved insulin sensitivity in individuals with elevated LDL-C concentrations. Furthermore, studies in type 2 diabetic patients, 5 g/day of flaxseed oil consumption has been associated with a significant reduction of plasmin alpha-2-plasmin inhibitor complex level, plasminogen activator inhibitor-1 activity and thrombin antithrombin III complex level after a two week of intervention (Toghi et al. 2004). In an animal model, Velasquez et al (2003) reported that flaxseed meals reduced proteinuria and ameliorated nephropathy in type 2 diabetes mellitus. Due to the strong association between obesity and T2D, caution should be exercised in recommendations for a potentially high fat diet. Studies examining the effects of flaxseed consumption on changes in body mass have not found evidence that flaxseed induces weight gain or increases in body fat (Faintuch et al. 2007; Nelson et al. 2007).

2.9 Consequences or Implications

The overall goal of this study is to contribute to the understanding of how flaxseed supplementation may help manage hyperglycemia in diabetes as well as improve vascular health. The results of this study will provide new insight into the feasibility of use regarding a plant-based dietary treatment for T2D that is predicted to have few, if any, side-effects and is affordable (as compared to oral hypoglycemic medications) which would also help to alleviate the economic burden of diabetes for many individuals from low socioeconomic backgrounds. Additionally, the findings of this study regarding the use of dietary interventions to reduce inflammation and oxidative stress which are similar

underlying factors of T2D, endothelial dysfunction, and cardiovascular disease will be applied to future work regarding the effects of diabetes on vascular health and the use of nutritional intervention therapy in an effort to further our understanding of the devastating effects of poor dietary habits and obesity on vascular health. The potential benefits of this study will be to establish and characterize how flaxseed supplementation may attenuate hyperglycemia and increased cardiovascular risks in non-insulin dependent diabetes patients and design future goals for implementing interventions with the aim of avoiding the adverse consequences of impaired insulin regulation, hyperglycemia and subsequent disease and help to establish optimal health.

CHAPTER 3

MATERIALS AND METHODS

3.1 Study Design

In this comparative single-blinded randomized double-arm parallel efficacy parallel trial, participants were randomly assigned to supplementation for eight weeks with 28 g/d of ground flaxseed (intervention) or the control, 9 g/d of ground psyllium husks, which was standardized to control for fiber content (Table 2). Furthermore, this was designed to be a pilot study to determine the following: 1) would participants adhere to the previously stated volumes of test foods supplemental to their normal diet for an eight week trial

period; 2) would test food volumes be adequate to elicit a detectable change in the measured variables; 3) would the trial length be long enough in order to elicit previously observed effects of either ground flaxseed supplementation or psyllium supplementation on measured outcomes including glucose regulation, lipid profiles, circulating inflammation concentrations, and markers of oxidative stress. Participants were asked to refrain from making any additional changes to their usual diet or activity levels throughout the duration of the entire study. The study was approved by Arizona State University's Institutional Review Board (Appendix A).

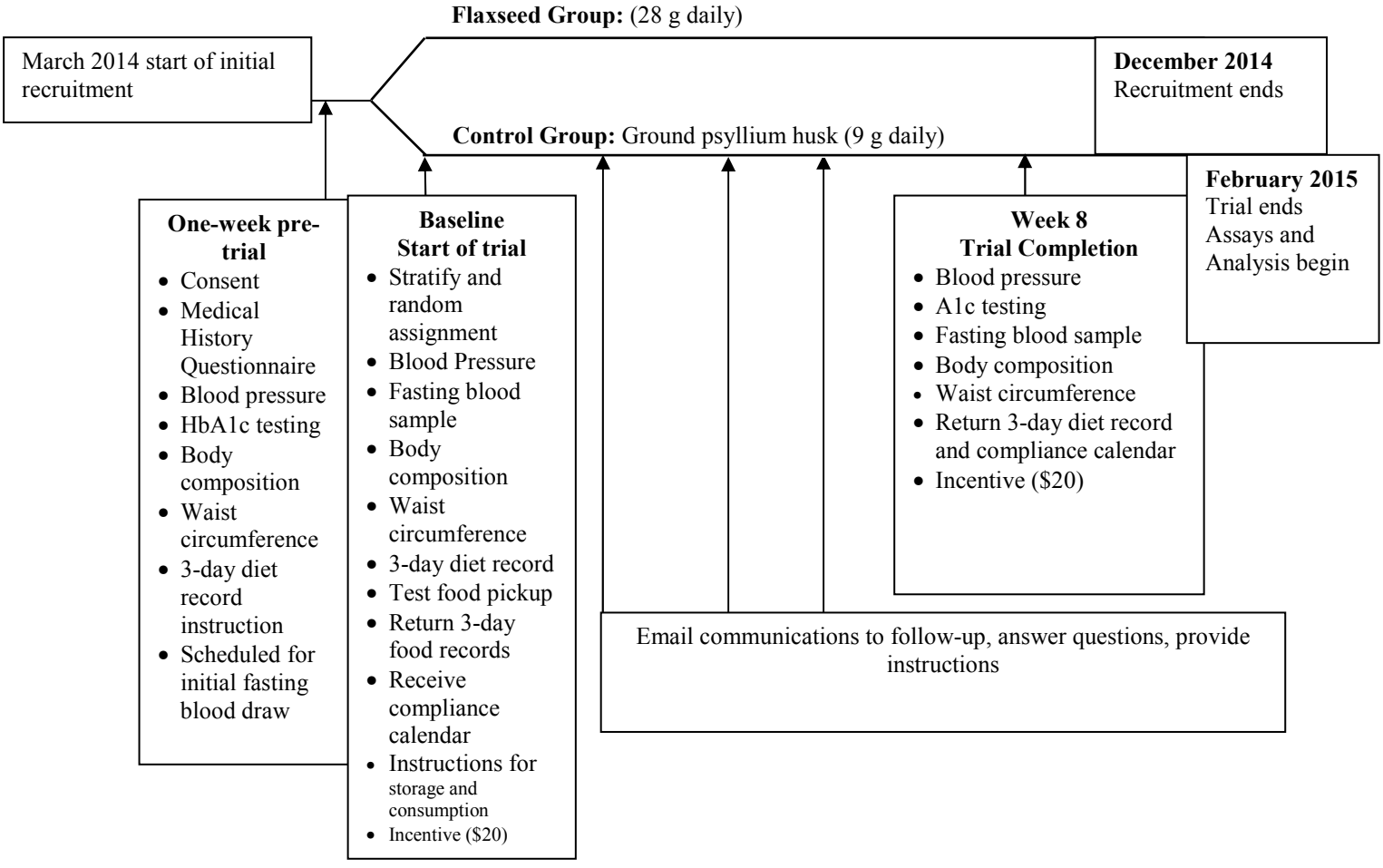

Figure 2. Study Timeline and Milestones for Eligible Participants.

3.2 Participants and Recruitment

3.2.1 Participants

Participants for this study included 17 adults (18 - 75y) who had a medical diagnosis of non-insulin dependent type 2 diabetes mellitus at least 6 months prior to enrollment. Subjects were recruited from the Phoenix Metropolitan area through flyers (Appendix B), diabetes support groups and events, health fairs, local hospitals, clinics and doctor's offices.

3.2.2. Initial Screening

Subjects were pre-screened by completing an online questionnaire (SurveyMonkey.com) (Appendix C) designed to assess the exclusion criteria.

3.2.3 Exclusion Criteria

Exclusion criteria included: history of flaxseed allergies, current fiber supplement use, insulin use, currently pregnant or planning to become pregnant, currently breastfeeding, active disease states (other than diabetes), and anticipated changes to diet or physical

activity levels. Prescription medication use by participants, including oral hypoglycemic agents, statins, and hypertensive medications were required to have been consistent prior to the trial and were to remain consistent throughout the study.

3.2.4 Rolling Enrollment

Participants were enrolled after successful completion of initial online survey. Participant inclusion into the 8 week trial was based upon a rolling recruitment. Enrollment began on March 2014 and was concluded on December 2014.

3.2.5 Consent Visit

A total of 66 potential participants responded to the online questionnaire (Fig. 3). Those subjects who passed the pre-screening stage were invited for an initial visit at the School of Nutrition and Health Promotion (SNHP) Research Facility in the Arizona Biomedical Collaborative Building (Phoenix, AZ). Informed consent was obtained during this visit (Appendix D and E) and each subject completed a medical history questionnaire (Appendix F). Following consent, glycated hemoglobin (HbA1c) was determined using a small blood sample collected by fingerprick (DCA Vantage autoanalyzer, Siemens, Washington, D.C.). Additionally, anthropomorphic measurements were recorded as described later (refer to *3.5.1)*.

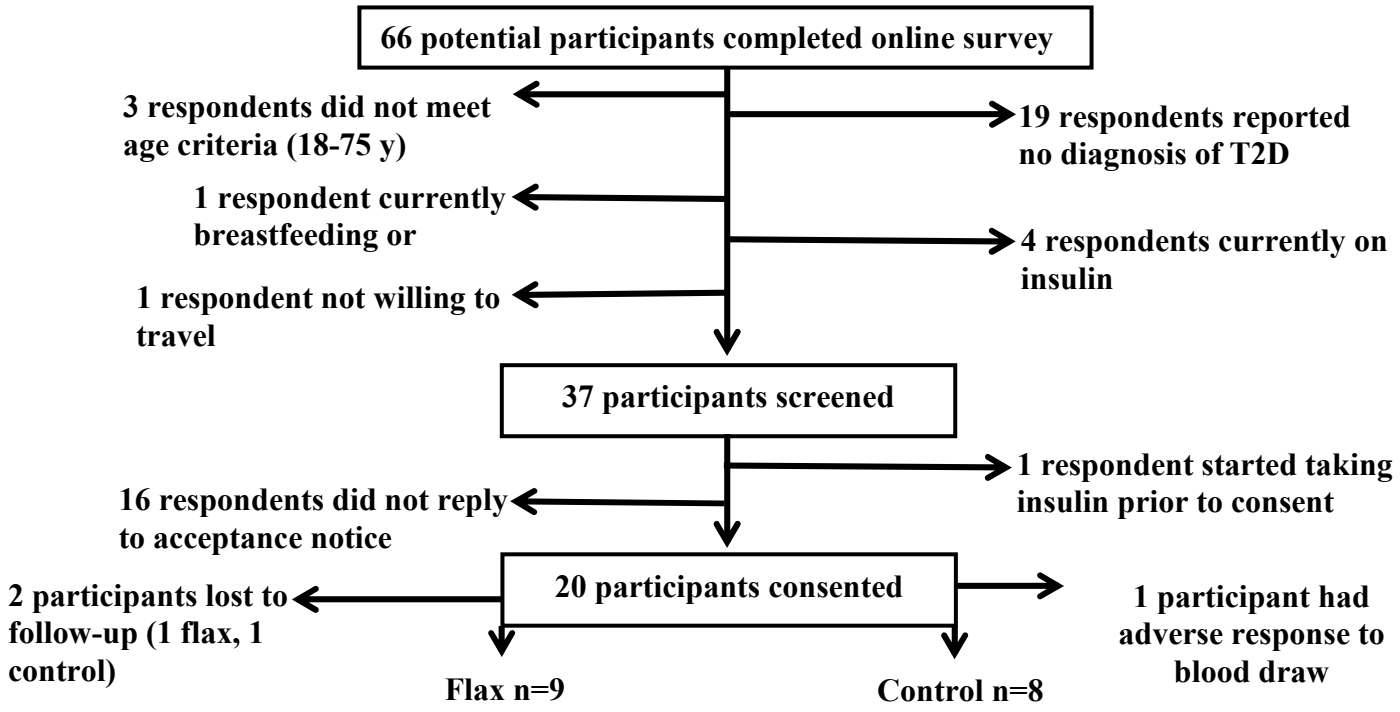

Figure 3. Study CONSORT flow diagram.

3.3 Treatment and Control Foods

The test foods consisted of ground flaxseed (Midwestern Flax, Valley City, ND) and ground psyllium husks (NOW Foods, Bloomindale, IL). Test foods were measured by weight and packaged in individual serving food grade plastic bags (American Plastics, Tracy, CA) in the Nutrition Metabolic Kitchen, located in the Arizona Biomedical Collaborative Building. Participants received 56 individually packaged servings for each day of the 8 week study intervention period. Subjects were given instructions on proper storage as well as suggestions on how to incorporate the fiber supplements into their daily diets (Appendix G). Participants were instructed to consume one individual package per day and asked to consume the supplement every day of the week and at minimum 5 days of the week. Furthermore, subjects were allowed to distribute the fiber supplement throughout the day as they wished in an effort to improve compliance. The selected dose of the treatment fiber, ground flaxseed (28 g/d) is equivalent to approximately one quarter of a cup. Ground psyllium husks was chosen for the control intervention due to its common use as a dietary fiber bulking agent in addition to its reported cholesterol and glucose lowering effects (Ziai et al. 2005). Psyllium has demonstrated outcome improvements are linearly related to the consumed dose and in clinical trials relatively low doses of psyllium without dietary instructions (< 9 g/d) are not significantly observed with improvements in glucose regulation (Anderson et al. 1999; Theuwissen and Mensink, 2008; Ziai et al. 2005). The amount of ground psyllium (9 g/d) used for the intervention is equivalent to approximately 1.5 teaspoons and was standardized according to fiber to ensure equal fiber content in both test food variables (Table 2). To monitor compliance and whether participants maintained their usual dietary habits, participants

were asked to complete a 3-day food record (Appendix H) 3 days prior to their first fasting blood draw and again 3 days prior to their final fasting blood draw. In addition, they were given calendars which corresponded to the intervention period and asked to check off the day the supplements were consumed (Appendix I). The 3-day food records were reviewed and analyzed using Food Processor software (Esha Research, Salem, OR). Dietary variables of interest were estimates of total energy intake, percentage of energy provided by macronutrients (carbohydrate, fat, and protein), and total fiber consumed by the participants.

Table 2. Nutrient Comparison of Test Foods

Nutrient	Ground Flaxseed (28g)	Psyllium (9g)
Total Energy (Kcals)	150	34
Total Fat (g)	11.8	0.0
Total ALA (g)	8.04	0.0
Total carbohydrates (g)	8.09	8.0
Total fiber (g)	7.6	7.0
Soluble fiber (g)	2.8	7.0
Total protein (g)	5	0.0
Thiamin (B1) (mg)	0.46	0.0
Riboflavin (B2) (mg)	0.045	0.0
Niacin (B3) (mg)	0.862	0.0
Vitamin B6 (mg)	0.132	0.0
Folate (DFE)	24.0	0.0
Calcium (mg)	71.0	30.0
Magnesium (mg)	9.63	0.0
Phosphorous (mg)	1079.0	0.0
Potassium (mg)	1366.0	0.0
Zinc (mg)	1.2	0.0
SDG content (mg)[a]	24.32	0.0

Source: NDSR, 2012

[a] SDG, Secoisolariciresinol digluscside

3.4 Blood Collection

Fasting blood samples were collected at the beginning and end of the 8 week dietary intervention period. Fasting blood draws occurred in the morning between 7:00 am and

10:00 am at both time points. Participants were asked to fast for 10-12 hours prior to their fasting blood draws at the SNHP research facilities For each fasting visit, blood was collected from a routine venous puncture procedure using a 23-gauge butterfly venous puncture needle. If a venous blood draw was not possible from the antecubital vein in either arm, blood was collected from a vein in the subject's non-dominant hand. Blood was collected into vacutainer tubes as follows: one separating tube containing serum-clotting factor (7 ml), one tube containing EDTA for plasma (10 ml), and one potassium oxalate and sodium fluoride containing vacutainer tube for plasma (4 ml). Blood was immediately centrifuged at 10,000 x g at 4°C for 20 minutes, and serum/plasma was separated, aliquoted, and stored at -80°C for future analyses.

3.5 Measurements

3.5.1 Anthropometric Data

Subject blood pressure was taken at one time-point during each visit prior to any additional measurements from the non-dominant arm (unless contraindicated) following a 5 to 10 minute seated rest period (both feet resting on floor) using a Medline MDS2001 automated blood pressure monitor (Medline Industries, Inc., Mundelein, IL). Height was measured in centimeters using a wall-mounted stadiometer. Body weight (in kilograms), percent body fat, and body mass index (BMI, kg/m^2) were measured using a calibrated Tanita body composition analyzer (model TBF- 300A, Tanita Corporation, Tokyo, Japan). Waist circumference (in centimeters) was measured in inches using a flexible tape measure at the naturally smallest point of the natural waist, between the lowest rib

and the iliac crest. All measurements were performed at a single time point by the same study investigator to increase reliability and validity.

3.5.2 Glucose and Lipids

Fasting glucose concentrations were measured using an automated chemistry analyzer (COBAS C111 chemistry analyzer, Roche Diagnostics, Indianapolis, IN). Glucose was measured in plasma which was collected into a vacutainer tube containing potassium oxalate and sodium fluoride to prevent glycolysis. Measurements of lipids which included total cholesterol, low density lipoprotein cholesterol (LDL-C), high density lipoprotein cholesterol (HDL-C), and total triglycerides, were measured in serum and determined enzymatically on a COBAS autoanalyzer (COBAS C111 chemistry analyzer, Roche Diagnostics, Indianapolis, IN). Very low density lipoprotein (VLDL)-cholesterol was calculated as total triglycerides/5.

3.5.3 Fasting Insulin and HOMA-IR

Insulin was measured in serum using a commercially available kit, ultra-sensitive human insulin radioimmunoassay kit (Cat. No. HI-14K; EMD Millipore, Billerica, MA). To determine insulin sensitivity, the homeostatic model assessment of insulin resistance (HOMA-IR) was calculated from fasting glucose and insulin values as follows: [fasting glucose (mg/dL) x fasting insulin (mg/dL)]/405. The calculated HOMA-IR value is highly correlated with the euglycemic clamp method (r^2=0.88) (Matthews et al. 1985). Higher levels of HOMA-IR indicate diminished insulin sensitivity.

3.5.5 Markers of Inflammation and Oxidative Stress

Increases in inflammatory markers, such as TNF-alpha, and reactive oxygen species (ROS) favor oxidative stress. Additionally, total nitrate and nitrite concentrations (i.e. NOx) are often utilized as a clinical marker for ROS generation and vascular dysfunction. TNF-alpha was measured in plasma using a commercially available ELISA kit (Cat. No. EH3TNFA; Life Technologies, Thermo Scientific, Waltham, MA) and measured at 450 nm on a Multiskan GO microplate spectrophotometer reader (Thermo Scientific, Waltham, MA). Lipid peroxidation, a marker of oxidative stress, was measured using a commercially available thiobarbituric acid reactive substances (TBARS) assay kit (Cat. No. 0801192; Zeptometrix, Buffalo, NY) using a miniaturized version of the standard protocol (Appendix J) based on the reaction between malondialdehyde (MDA) and thiobarbituric acid (TBA). Absorbance was measured spectrophotometrically at 532 nm on a microplate absorbance reader (iMark plate reader; BioRad, Hercules, CA). Plasma levels of TBARS are expressed as nM MDA/L. Additionally, plasma concentrations of NOx were measured in EDTA plasma with a commercially available Greiss assay kit (Cat. No. 780001; Cayman, Ann Arbor, MI) according to the manufacturer's protocol. Total plasma NOx activity was measured at wavelengths 450 nm and 550 nm (Multiskan Go; Thermo Scientific, Waltham, MA). Measurements at 550 nm were subtracted from measurements taken at 450 nm to correct for imperfections in the microplate. Plasma concentrations of NOx are expressed in nM/L.

3.6 Sample Size Calculation

This was designed to be a pilot study to determine the following: 1) would participants adhere to the previously stated volumes of test foods supplemental to their normal diet for an eight week trial period; 2) would test food volumes be adequate to elicit a detectable change in the measured variables, and 3; would the trial length be long enough to observe significant changes in previously described endpoint measurements. The primary endpoints were absolute change from baseline to week 8 in HbA1c (%) and plasma glucose. Secondary endpoints included: absolute changes in BMI (kg/m^2), waist circumference, body fat %, fasting insulin, HOMA-IR, serum triglycerides, total cholesterol, HDL-C, measured LDL-C, calculated VLDL-C, plasma oxLDL (i.e. TBARS), serum TNF-alpha, and total plasma NOx. As previously stated, this study was a pilot trial in order to assess feasibility and generate data for future large scale clinical interventions. Conducting a pilot trial before a main study may potentially help to avoid erroneous main trials due to flaws in the study design and may increase the likelihood the main study will succeed. A modified power analysis for feasibility and pilot studies was performed to determine the sample size necessary to detect significant changes in the primary endpoints.

Based upon published data (Hutchins et al. 2013; Mani et al. 2011), it was determined that a total of 50 participants would be needed to provide at least 80% power (at a significance level of 0.05) to observe an estimated 19.7% reduction in plasma glucose and 15.6% reduction in HbA1c for a large scale study design. This sample size was modified based upon recommendations proposed by Stallard (2012) and Chow

(2011) who collectively proposed that an appropriate sample size could be achieved by acquiring 8 to 15 participants per sample group to sufficiently mirror a large clinical trial with similar endpoints. A sample size of 16 total participants (8 per sample group) was constructed upon these recommendations. To account for an expected 20% attrition rate at follow-up additional participants were recruited (Fig. 4).

3.7 Statistical Analyses

Data was analyzed using SPSS 22.0 (IBM, 2014, Chicago, IL). Differences in baseline measurements between the flaxseed treatment group and the control group were determined using a student independent samples t-test. Between group changes from baseline to week 8 were determined by analyzing the absolute net change (week 8 values minus baseline values, Δ) with Mann Whitney U non-parametric analyses. Results are expressed as means ± standard deviation (SD). Results were considered significant at 95% or above (p-value of ≤ 0.05).

CHAPTER 4

RESULTS

4.1 Baseline Participant Characteristics and Dietary Intake

Seventeen of the 20 enrolled participants (85%) completed all study related visits (Figure 3). Baseline characteristics of the enrolled subjects are presented in Table 3. There were no significant differences in baseline measurements between the flaxseed and control groups ($p>0.05$). The mean age of participants in this study was 59.1 ± 7.8 years of age.

Additionally, the mean BMI (kg/m^2) of the participants was 30.4 ± 5.8 and mean percent body fat was 34.9 ± 8.9%. Mean waist circumference (cm) for study participants was 102.6 ± 16.4, both mean baseline measurements for waist circumference in women (98.6 ± 16.8 cm) and men (106.4 ± 16.0 cm) participants in this study were above the recommended healthy ranges for their respective gender (88 cm for women and 100 cm for men). Additionally, mean systolic blood pressure for participants (141.4 ± 13.9 mm Hg) fell within the American Heart Association's diagnostic criteria for hypertension stage 1 (140-159 mm Hg) while the mean diastolic blood pressure of 77.1 ± 5.6 mm Hg was within the normal range (< 80 mm Hg). Finally mean baseline glycated hemoglobin (HbA1c) was 6.91 ± 1.7% which is considered within the diagnostic criteria for diabetes (> 6.5%).

Table 3. Baseline Characteristics and Measurements for Study Participants

Subject Characteristics	**Flaxseed (*n=9*)**	**Control (*n=8*)**	***p-value*[a]**
Age (mean ±SD), y	59.7±7.9	58.5±9.4	*0.802*
Male sex, n	5	4	*N/A*
Female sex, n	4	4	*N/A*
Race/Ethnicity			
White, n (%)	8	7	
Non-Hispanic, n (%)	7	5	
Black, n (%)	1	0	
Asian, n (%)	0	1	
Other, n (%)	0	0	
BMI (mean±SD), kg/m^2	31.7±4.2	29.03±6.7	*0.335*
Body Mass (mean±SD), kg	94.3±15.2	85.88±21.1	*0.358*
Waist Circumference (mean ±SD), cm	105.0±12.7	100.0±20.3	*0.548*
HbA1c (mean ±SD), %	7.1±1.5	6.7±2.0	*0.333*

Body Fat (mean±SD), %	36.4±3.2	33.3±2.9	*0.483*
Systolic BP (mean±SD), mm Hg	141.8±14.4	141.0±14.0	*0.912*
Diastolic BP (mean±SD), mm Hg	76.1±4.0	78.3±7.1	*0.448*
Current Smokers, n (%)	0	0	*N/A*

[a] Between group baseline measurements were analyzed using Student's Independent samples t-test. Significance was set at $\alpha < 0.05$.
Abbreviations: HbA1c-glycated hemoglobin; BMI- body mass index; BP-blood pressure

4.2 Nutrient Intake

Analysis of nutrient intake from 3-day food records completed by all subjects the 3 days prior to baseline fasting blood draw and the 3 days prior to week 8 fasting blood draw showed no significant differences in nutrient intake between groups at baseline or at 8 weeks (Table 4). Prior to the start of the nutrition intervention, participants were given compliance calendars to keep a tally of the days test foods were consumed. Based upon compliance records returned by 16 of the 17 participants, compliance with the study protocol was very high (92.8 ± 8 %). Additionally, there were no reported differences in adherence between the flaxseed (92.3 ± 10%) and control groups (93.3 ± 6%) during the 8 week trial period. Data on compliance was missing in 1 subject (control group) which was not included in the subject adherence analysis.

Table 4. Baseline and Week 8 Nutrient Intakes for Flaxseed and Control Groups.

	Flaxseed *n = 9*			**Control *n = 8***			**Between Groups**
Variable[a]	**Baseline**	**Week 8**	**Δ[b]**	**Baseline**	**Week 8**	**Δ[b]**	***p-value*[c]**
***Energy (kcal)*[d]**	1925±394	1862±334	-63	1937±474	1830±444	-107	***0.630***
***Total Fat (g)*[d]**	59±31	62±25	+3	53±26	55±145	+2	***0.847***
***Fiber (g)*[d]**	22±6	28±5	+5	26±5	31±4	4.1	***0.191***
***Sodium (mg)*[d]**	2148±619	1851±672	-296	2338±936	2185±662	-153	***0.700***

[a] Data is represented as Mean±SD.
[b] Δ represents absolute net change from baseline to week 8.
[c] Data was analyzed as week 8 values minus baseline values (absolute net change); *p-value* represents Mann-Whitney U non-parametric test.

[d] Student's independent t-test analysis determined there were no significant differences at baseline between the flaxseed and control groups for kcals (p=0.958), fat (p-0.683), fiber (p=0.159), or sodium (p=0.624)

4.3 Anthropometric Characteristics

The effects of the 8 week intervention on anthropometric measurements are shown in Table 5. Body mass (kg), BMI (kg/m^2), waist circumference (cm), and percent body fat were not significantly different between the flaxseed and control groups throughout the trial period. Additionally, there were no significant changes within groups. Both systolic and diastolic blood pressures were comparable between groups from baseline to week 8.

Table 5. Anthropometric Measurements from Baseline to Week 8.

	Flaxseed *n = 9*			Control *n = 8*			Between Groups
Variable[a]	**Baseline**	**Week 8**	**Δ[b]**	**Baseline**	**Week 8**	**Δ[b]**	***p-value*[c]**
Body Mass (kg)	94.3±15.2	93.5±14.7	-0.8	85.9±21.2	85.8±21.4	-0.1	***0.665***
***BMI (kg/m^2)*[d]**	31.7±4.2	31.4±4.1	-0.3	29.0±6.7	28.9±6.6	-0.1	***0.152***
Waist Circumference (cm)	104.9±12.7	102.2±11.9	-2.8	100.0±20.3	98.82±20.3	-2.5	***0.809***
Body Fat (%)	36.4±9.5	37.4±8.9	+1.0	33.3±8.2	33.9±9.1	+0.7	***0.700***
***Systolic BP (mm Hg)*[d]**	141.8±8.9	140.4±11.6	-1.3	141.0±14.0	139.6±12.5	-1.4	***0.735***
***Diastolic BP (mm Hg)*[d]**	76.1±3.9	74.6±3.9	-1.6	78.3±7.1	76.6±6.2	-1.6	***0.961***

[a] **Data is represented as Mean±SD.**
[b] **Δ represents absolute net change from baseline to week 8.**
[c] **Data was analyzed as week 8 values minus baseline values (absolute net change); *p-value* represents Mann-Whitney U non-parametric test.**
[d] **Abbrevations: BMI, body mass index; BP, blood pressure.**

4.4 Biomarkers of Glucose Regulation

Change in biomarkers of glucose regulation (fasting plasma glucose and insulin, HbA1c, and HOMA-IR) did not differ between groups (Table 6) from baseline to week 8. However, HbA1c, a measure of long term glucose control (2 to 3 months) demonstrated a modest decrease in the flaxseed group ($p = 0.099$) though not significant.

Table 6. Changes in Markers of Glucose Regulation from Baseline to Week 8.

	Flaxseed ***n = 9***			**Control** ***n = 8***			**Between Groups**
Variable[a]	**Baseline**	**Week 8**	**Δ[b]**	**Baseline**	**Week 8**	**Δ[b]**	***p-value*[c]**
HbA1c	7.1±1.5	6.6±1.1	-0.5	6.7±2.0	6.8±2.6	+0.1	***0.099***
Fasting Glucose (mg/dL)	119.5±32.7	134.9±55.1	+15.4	130.7±62.2	129.1±69.1	-1.6	***0.248***
Fasting Insulin (mg/dL)	13.6±3.7	17.5±7.8	+4.0	14.6±5.8	19.2±13.5	+4.6	***0.773***
***HOMA-IR*[d]**	4.1±1.6	6.3±4.1	+2.2	5.0±3.1	6.8±6.6	+1.8	***0.336***

[a] **Data is represented as Mean±SD.**
[b] **Δ represents absolute net change from baseline to week 8.**
[c] **Data was analyzed as week 8 values minus baseline values (absolute net change); *p-value* represents Mann-Whitney U non-parametric test.**
[d] **HOMA-IR was calculated as (fasting glucose mg/dL x fasting insulin mg/dL)/405.**

4.5 Serum Lipid Profile

Comparisons of serum lipid profiles from baseline to week 8 between the treatment and control groups are shown in table 7. There were no significant differences found for any of the serum lipid markers including total cholesterol, LDL-C, HDL-C, total triglycerides, calculated VLDL-C (total triglycerides/5), or HDL:LDL ratios.

Table 7. Changes in Measurements of Serum Lipids from Baseline to Week 8.

	Flaxseed *n = 9*			Control *n = 8*			Between Groups
Variable[a]	**Baseline**	**Week 8**	**Δ[b]**	**Baseline**	**Week 8**	**Δ[b]**	***p-value*[c]**
Cholesterol (mg/dL)	167.7±45.7	159.6±28.9	-8.1	173.5±43.7	166.1±40.8	-7.4	***0.923***
LDL-C (mg/dL)	105.5±53.7	100.0±36.2	-5.5	105.5±46.1	96.4±28.0	-9.1	***0.773***
HDL-C (mg/dL)	55.3±19.7	49.2±22.9	-6.1	58.0±56.6	56.6±36.2	-1.4	***0.470***
VLDL-C*[d] *(mg/dL)	21.7±12.1	24.3±15.0	+1.5	20.1±6.5	22.5±5.6	+2.3	***0.294***
Triglycerides (mg/dL)	108.3±57.4	121.3±75.2	+13.0	128.9±76.9	134.4±138.6	+5.5	***0.248***
HDL:LDL (mg/dL)	0.6±0.2	0.6±0.2	+0.0	0.7±0.4	0.7±0.4	-0.1	***0.149***

[a] Data is represented as Mean±SD.
[b] Δ represents absolute net change from baseline to week 8.
[c] Data was analyzed as week 8 values minus baseline values (absolute net change); *p-value* represents Mann-Whitney U non-parametric test.
[d] VLDL-C was calculated as total triglycerides/5.

4.6 Markers of Inflammation and Oxidative Stress

There was a trend for decreased serum TNF-α concentrations in the flaxseed group as compared to the control ($p = 0.060$) from baseline to week 8 suggesting a tendency for decreased inflammation. There was also a modest decrease, though not significant observed between the flaxseed group and the control group from baseline to week 8 for TBARS, a marker of lipid peroxidation and oxidative stress ($p = 0.083$). A significant difference for NOx between groups from baseline to week 8 was not established. However, the flaxseed group had slightly higher concentrations of NOx at week 8 as compared to baseline (Δ = +5.7 nM/L vs. -2.41 nM/L for the control group). Measurements are shown below in table 8.

Table 8. Changes in Markers of Inflammation and Oxidative Stress from Baseline to Week 8.

	Flaxseed *n = 9*			Control *n = 8*			Between Groups
Variable[a]	**Baseline**	**Week 8**	**Δ[b]**	**Baseline**	**Week 8**	**Δ[b]**	***p-value*[c]**
***TNF-α (pg/mL)*[d]**	0.1±0.0	0.06±0.02	-0.0	0.1±0.0	0.1±0.0	-0.0	***0.060***
***TBARS (nM MDA/L)*[d]**	6.7±5.0	3.9±2.0	-2.8	2.9±1.4	2.8±2.0	-0.1	***0.083***
***NOx (nM/L)*[d]**	5.9±1.6	11.7±3.5	+5.7	7.1±2.9	4.7±2.2	-2.4	***0.092***

[a] Data is represented as Mean±SD.
[b] Δ represents absolute net change from baseline to week 8.
[c] Data was analyzed as week 8 values minus baseline values (absolute net change); *p-value* represents Mann-Whitney U non-parametric test.
[d] Abbrevations: TNFα, tumor necrosis factor-alpha; TBARS, thiobarbituric acid reactive substances; NOx, total nitrate/nitrites.

4.7 Safety and Tolerability

There were no reported issues with any complications arising from the test food volumes participants consumed during this nutrition intervention. The flaxseed group consumed 28 g/d of ground flaxseed while the control group consumed 9 g/d of ground psyllium husk. All participants were instructed to report any discomfort, irritation, or aversions to the test foods at any time point throughout the study. No aversions to either test food or physical discomfort was reported.

CHAPTER 5

DISCUSSION

Lifestyle changes such as diet modifications and exercise as well as pharmaceutical interventions (i.e., insulin and hypoglycemic) are often implemented to control glucose metabolism during the initial treatment stages of T2D (i.e., stage I and stage II interventions, Lebovitz, 1999). In recent years there has been much attention paid to the potential benefits of various foods and their nutritive components in the management of T2D symptoms and associated outcome risks. Studies examining the effects of flaxseed consumption in its various forms (oil, ground, and whole seed) have provided mixed results in regards to these outcomes. Very few clinical trials looking at the impact of glycemic control and markers of inflammation in T2D participants have been conducted (Pan et al. 2007; Taylor et al. 2010). Furthermore, a comparison of the effectiveness of ground flaxseed as compared to psyllium supplementation in addition to a habitual diet in adult non-insulin dependent T2D participants has not been investigated. To the best of our knowledge, the present study is the first to investigate the effect of a modest amount of ground flaxseed compared to a fiber-matched control in conjunction to a person's normal daily nutrient intake on glycemic control, lipid profiles, inflammation and vascular function in non-insulin dependent T2D patients. We demonstrated that eight weeks of supplementation with 28 g/d ground flaxseed in conjunction to an individual's habitual diet demonstrated a mild increase in total NOx ($p = 0.099$) as well as a slight reduction in markers of inflammation (TNF-alpha, $p = 0.060$) and oxidative stress (TBARS, $p = 0.083$). Additionally, our findings resulted in a modest (though not

significant) decrease in HbA1c *(p* = 0.099) as compared to the control group (9 g/d ground psyllium husk).

5.1 Measurements of Body Composition and Blood Pressure

No changes in measurements of body composition (Table 5) were observed in either the flaxseed group or the control group from baseline to week 8. Our results confirm previous studies which failed to report any significance in measurements of body mass, BMI, body fatness, or waist circumference associated with flaxseed consumption (Hutchins et al. 2011; Rhee and Brunt, 2011) or lower doses (less than 12 g/d) of psyllium (Pal et al, 2011; Pittler and Ernst, 2004). Additionally, there were no changes in systolic or diastolic blood pressure of the non-insulin T2D participants enrolled in this study. To date, findings regarding the effects of flaxseed consumption on blood pressure have been inconclusive. Studies which have reported decreases in systolic BP, diastolic BP, or both have only done so in participants with baseline blood pressure values that were considered hypertensive (Paschos et al. 2007; Dupasquier et al. 2006).

5.2 Glucose Regulation

Type 2 diabetes is a chronic disease characterized by insulin insensitivity and hyperglycemia. Treatment and management of this disorder aims to control glucose regulation in the body, specifically to attenuate hyperglycemia in the bloodstream, as well as prevent the damage associated with prolonged tissue exposure to elevated blood glucose concentrations. Associated complications of T2D include macrovascular

complications (coronary artery disease, peripheral arterial disease, and stroke) and microvascular complications (diabetic nephropathy, neuropathy, and retinopathy). HbA1c, also known as glycated hemoglobin, is an indicator of long-term glycemic control over the past two to three months and is strongly associated with vascular complications, both macro- and micro-vascular (Fowler, 2008).

While results from our current study suggested a modest decrease in HbA1c concentrations, these results were not significant ($p = 0.099$) and did not ascertain any major link between ground flaxseed consumption and long term glucose regulation. Moreover, since HbA1c is a long term marker of glucose control and reflects glucose regulation of the previous 2 to 3 months, short trials may find very little change in measured outcomes. Interestingly, one study that examined the effects of replacing a standard chow diet consisting of corn oil (1.2. mg/kg/d) with flaxseed oil (1.2 ml/kg/d) in streptozotocin (STZ)-induced diabetic rats and non-diabetic rats observed a significant decrease in fasting glucose and HOMA-IR levels in the STZ-treated diabetic group as compared to the STZ corn oil group and the control. However, the flaxseed supplemented STZ group did have increased (N.S.) fasting insulin levels compared to these same groups (Hussein et al. 2012). Several clinical trials have failed to establish any link between low or high doses of flaxseed oil and glycemic control in type 2 diabetic patients (Barre et al. 2008; Goh et al. 1997; McManus et al. 2006). High doses of EPA and DHA, which as previously discussed can be metabolized from ALA, have been associated with deteriorated glycemic control in T2D (Glauber et al. 1998; Nettleton et al. 2005; Woodman et al. 2002).

Current research in animal and human studies (Hussien et al. 2012; Hutchins et al, 2008; Rhee et al. 2011 Velqasquez et al. 2003) suggests the ground form of flaxseed, the form used in this current study, which is also rich in the ALA, as well as fibers, lignans, and folic acid may be useful in reducing hyperglycemia. Recent clinical trials have shown that ingestion of ground flaxseed (50 g/d, 10 weeks) resulted in statistically significant improvements in glycemic control in T2D patients as measured by fasting glucose, fasting insulin, and HOMA-IR (Bloedon et al. 2008). Furthermore, previous studies have demonstrated that ground flaxseeds improve glucose metabolism in healthy participants. In one study, healthy participants who consumed a baked product containing 50 g of flaxseed daily for 4 weeks had a 27% decrease in post-prandial glucose concentrations (Cunnane et al. 1993). Lemay (2002) found that ground flaxseed consumption (40 g/d for 8 weeks) in post-menopausal hypercholestorelemic women resulted in significantly reduced blood glucose concentrations. Additionally, Hutchins et al, (2012) found that in obese pre-diabetic participants given 13 g/d or 26 g/d ground flaxseed, the 13 g/d group had improved fasting insulin levels as compared to the control (0 g/d) and higher flaxseed group (26 g/d), however, improvement were not seen in fasting glucose or HOMA-IR. Dahl (2005) demonstrated that healthy subjects who consumed flaxseed fiber in conjunction with their normal diet displayed attenuated peak glucose concentrations. In contrast, data from this current study did not establish any major effect on fasting glucose and insulin concentrations, or HOMA-IR. The focus of this trial was to examine the effectiveness of ground flaxseed (28 g/d) consumption as compared to the control test food (psyllium, 9 g/d) supplementation in non-insulin T2D participants as opposed to

previous trials which examined obese, pre-diabetic, or healthy participants which may account for the discrepancies in observed outcomes. Additionally, due to lack of research establishing an optimal dose for either dietary supplement as well as no known comparative trials examining the efficacy of flaxseeds versus psyllium, we have a lack of studies that we can directly compared our outcomes against. Furthermore, the mechanisms by which flaxseed exerts its control over glucose metabolism in the body have yet to be elucidated. Both the fiber components of flaxseed as well as the lignan concentration (i.e., SDG) may be responsible for results observed in prior studies.

5.3 Serum Lipids

Several studies have reported a positive impact of flaxseed consumption on lipid metabolism. Previous animal studies suggest that flaxseed or flaxseed derived lignans reduce both total and LDL-C as well as attenuate the progression of atherosclerosis (Lucas et al. 2004; Prasaad, 2008; Prasaad, 1999; Prasaad, 1997). In clinical trials, flaxseed consumption on blood lipid concentrations appear to be much more modest and the results lack consistency. Studies observing the effects of flaxseed oil in lipids profiles have reported decreases in total cholesterol and LDL-C. Harper et al. (2006) reported that 3 g/d of flaxseed-derived ALA resulted in no significant differences between LDL-C levels, however, LDL-C subfractions LDL_1 and LDL_2, which are considered large buoyant LDL particles were significantly increased as compared to the smaller dense LDL subfractions which have a greater likelihood of oxidation and contributing to the progression of atherosclerosis. In hypercholesterolemic participants, ground flaxseed

supplementation (30 g/d, 12 weeks) and flaxseed-derived lignan supplementation (600 mg/d, 8 weeks) has been associated with decreases in total cholesterol (7% and 22%, respectively) and LDL-C concentrations (10% and 24%, respectively). Most studies to date have not shown any impact of flaxseed consumption in any of its forms on HDL-C concentrations (Lemay et al. 2002; Harper et al. 2006; Stuglin et al. 2005), with the exception of one trial (Bloedon et al. 2008) which reported a decrease in HDL-C in hypercholesterolemic men. A meta-regression analysis of flaxseed supplementation in its various forms (Pan et al. 2009) analyzing a total of 28 clinical trials (overall participants = 1539) demonstrated that of the 13 out of 28 trials which used flaxseed oil, no significant changes in total and LDL cholesterol were detected. Flaxseed in whole or ground form were used for ten of these 28 studies and flaxseed derived lignan trials comprised five of the 28 studies. Whole flaxseed and flaxseed lignans demonstrated a greater impact on serum lipids as compared to ground flaxseed. Of the reported trials which used flaxseed in its whole form or lignans derived from flaxseed there were significant decreases in total cholesterol and LDL-C concentrations, however this reduction was much greater in studies including post-menopausal women and these reductions were only moderate for trials that included both women and men. In agreement with our findings the authors found no association between flaxseed in any form or flaxseed derived lignans on HDL-C concentrations. Results from this analysis suggested that sex, type of intervention (whole flaxseed, flaxseed oil, or lignan supplement), and initial lipid concentrations (i.e., healthy subjects vs. hypercholestorelemic subjects) influenced the net changes in total and LDL cholesterol.

Trials examining the effects of flaxseed consumption have also displayed varying outcomes. While epidemiological data reports higher levels of ALA consumption associated with lower total triglyceride levels (Dejousse et al. 2005), flaxseed clinical trials have reported an increase (Cunnane et al, 1995), decrease (Dejousse et al. 2003; Zhao et al. 2004), or no change (Rallidis et al. 2003; Paschos et al. 2007) on total triglyceride levels.

5.4 Inflammation and Oxidative Stress

Oxidative stress resulting from increases in inflammation, dyslipidemia, and hyperglycemia is also strongly associated with worsening of T2D symptoms and associated complications. There is strong evidence of increased platelet aggregation in type 2 diabetes which may be a result of ROS generation and oxidative stress (Ferroni et al. 2004). As we demonstrated in this study there was a trend for decreased serum concentrations of the inflammatory marker TNF-alpha in the flaxseed group ($p = 0.060$) as well as a modest decrease in plasma concentrations of TBARS ($p = 0.083$) which suggests decreased lipid peroxidation. While not significant, possibly due to the relatively small sample size or participant characteristics, these results appear to corroborate with previous findings which demonstrated flaxseed consumption in animal models (Dupasquier et al. 2007; Hussein et al. 2012) and clinical trials (Allman et al. 1995; Bierenbaurn et al. 1993; Freese et al. 1994; Pilar et al. 2014; Rhee and Brunt, 2011) act to decrease concentrations of platelet aggregation (i.e., VCAM, ICAM, VEGF), inflammation (TNF-alpha, IL-6) and oxidative stress (TBARS) in healthy, obese, and

glucose-intolerant individuals. Findings from the previous studies suggest a protective or antioxidant effect of flaxseeds which is in agreement with the findings of the present study. Increased glucose oxidation and lipid peroxidation as a result of hyperglycemia and obesity increases inflammation, ROS generation, and oxidative stress (Furukawa et al. 2004). Increased oxidative stress may inhibit proper phosphorylation of the insulin receptor or decrease the translocation of GLUT4 on the cell membrane through impaired insulin signaling intermediates (Hoehn et al. 2009; Rudich et al. 1998). It was found that antioxidants attenuate impaired GLUT4 translocation and increase glucose uptake (Estrada et al. 1996; Shin et al. 2006). Increases in ROS, such as superoxide lead to increases in oxidative stress through scavenging of nitric oxide (NO) to form peroxynitrite, which is strongly associated with vascular dysfunction. Furthermore, cells other than the endothelium, which produces NO as a vasodilator for vascular smooth muscle, such as macrophages, can produce NO in very small quantities as a defense against inflammation and oxidative stress. High physiological concentrations of NO in the body favor increases in oxidative stress thus worsening symptoms associated with T2D. We measured total plasma concentrations of nitrates and nitrites (NOx) since nitrates are rapidly metabolized to nitrites *in vivo*. Consistent with our findings for TNF-alpha and TBARS concentrations, there was a slight increase in plasma concentrations of NOx ($p = 0.099$) following the 8 week supplementation with ground flaxseed. These modest reductions in levels of inflammation and oxidative stress may be attributed to the SDG content in flaxseed which previous studies have reported to decrease lipid peroxidation due to its ability to scavenge ROS (Houstis et al. 2006). This present study

did not specifically examine serum concentrations of SDG in the flaxseed group as compared to the control and only a few clinical trials have examined SDG in flaxseeds on oxidative stress, thus greater investigation is warranted.

5.5 Study Limitations and Strengths

The current study did have some limitations. Few studies have been conducted in regards to flaxseed and T2D. It is possible that our study may have lacked statistical power to detect changes in outcomes of interest. The sample size calculation estimated that at least 16 subjects would be necessary for this study to have sufficient statistical power for detecting changes in HbA1c and fasting plasma glucose. Recruitment was increased in order to assure that the minimum sample size was achieved. The sample size calculation was based on two previous studies Hutchins et al. (2012) and Pan et al. (2007) which specifically examined the effects of ground flaxseed consumption in diabetic participants on measures of glycemic control. It is possible that the effect sizes based on that study may have been overestimated due to differences in study design (parallel arm trial as compared to randomized crossover study).

Our study failed to achieve an effect size greater than 55% using a Mann-Whitney U analysis, which strongly suggests that additional participants were needed to truly observe any valid changes between groups. Additionally, of the few clinical trials that have looked at the effects of whole or ground flaxseed consumption on similar outcome variables, dosage of flaxseed ranged from 20 g/d to 50 g/d and lasted from 2 weeks up to 12 weeks in length, thus a known optimal dosage has yet to be established. This study included participants who were on oral hypoglycemic medications (i.e., metformin, flaxseed 5/9; control 6/80) and allowed for participants to be on statins or fibrolytics (flaxseed, 7/9; control 6/8) which may have effected changes seen in lipid variables, although participants were instructed to maintain consistent use of medications to avoid this issue. The majority of previous trials examining the effects of flaxseeds on serum lipids had participants with higher total-and LDL-C concentrations at baseline in addition to specifically examining hypercholestorelemic populations. When performing a statistical analysis for changes in the primary outcomes (HbA1c and fasting plasma glucose) from the present study to conduct a power analysis, the suggested sample size for having a statistical power (at a 0.05 significance level and power > 0.80) was 15 participants in both the flaxseed and control group (N = 30). Since two to three months are often necessary to observe changes in HbA1c it is possible that greater effects would have been observed if the participants had consumed the flaxseed for a long period of time. A longer intervention time would have also provided more information regarding adherence of individuals to flaxseeds supplemental to a normal diet. Additionally, we relied on self-reported nutrient intake data to estimate dietary measurement using 3-day

food records. While this questionnaire has been validated (Pietinen et al. 1988) and is not reliant on memory recall there may be issues with participants underestimating food quantities and underreporting portions. Other clinical nutrition interventions have circumvented this issue by structuring a complete diet program for study participants as well as providing comprehensive dietary instructions (Serra-Majem et al. 2006). The present study has a number of strengths worth discussing. For all subjects who completed the baseline fasting blood draw there was minimal attrition (17 out of 19, 85%) from baseline to week 8 which is less than the anticipated participant attrition rate (20%). Additionally, in this randomly controlled trial, subjects (all non-smokers with a diagnosis of T2D ≥ six months) were matched based upon baseline height, weight, HbA1c, sex, and age then randomly assigned to their respective group to eliminate confounding variables affecting between group measured outcomes. Subjects were blinded to their study condition which further decreases likelihood of performance bias or attrition bias disrupting the validity of this intervention trial. Furthermore, body composition and nutrient intakes for each group was comparable within group and between groups from baseline to week 8 reducing the likelihood of an interaction effect of weight loss or changes in body fatness on measures of glycemic control, serum lipids, and inflammation. Additionally, there was little between group variations which increased validity of measured outcome data between groups.

5.6 Future Research

This current study was conducted in order to investigate the effects of flaxseed use on complications of T2D. Future studies should include a larger sample size in addition to a tightly structured randomized control trial with an expanded participant sample population to include subjects with pre-diabetes and MetS to determine variations in the effectiveness of flaxseeds on specific populations. Additionally, this study did not measure plasma concentrations of polyunsaturated fatty acids ALA, EPA, DHA, or the flaxseed-derived lignan SDG. As these bioactive components have been proposed as potential mechanisms by which flaxseeds may exert their effect, future analyses should include these measures. Additionally, plasma levels of alpha-tocopherols should be measured in future studies to elucidate the potential antioxidant protective effects of flaxseeds in the progression of T2D.

CHAPTER 6

CONCLUSIONS

Due to the prevalence and economic burden of diabetes and its associated CVD risks, research is needed to assess the safety and effectiveness of treatment strategies. Nutrition interventions that are simple and effective in reducing symptoms of diabetes are an area of great interest due to the relative low cost and accessibility to the general public. However, diets are not limited to a single food; likewise, foods such as flaxseeds are not limited to a single nutrient thus complicating the mechanistic properties and health benefits of a particular food on any given population. The primary aims of this study were to investigate the effects of consuming 28 g/d ground flaxseed in addition to a participant's habitual diet for eight weeks on markers of glycemic control in T2D. Additionally, we further investigated the effects of ground flaxseeds on serum lipid concentrations as well markers of inflammation and oxidative stress in subjects with non-insulin dependent T2D. Due to the lack of knowledge regarding the use and efficacy of modest consumption of ground flaxseeds at attenuating symptoms associated with T2D, additional exploratory outcomes included evaluating compliance as well as the efficacy of incorporating a moderate amount of flaxseed into the daily diets of individuals with non-insulin T2D as compared to previous studies which used larger volumes (50 g/d) of ground flaxseed.

To our knowledge this study is the first to examine a moderate amount of ground flaxseeds on glycemic control, serum profiles, and markers of inflammation and oxidative stress in T2D participants. We did not confirm the main hypothesis that 28 g/d

of flaxseed intake would result in significantly improved glucose regulation, however, we did demonstrate a trend towards reduced markers of inflammation (NOx, TNF-alpha) and oxidative stress (TBARS) which is consistent with previous literature. Additionally, it should be noted 28 g/d of ground flaxseed did not result in any negative effects and was favorably reported by study participants thus increasing the likelihood that the dosage was reasonable and could be assimilated into a habitual diet. Lack of significant findings in this study may be related to the small study sample size, length of trial, or mechanism by which flaxseeds exert their greatest effects. The latter requires further investigation to elucidate the populations that may potentially benefit the greatest from ground flaxseed interventions.

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APPENDIX A

APPROVED ASU IRB PROTOCOL

ASU Knowledge Enterprise Development

Office of Research Integrity and Assurance

To:	Karen Sweazea LSC 552A
From:	Carol Johnston, Chair Biosci IRB
Date:	05/15/2013
Committee Action:	**Expedited Approval**
Approval Date:	05/15/2013
Review Type:	Expedited F2 F7
IRB Protocol #:	1304009144
Study Title:	PotentialTherapeutic Benefits of Flaxseeds in the Treatment of Type 2 Diabetes Symptoms
Expiration Date:	05/15/2014

C. Johnston, ASU IRB

The above-referenced protocol was approved following expedited review by the Institutional Review Board.

It is the Principal Investigator's responsibility to obtain review and continued approval before the expiration date. You may not continue any research activity beyond the expiration date without approval by the Institutional Review Board.

Adverse Reactions: If any untoward incidents or severe reactions should develop as a result of this study, you are required to notify the Biosci IRB immediately. If necessary a member of the IRB will be assigned to look into the matter. If the problem is serious, approval may be withdrawn pending IRB review.

Amendments: If you wish to change any aspect of this study, such as the procedures, the consent forms, or the investigators, please communicate your requested changes to the Biosci IRB. The new procedure is not to be initiated until the IRB approval has been given.

Please retain a copy of this letter with your approved protocol.

APPENDIX B

ADVERTISEMENT FOR FLAXSEED RECRUITMENT

The ASU Nutrition Program is recruiting adults with type 2 diabetes (18-75 y of age) who are willing to consume ground flaxseeds or psyllium fiber for 8 weeks. Participation includes traveling to the downtown ASU research site for 3 visits total. Two of these visists will require fasting for 8-12 hours prior to appointment. On each of the 3 visits a finger prick will be performed. Participants will receive flaxseeds and psyllium fiber as well as up to $40 in cash incentives for the study. If you are a non-insulin dependent type 2 diabetic who is generally healthy and interested in joining our study, please visit our recruitment site: https://www.surveymonkey.com/s/ASU_Flaxseeds_Study

APPENDIX C

ONLINE SCREENING QUESTIONNAIRE FOR FLAXSEED PARTICIPANT RECRUITMENT

*** 1. Please provide your email address.**

2. Has your physician diagnosed you with type 2 diabetes?

- Yes
- No
- Not sure

3. Do you know your glycosylated hemoglobin A1c level?

- Yes
- No
- Not sure

If yes, what is it?

4. Are you between 18 and 75 years old?

- Yes
- No

5. Are you currently taking insulin?

- Yes
- No
- Not sure

6. Are you currently taking any oral diabetic medications (e.g., metformin)?

- Yes
- No

7. Do you take any of the following medications: e.g. beta-blockers, ACE inhibitors, diphenhydramine or cyproheptadine (allergy medications), lithium carbonate, corticosteroids, thiazolidinediones (Actos, Avandia or Avandamet), sulfonylureas, biguanides, meglitinides, incretins, sodium valproate, or thyroid replacement therapy?

- Yes
- No
- Not sure

8. Are you currently pregnant or breast-feeding?

- ☐ Yes
- ☐ No

9. Do you have any known food allergy?

- ☐ Yes
- ☐ No
- ☐ Not Sure

If yes, please specify

10. Would you be willing to consume 4 tablespoons of ground flaxseeds or 1.5 tablespoons pysllium fiber daily for 8 weeks?

- ☐ Yes
- ☐ No

11. Will you be able to maintain your current diet and physical activity for a consecutive 8 weeks?

- ☐ Yes
- ☐ No
- ☐ Not sure

12. Do you train athletically to compete?

- ☐ Yes
- ☐ No

13. Are you willing and are able to travel to the ASU Downtown Campus to meet with the research investigators on three separate mornings?

- ☐ Yes
- ☐ No

14. Are you willing to have a fasting blood draw (fast 10-12 hours prior to blood draw) on 2 separate occasions?

- ☐ Yes
- ☐ No
- ☐ Not Sure

15. Where did you hear about this survey?

APPENDIX D

APPROVED ASU IRB CONSENT FORM

Informed Consent

Promoting healthy eating behaviors in older adults with type 2 diabetes (T2D).

INTRODUCTON
The purposes of this form are (1) to provide you with information that may affect your decision as to whether or not to participate in this research study, and (2) to record your consent if you choose to be involved in this study.

RESEARCHERS
Drs. Karen Sweazea and Kristin Ricklefs, Nutrition professor at Arizona State University Downtown Campus, and Kristin Ricklefs, a nutrition doctoral student, have requested your participation in a research study.

STUDY PURPOSE
The purpose of the research is to examine the effects of simple diet alterations on heart disease risk factors in individuals with type 2 diabetes.

DESCRIPTION OF RESEARCH STUDY
You have indicated to us that you are 25-75 years of age and have been diagnosed by a physician with type 2 diabetes. It is our understanding that you do not take insulin for your condition but you may take other prescription medications. If there is a change to your prescription medication use during the 8-week study, please notify study personnel. This study will initially involve the completion of a brief medical history questionnaire and a short dietary questionnaire. At this time, your weight, height, and girth will be measured; your blood pressure will be taken; and, a drop of blood from a finger prick will be used to measure hemoglobin A1c. You will be instructed to record all food and beverage intake on 6 days during the 8-week study. This first meeting will take about 30 minutes. There are two additional visits (study visits 1 and 2) that will last about 30 minutes and scheduled eight weeks apart. On test days, you will travel to ASU (the Nutrition labs at the ASU Downtown campus) early in the morning and in a fasted state (no food or drink with the exception of water for >8 hours.)

On the test days, you will have a blood sample drawn from an arm vein, and we will measure blood pressure, weight, and girth. Food records will be collected at each test visit, and study foods will be provided. About 20 subjects will participate in this study.

A research nurse will draw blood using standard, sterile techniques. Finger pricks will be conducted under sterile conditions using disposable, retractable lancets. Blood samples will be analyzed for biomarkers that are associated with the diabetic condition including glucose, insulin, cholesterol, and inflammation.

RISKS
Bruising of the skin or a feeling of faintness is possible during the blood draws. A registered nurse will draw the venous blood sample under sterile conditions and is trained to minimize these risks. Although participants are screened for food allergies, it is possible that individuals may have allergic reactions to the flaxseeds and psyllium powder used in the study. This dietary intervention may cause loose stools or gas. Please inform the researchers if you experience either of these discomforts and/or wish to withdraw from the study.

BENEFITS
This study will provide information regarding the usefulness of simple diet alterations for improving markers of heart disease risk in individuals with type 2 diabetes. If desired, you will be provided with study results and your personal blood data at the end of the study.

NEW INFORMATION
If the researchers find new information during the study that would reasonably change your decision about participating, then they will provide this information to you.

CONFIDENTIALITY
All information obtained in this study is strictly confidential unless disclosure is required by law. The results of this research study may be used in reports, presentations, and publications, but your name or identity will not be revealed. In order to maintain confidentiality of your records, the investigators will use subject codes on all data

collected, maintain a master list separate and secure from all data collected, and limit access to all confidential information to the study investigators. Plasma from blood samples will be stored for 5 years in freezers in the laboratories of the Nutrition Program at Arizona State University Downtown Campus after which time they will be disposed of as biohazard waste.

WITHDRAWAL PRIVILEGE

You may withdraw from the study at any time for any reason without penalty or prejudice toward you. Your decision will not incur negative treatment to you by the researchers.

COSTS AND PAYMENTS

All study foods will be given to you during the study free of charge. You will also receive a cash incentive at both study visits (total amount, $40).

COMPENSATION FOR ILLNESS AND INJURY

If you agree to participate in the study, then your consent does not waive any of your legal rights. However, in the event of harm, injury, or illness arising from this study, neither Arizona State University nor the researchers are able to give you any money, insurance coverage, free medical care, or any compensation for such injury. Major injury is not likely but if necessary, a call to 911 will be placed.

VOLUNTARY CONSENT

Any questions you have concerning the research study or your participation in the study, before or after your consent, will be answered by Dr. Karen Sweazea [480-965-6025] or Kristin Ricklefs [785-317-0263].

If you have questions about your rights as a subject/participant in this research, or if you feel you have been placed at risk, you can contact the Chair of the Human Subjects Institutional Review Board, through the ASU Research Compliance Office, at 480-965 6788.

This form explains the nature, demands, benefits and any risk of the project. By signing this form you agree knowingly to assume any risks involved. Remember, your participation is voluntary. You may choose not to participate or to withdraw your consent and discontinue participation at any time without penalty or loss of benefit. In signing this consent form, you are not waiving any legal claims, rights, or remedies. A copy of this consent form will be given to you.

Your signature below indicates that you consent to participate in the above study.

Subject's Signature	Printed Name	Date
Contact phone number	Email	

ARIZONA STATE UNIVERSITY IRB
APPROVED BY IRB
DATE: 5/15/13 – 5/14/14
NAME:

INVESTIGATOR'S STATEMENT

"I certify that I have explained to the above individual the nature and purpose, the potential benefits, and possible risks associated with participation in this research study, have answered any questions that have been raised, and have witnessed the above signature. These elements of Informed Consent conform to the Assurance given by Arizona State University to the Office for Human Research Protections to protect the rights of human subjects. I have provided the subject/participant a copy of this signed consent document."

Signature of Investigator_____ Date_____

APPENDIX E

APPROVED MODIFIED ASU IRB CONSENT FORM

Promoting healthy eating behaviors in older adults with type 2 diabetes (T2D).

INTRODUCTON
The purposes of this form are (1) to provide you with information that may affect your decision as to whether or not to participate in this research study, and (2) to record your consent if you choose to be involved in this study.

RESEARCHERS
Dr. Karen Sweazea, Nutrition professor at Arizona State University Downtown Campus, and Kristin Ricklefs, a nutrition doctoral student, have requested your participation in a research study.

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The purpose of the research is to examine the effects of simple diet alterations on heart disease risk factors in individuals with type 2 diabetes.

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You have indicated to us that you are 18-75 years of age and have been diagnosed by a physician with type 2 diabetes. It is our understanding that you do not take insulin for your condition but you may take other prescription medications. If there is a change to your prescription medication use during the 8-week study, please notify study personnel. This study will initially involve the completion of a brief medical history questionnaire and a short dietary questionnaire. At this time, your weight, height, and girth will be measured; your blood pressure will be taken; and, a drop of blood from a finger prick will be used to measure hemoglobin A1c. You will be instructed to record all food and beverage intake on 6 days during the 8-week study. This first meeting will take about 30 minutes. There are two additional visits (study visits 1 and 2) that will last about 30 minutes and scheduled eight weeks apart. On test days, you will travel to ASU (the Nutrition labs at the ASU Downtown campus) early in the morning and in a fasted state (no food or drink with the exception of water for >8 hours.)

On the test days, you will have a blood sample drawn from an arm vein, and we will measure blood pressure, weight, and girth. Food records will be collected at each test visit, and study foods will be provided. About 20 subjects will participate in this study.

A research nurse will draw blood using standard, sterile techniques. Finger pricks will be conducted under sterile conditions using disposable, retractable lancets. Blood samples will be analyzed for biomarkers that are associated with the diabetic condition and cardiovascular disease including markers of blood sugar regulation, cholesterol, inflammation, estrogen and testosterone.

RISKS
Bruising of the skin, discomfort at site of needle puncture, muscle soreness, or a feeling of faintness is possible during the blood draws. A registered nurse will draw the venous blood sample under sterile conditions and is trained to minimize these risks. Although participants are screened for food allergies, it is possible that individuals may have allergic reactions to the flaxseeds and psyllium powder used in the study.

BENEFITS
This study will provide information regarding the usefulness of simple diet alterations for improving markers of heart disease risk in individuals with type 2 diabetes. If desired, you will be provided with study results and your personal blood data at the end of the study.

NEW INFORMATION
If the researchers find new information during the study that would reasonably change your decision about participating, then they will provide this information to you.

CONFIDENTIALITY
All information obtained in this study is strictly confidential unless disclosure is required by law. The results of this research study may be used in reports, presentations, and publications, but your name or identity will not be revealed. In order to maintain confidentiality of your records, the investigators will use subject codes on all data

collected, maintain a master list separate and secure from all data collected, and limit access to all confidential information to the study investigators. Plasma from blood samples will be stored for 5 years in freezers in the laboratories of the Nutrition Program at Arizona State University Downtown Campus after which time they will be disposed of as biohazard waste.

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All study foods will be given to you during the study free of charge. You will also receive a cash incentive at both study visits (total amount, $40).

COMPENSATION FOR ILLNESS AND INJURY

If you agree to participate in the study, then your consent does not waive any of your legal rights. However, in the event of harm, injury, or illness arising from this study, neither Arizona State University nor the researchers are able to give you any money, insurance coverage, free medical care, or any compensation for such injury. Major injury is not likely but if necessary, a call to 911 will be placed.

VOLUNTARY CONSENT

Any questions you have concerning the research study or your participation in the study, before or after your consent, will be answered by Dr. Karen Sweazea [480-965-6025] or Kristin Ricklefs [785-317-0263].

If you have questions about your rights as a subject/participant in this research, or if you feel you have been placed at risk, you can contact the Chair of the Human Subjects Institutional Review Board, through the ASU Research Compliance Office, at 480-965 6788.

This form explains the nature, demands, benefits and any risk of the project. By signing this form you agree knowingly to assume any risks involved. Remember, your participation is voluntary. You may choose not to participate or to withdraw your consent and discontinue participation at any time without penalty or loss of benefit. In signing this consent form, you are not waiving any legal claims, rights, or remedies. A copy of this consent form will be given to you.

Your signature below indicates that you consent to participate in the above study.

Subject's Signature	Printed Name	Date

Contact phone number	Email

INVESTIGATOR'S STATEMENT

"I certify that I have explained to the above individual the nature and purpose, the potential benefits, and possible risks associated with participation in this research study, have answered any questions that have been raised, and have witnessed the above signature. These elements of Informed Consent conform to the Assurance given by Arizona State University to the Office for Human Research Protections to protect the rights of human subjects. I have provided the subject/participant a copy of this signed consent document."

Signature of Investigator_____ Date_____

APPENDIX F

MEDICAL HISTORY QUESTIONNAIRE

Medical History Questionnaire

To be completed by investigator

ID#_____

Age:_____

Height:_____ft. _____in.

Waist:_____in.

Gender (please circle): Female Male Weight:_____lbs.

Smoker (please circle): Yes No

1. Are you taking any medications regularly? (Including aspirin, steroids, birth control, etc.) Y N

If yes, what medications and how often?

---

2. Do you currently take supplements? (vitamins, minerals, herbs, etc.)

Y N

If yes, what supplements and how often?

---

3. Do you take insulin or an oral diabetic control medication (e.g., metformin)?

Y N

4. Do you know what your glycosylated hemoglobin A_1C (HbA1c) level is?

Y N

If so, what is it?_____

5. Has a doctor ever told you that you have any of the following conditions?

Heart disease? Y N Thyroid problems?
Y N

Kidney disease? Y N Cancer?
Y N

Liver disease? Y N High blood pressure?
Y N

Food Allergy? Y N (if yes, what type?)_____

Type 2 Diabetes? Y N (if yes, when were you diagnosed?)_____

Other chronic conditions?_____

6. Are you pregnant or planning on becoming pregnant in the next 16 weeks?
Y N

7. Are you currently breast-feeding?
Y N

8. Have you ever fainted at a blood draw?
Y N

Are you willing to participate in a blood draw?
Y N

Have you donated blood in the past 8 weeks?
Y N

9. Will you be willing to consume 4 tablespoons (40 g) of flaxseeds 5-7 times per week for 8 weeks?
Y N

10. Would you be willing to consume 1.5 tablespoons (18 g) of pysllium powder 5-7 per week for 8 weeks?
Y N

11. Do you follow a specific diet? (weight loss/gain, vegetarian, low-fat, etc.)

Y N

12. Are you willing to drive or take the Phoenix light rail (metro system) to the ASU downtown Phoenix campus for a fasting blood draw on 2 separate occasions?

Y N

13. Will you have a problem fasting overnight (10-12 hr) prior to the blood draw?

Y N

14. Will you be able to maintain your typical lifestyle/activities during the trial?

Y N

15. Over a 7 day period, how often do you engage in any regular activity long enough to work up a sweat

(e.g., heart beats rapidly)?_____ How often do you exercise moderately per

week?_____

16. Please circle the total time you spend in each category for an average week.

Light activities such as: slow walking, golf, easy swimming, gardening, etc.

Hours per week: 0 1 2 3 4 5 6 7 8 9 10+

Moderate activities such as: moderate walking, cycling, swimming, weight lifting, etc.

Hours per week: 0 1 2 3 4 5 6 7 8 9 10+

Vigorous activities such as: fast walking, jogging, cycling, heavy/intense weight lifting, etc.

Hours per week: 0 1 2 3 4 5 6 7 8 9 10+

17. Please describe any other medical conditions or situations that may affect you ability to participate in a research trial (i.e., pregnancy, infections, travel, deadlines, etc.).

APPENDIX G

INSTRUCTIONS FOR TEST FOOD STORAGE AND CONSUMPTION

Consuming plant-based fibers

1. Store the fiber supplement in the freezer, refrigerator, or other cool dry area such as the pantry. Try to avoid exposure to direct sunlight.
2. Consume 1 package of the fiber supplement 5-7 days per week for the eight weeks.
3. Fiber can be consumed at one time or split up throughout the day (e.g., 1 T in breakfast oatmeal, 2 tsp in lunch salad dressing, 1 T in dinner chili, and 2 tsp sprinkled over ice cream). You can divide up the fiber any way you choose as long as you consume the entire package during the course of the day.

Adding ground fibers to your daily diet

1. Add ground flaxseed or psyllium powder to yogurt, applesauce, soups, or smoothies. 1-2 tablespoons does not alter the flavor very much of the yogurt or smoothie. This will also add fiber to your yogurt or smoothie.
2. Bake with flaxseed or psyllium powder. Both fibers go well in baked goods at small or larger amounts. It imparts a toasted nut flavor that matches well in sweet or savory baked goods. Some popular baked uses of flaxseed are in breads or muffins. It has a good heat stability so all the nutrients are available after the baking process and is a good way to get extra fiber in a baked item without affecting texture and taste dramatically.
3. Add one or two spoonfuls into chili, spaghetti sauce, stew, or gravy.
4. Add to commonly used condiments. Each blends well in mayonnaise, mustard and ketchup. It also goes great in salad dressings and as a salad topping. You will not need to add very much (1 tablespoon or less will suffice).
5. Mix into oatmeal or cream of wheat cereal.
6. Sprinkle on top of toast with peanut butter and bananas or jelly.
7. Sprinkled on top of salads, mixed in to mashed potatoes, etc.
8. Add to drinks. Using smaller amounts throughout the day in whatever you are drinking will give just as much, without the thickness from one larger dose.
9. Stir a little into juice and drink up.
10. Sprinkled on top of fruit, pudding, or ice cream for dessert.

APPENDIX H

3-DAY FOOD RECORDS

DIETARY INTAKE RECORD

ASU Flaxseed Study

Day of Week: Su M T W Th F Sat (circle one)

TIME OF MEAL	FOOD ITEM	DESCRIPTION (how was it prepared or where was it purchased?)	AMOUNT (cups, oz, tsp, etc.)
TIME OF MEAL	FOOD ITEM	DESCRIPTION (how was it prepared or where was it	AMOUNT (cups, oz, tsp,

		purchased?)	**etc.)**

***Supplements Taken** – List brand, number of tablets/amount:

APPENDIX I

COMPLIANCE CALENDAR

September

2014

Sun	Mon	Tue	Wen	Thu	Fri	Sat
1	2	3	4	5	1	2
3	4	5	6	7	8	9
10	11	12	13	14	15	16
17	18	19	20	21	22	23
24	25	26	27	28	29	30
31						

INSTRUCTIONS

1. Check off each day indicating that the study food was consumed as instructed.
2. Arrive at the Nutrition labs at scheduled times for testing.
3. 12 hours prior to your blood draws:
    - Do not exercise strenuously although you may walk the dog and carry out routine activities.
    - Beginning at about 10:00 pm, do not eat or drink anything other than water.
4. At study weeks 0 and 8, bring your diet records with you to the lab.

APPENDIX J

MODIFIED TBARS PROTOCOL

TBARS Assay (Zeptometrix, Buffalo, NY) can be used with either plasma or serum (both heparin and EDTA)
Plasma: Collect fasting heparinized whole blood. Centrifuge at 3500 rpm for 10 min at 5-10°C, carefully remove plasma and place on ice for immediate analysis or freeze several aliquots at -70°C for later analysis. Samples can be safely stored for 1-2 months. Process as described below for serum.
Serum: Collect fasting whole blood in a red top vacutainer®. Incubate at room temperature for at least 30 min for clots to form. Centrifuge at 3500 rpm for 10 min. Carefully remove serum and place on ice for immediate analysis or freeze aliquots at -70°C for later analysis.
Protocol: All solutions must be at room temperature before performing the assay. If using stored samples, thaw on ice. Label tubes for standards (0-4) as well as samples. Poke holes in the top to avoid excessive pressure build-up during heat block phase.

Using the Zeptometrix TBARS assay kit prepare the following:

Set the heat block to 95^{o}C

To make TBA buffer:

- To 10 mL Diluent 1 add 106 mg TBA powder (cover with parafilm)
- Mix on a hot plate at a low temperature (45-55^{o}C)
- Turn off the plate (allow to cool), add 10 mL of Diluent 2, mix for 10 min on stir plate with heat off.

Prepare Standards:

- Prepare standards according to Table 1 (make sure to add MDA standard to Diluent).

Standard	MDA standard (uL)	MDA Diluent (uL)	Final Conc
0	0	100	0
1	12.5	87.5	12.5
2	25	75	25
3	20	50	50
4	100	0	100

- Once samples are prepared place on ice, they can now be treated the same as the samples.

Prepare Samples:

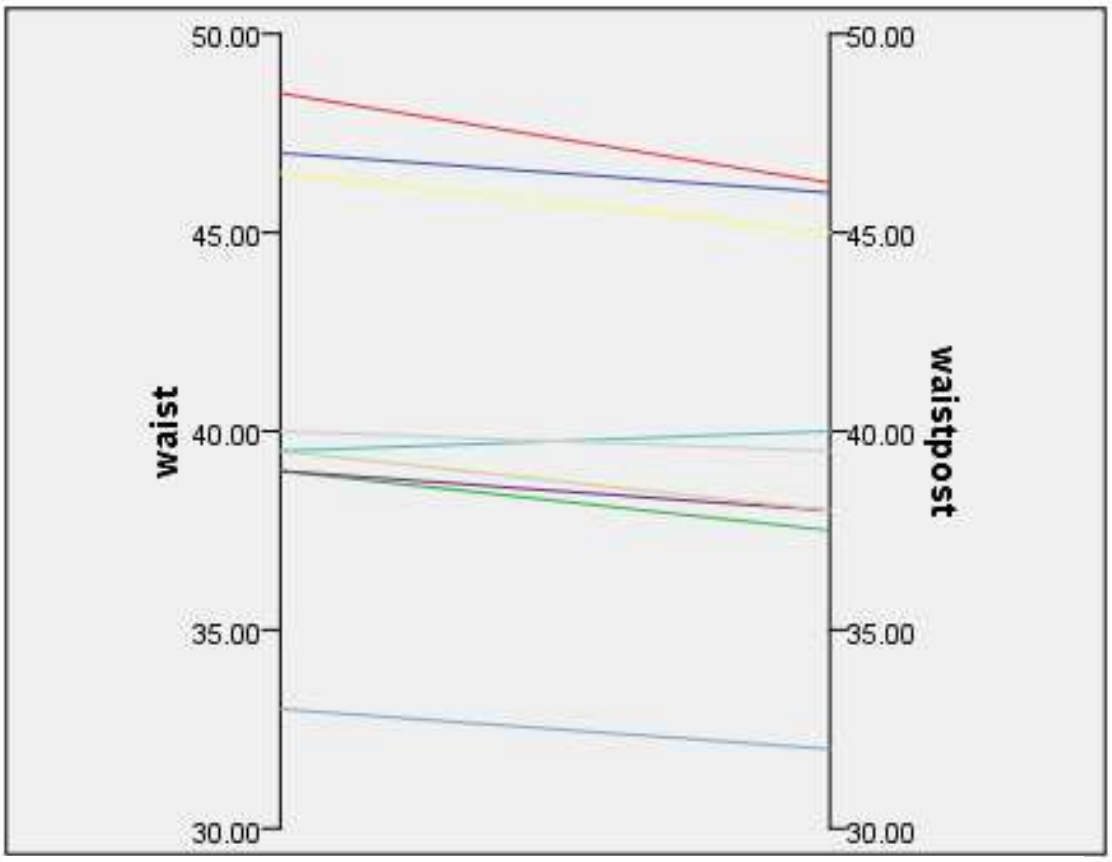
1. In labeled eppendorf tubes with hole (use 18-20G needle) prepared sample
2. Mix sample for duplicate runs
3. Add 30 uL of sample
4. Add 30 uL of SDS buffer
5. Add 750 uL of TBA buffer
6. Vortex tubes
7. Place on heat block @ 95^{o}C for 60 minutes
8. Place on ice for 10 minutes
9. Centrifuge samples (NOT STANDARDS) @ 3000 rpm for 15 minutes @ RT

Load 96 well plate:

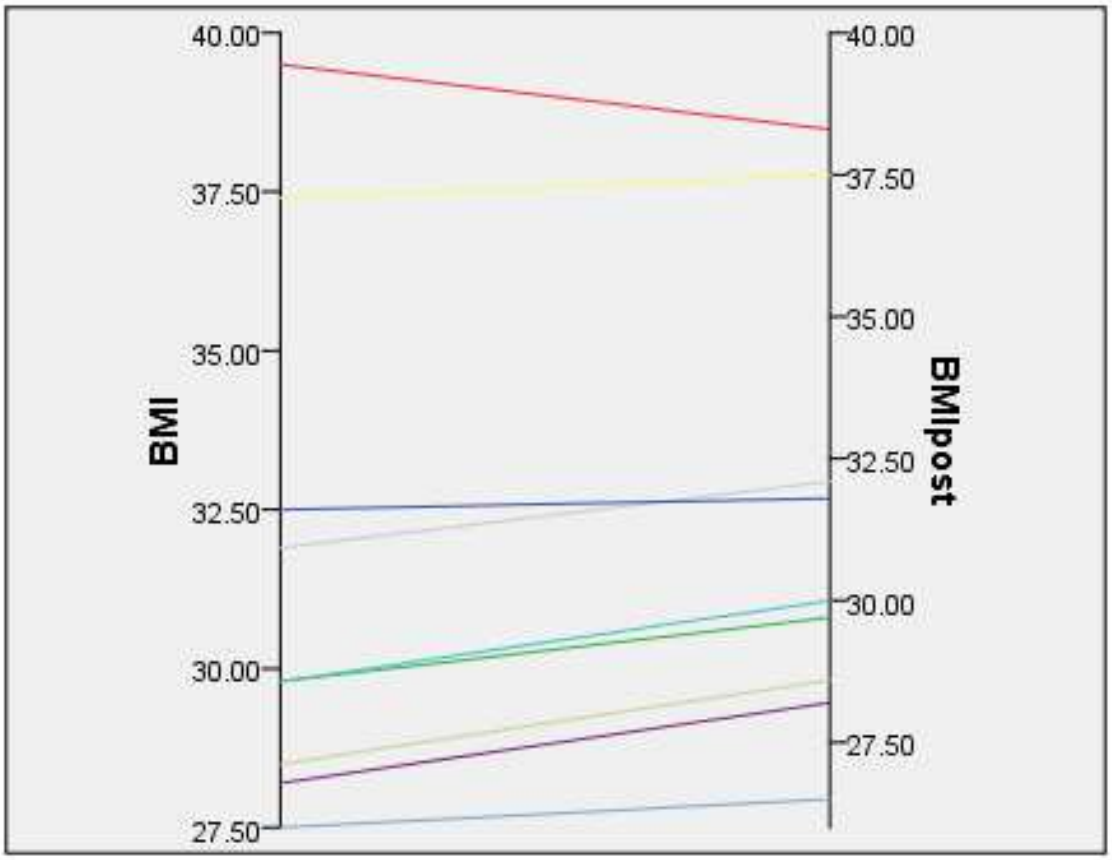
a. Add 200 uL of supernatant to plate well in duplicates (making sure not to disturb the pellet formed on the bottom of the tube)
b. Absorbance reading: read supernatants on plate reader @ 532 nm

APPENDIX K

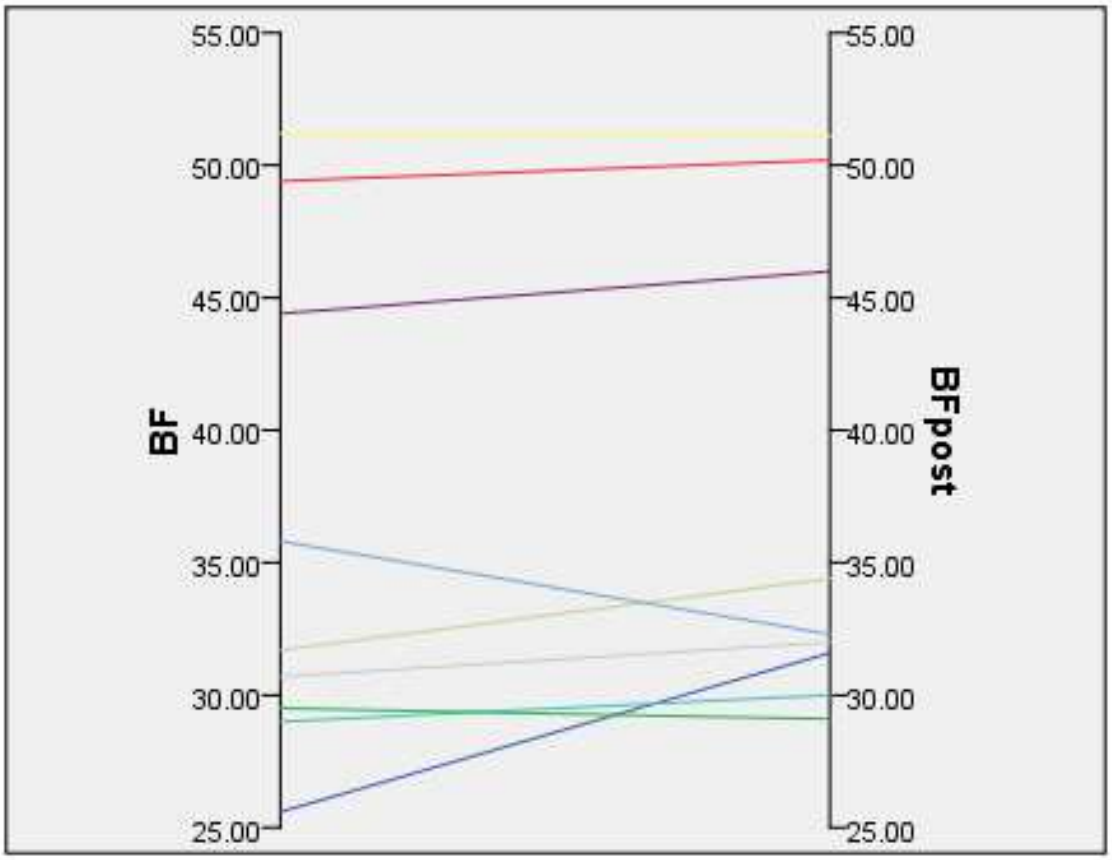
INDIVIDUAL VARIATIONS IN MEASURES OF BODY COMPOSITION, BLOOD PRESSURE, AND NUTRITENT INTAKE FOR FLAXSEED GROUP



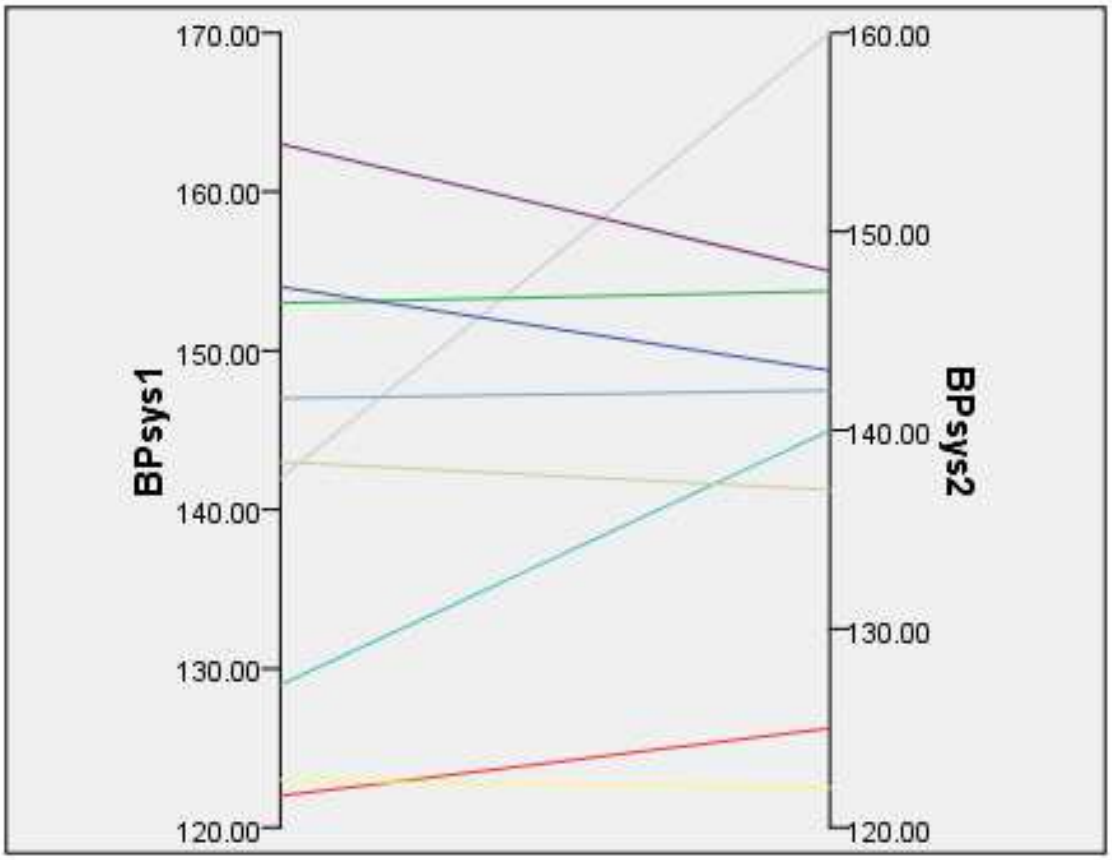
A. Individual changes in waist circumference (in) from baseline to week 8.



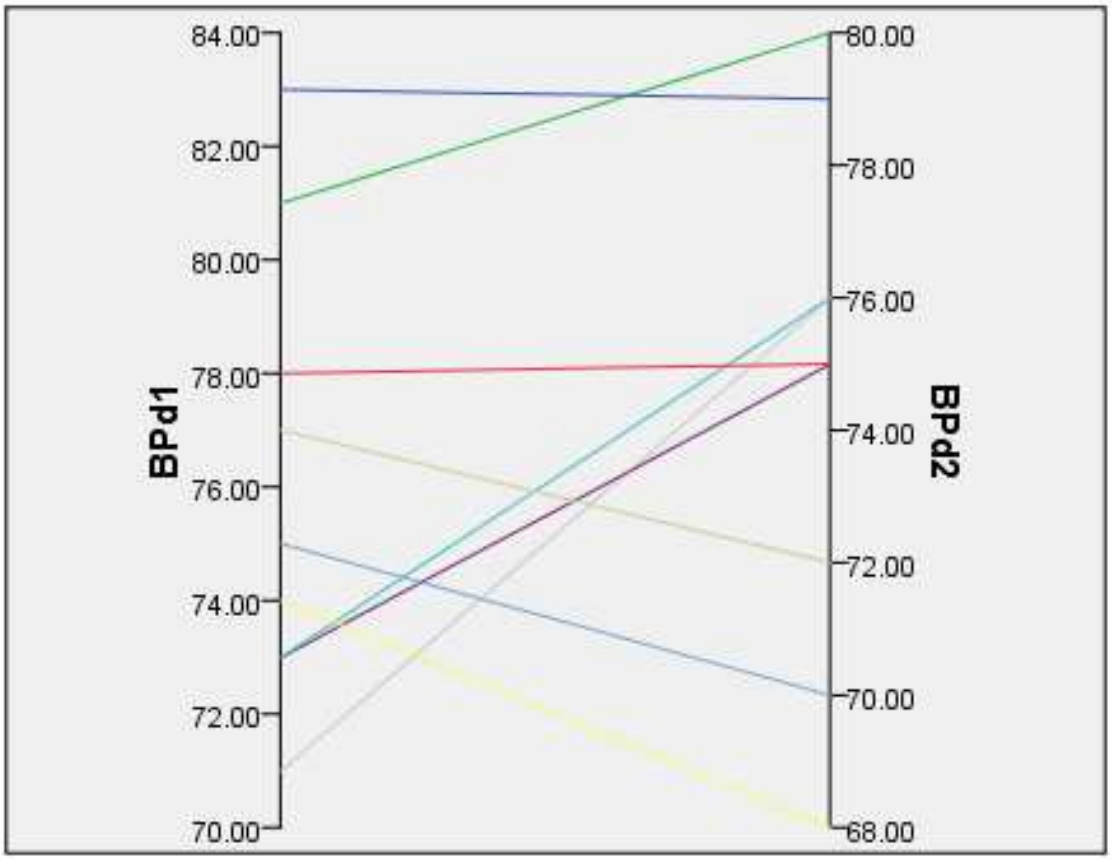
B. Individual changes in BMI (kg/m^2) from baseline to week 8.



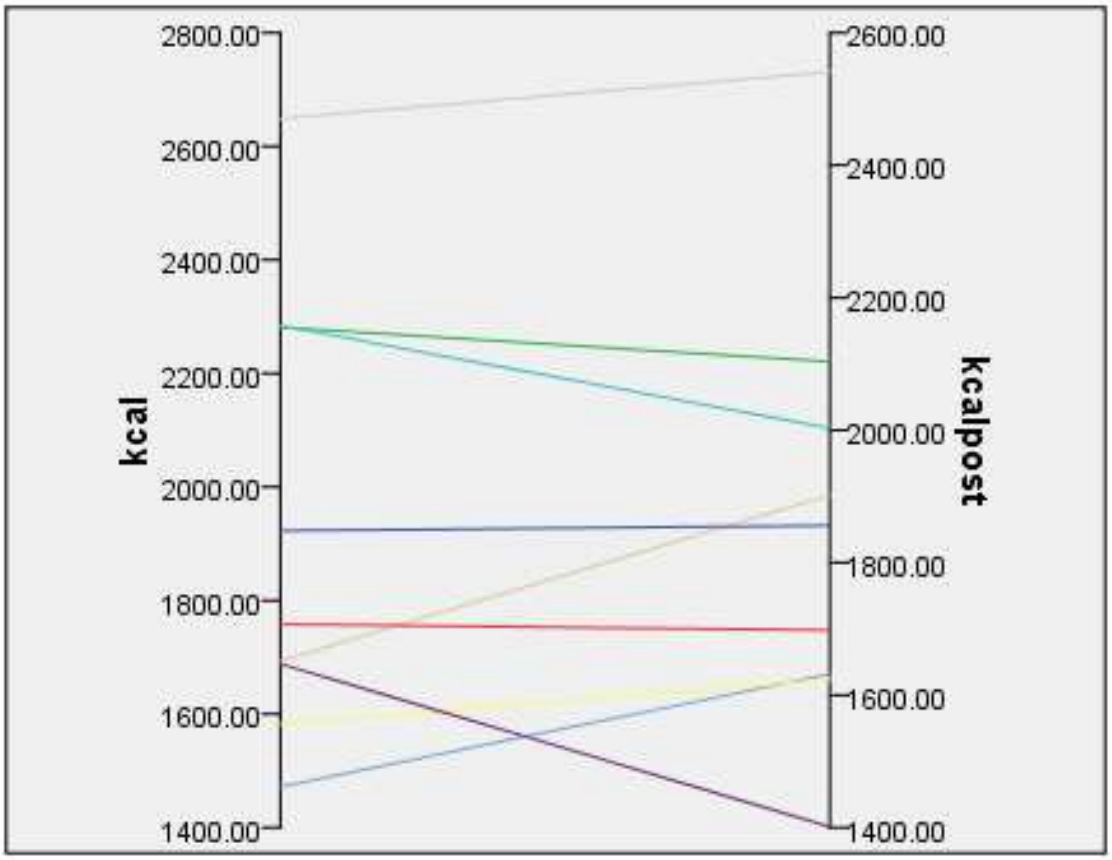
B. Individual changes in body fatness (%) from baseline to week 8.



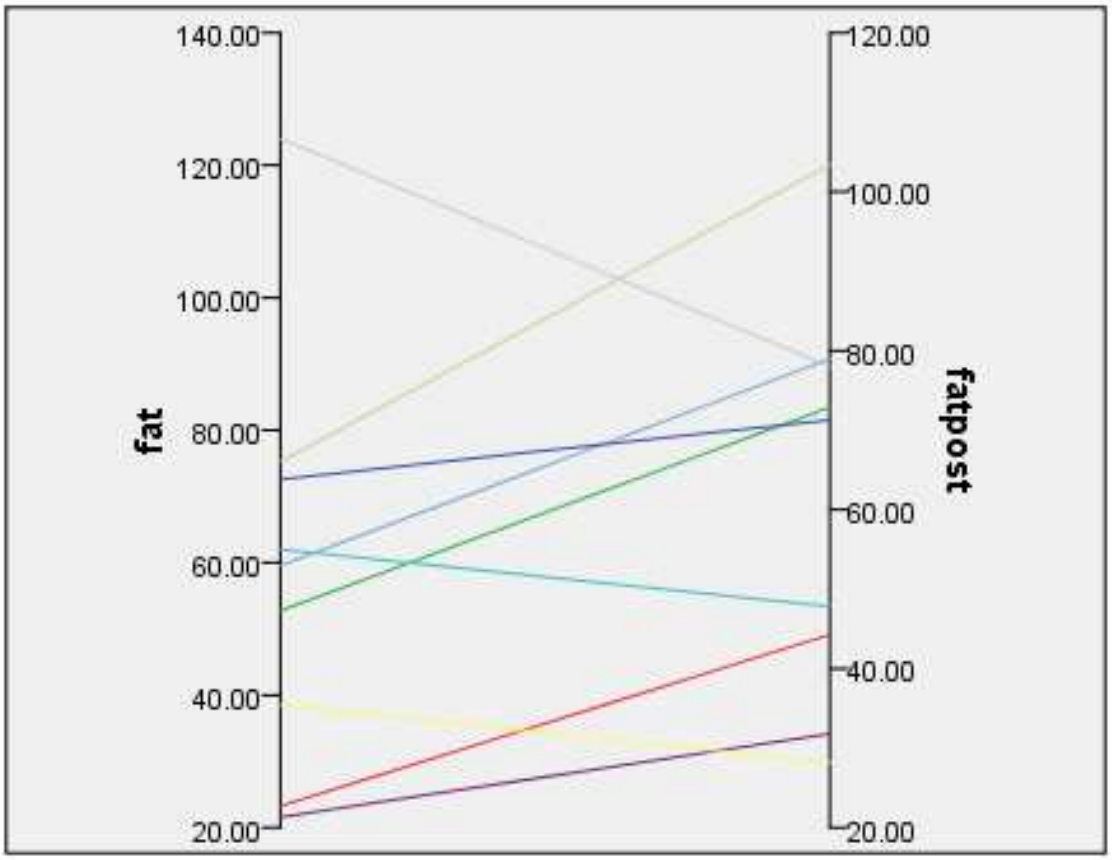
D. Individual changes in systolic blood pressure (mm Hg) from baseline to week 8.



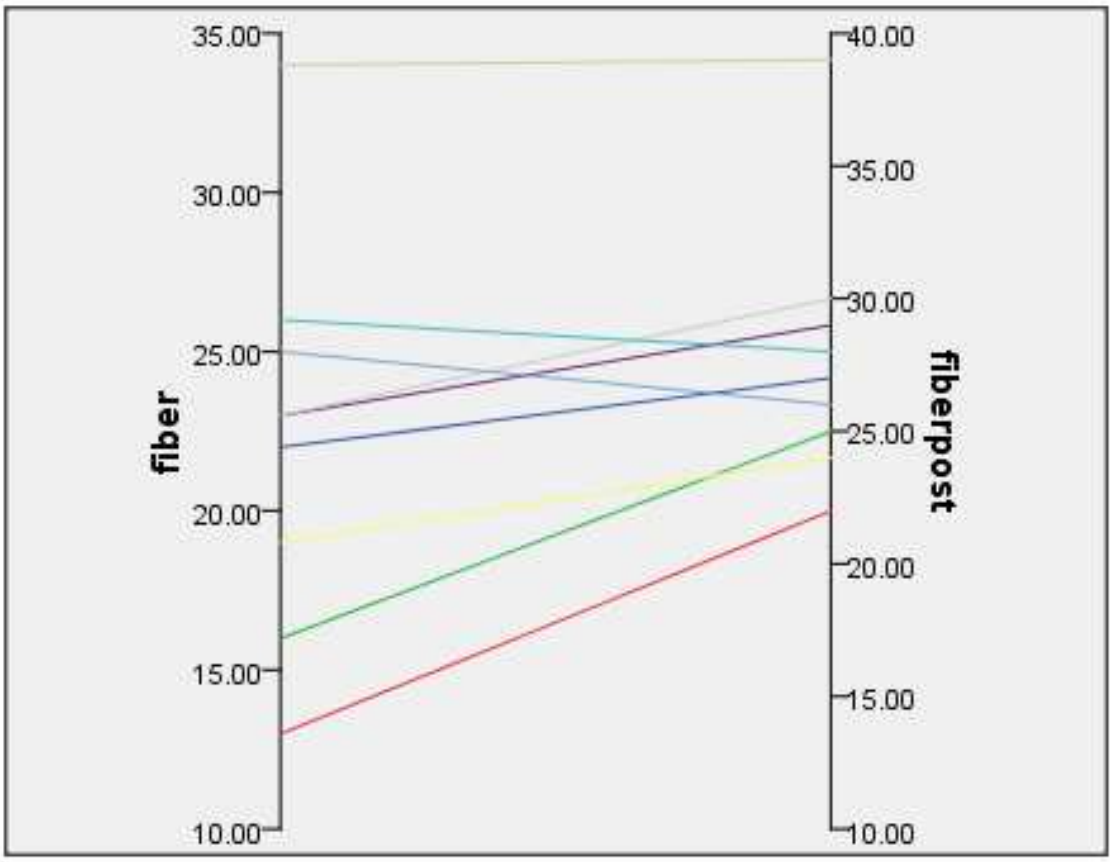
E. Individual changes in diastolic blood pressure (mm Hg) from baseline to week 8.



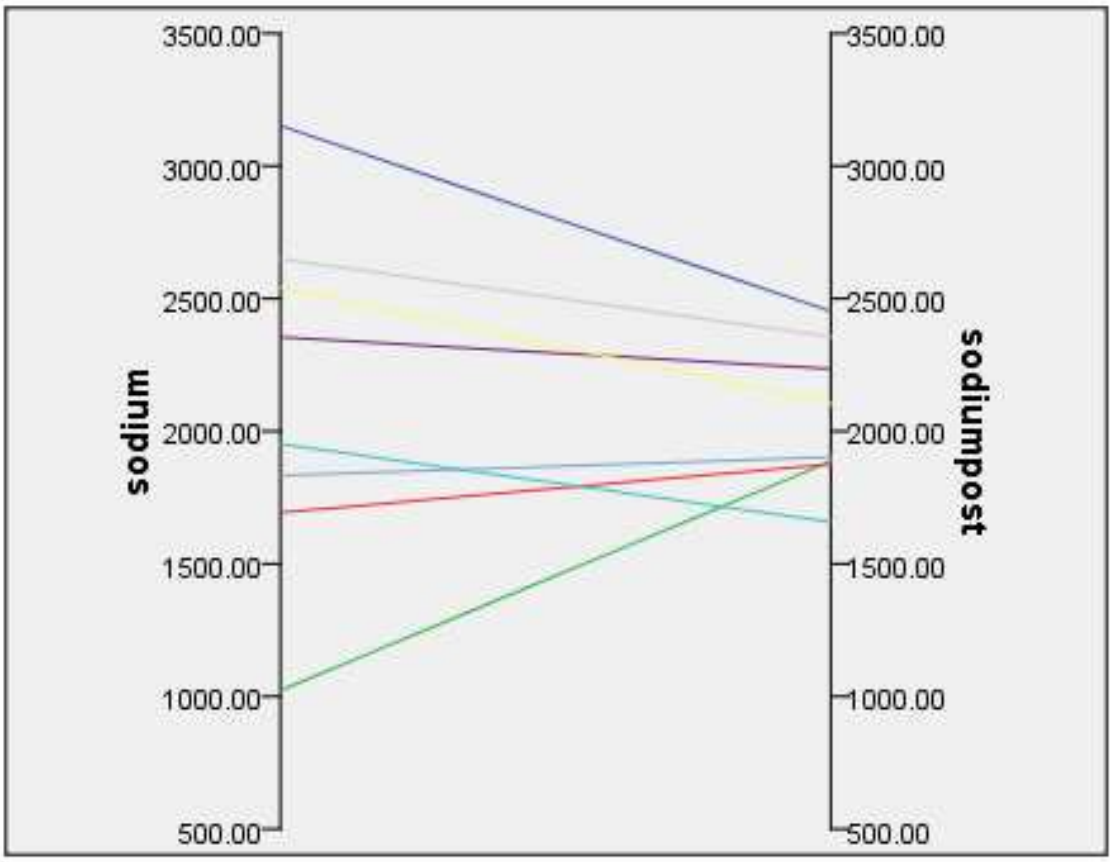
F. Individual changes in total energy (kcal) intake from baseline to week 8.



G. Individual changes in total fat (g) intake from baseline to week 8.



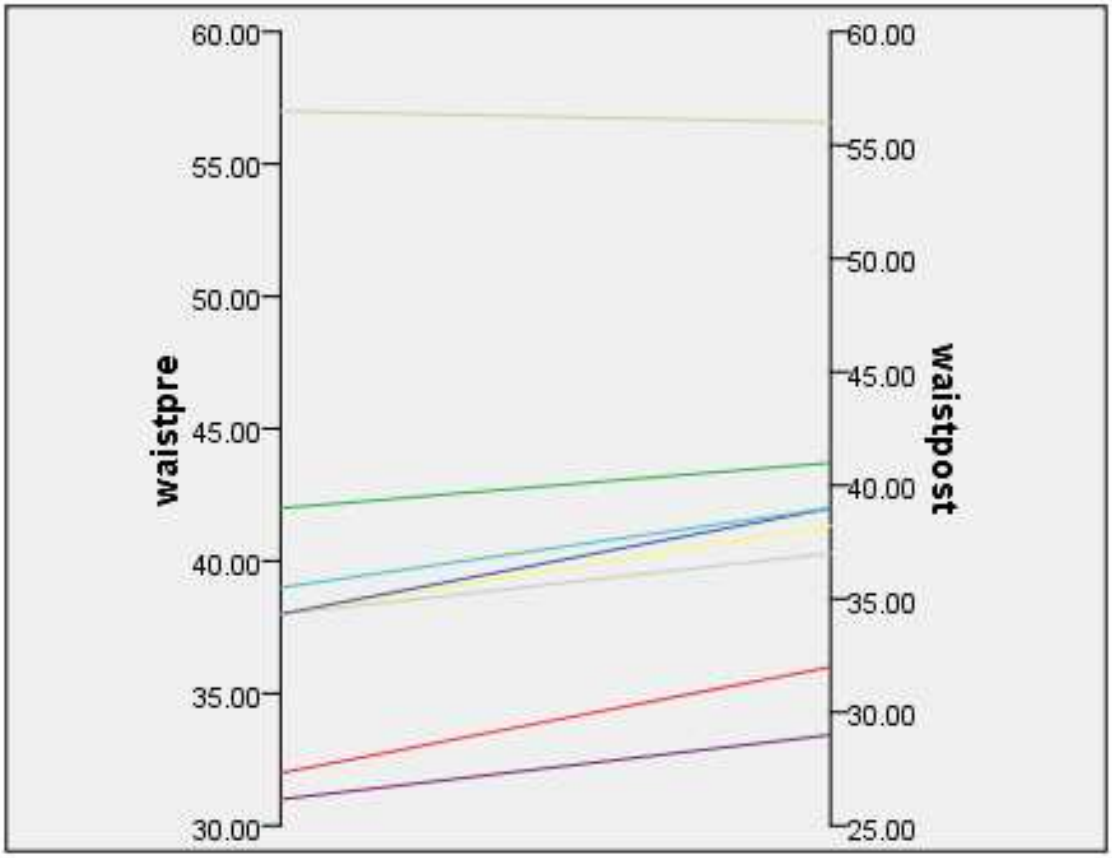
H. Invidual changes in total fiber (g) intake from baseline to week 8.



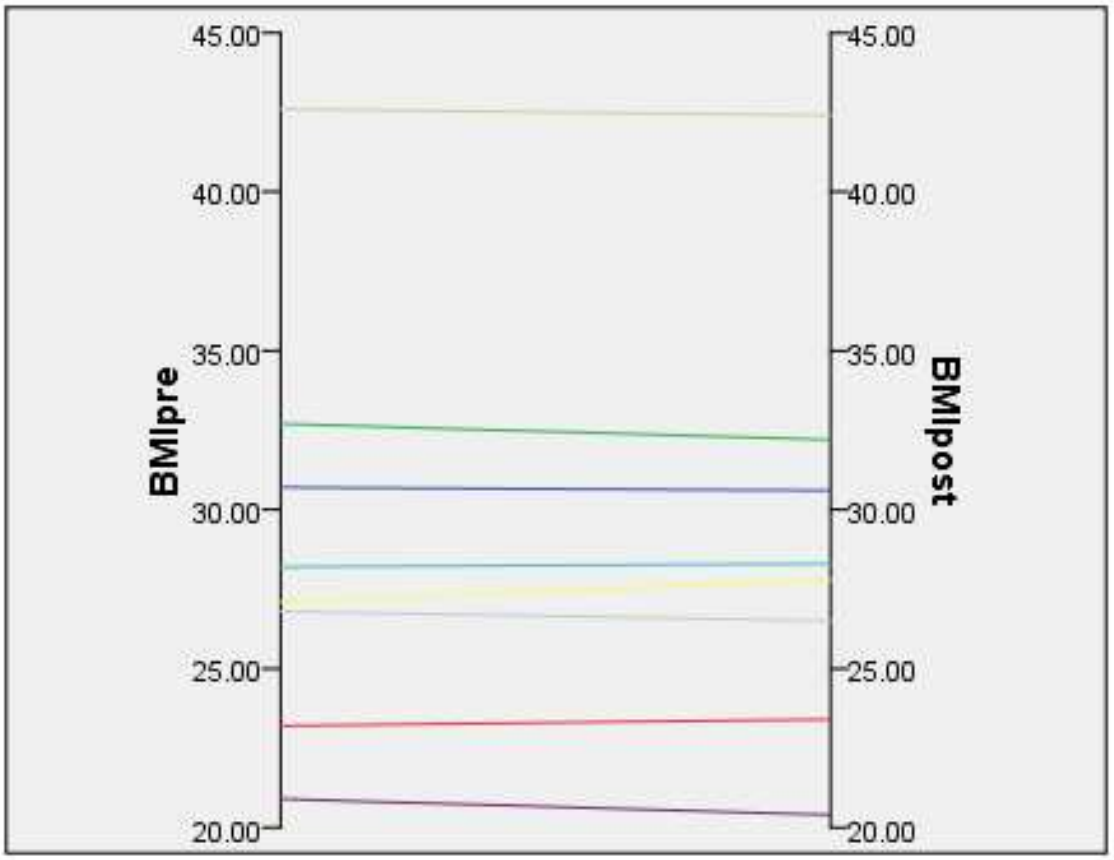
I. Individual changes in sodium (mg) intake from baseline to week 8.

APPENDIX L

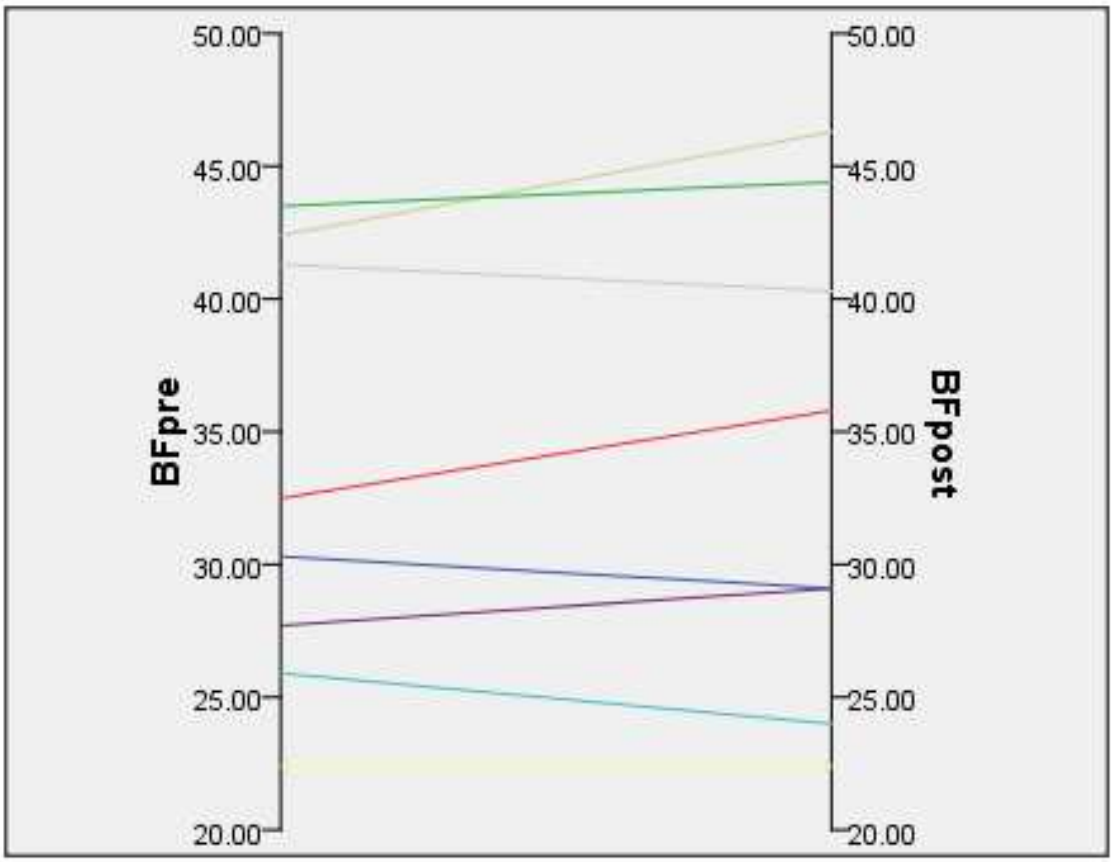
INDIVIDUAL CHANGES FOR MEASUREMENTS OF BODY COMPOSITION, BLOOD PRESSURE, AND NUTRIENT INTAKE FOR CONTROL GROUP



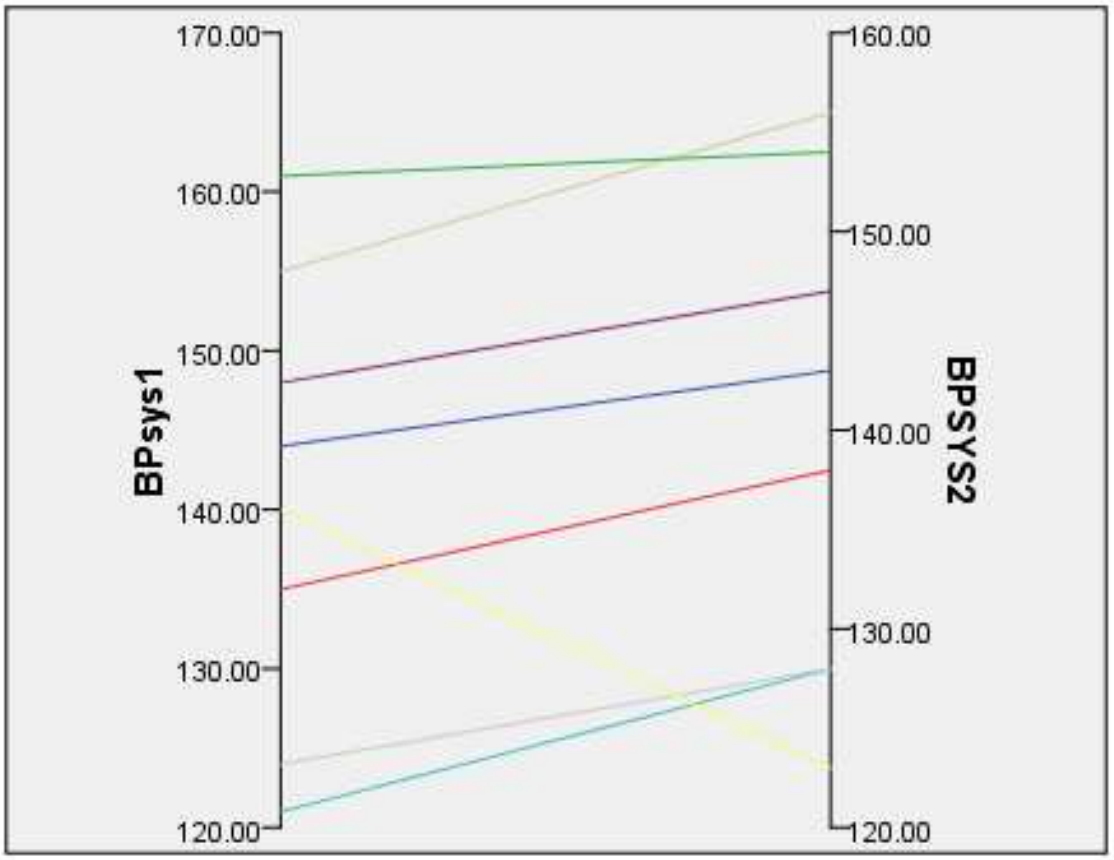
A. Individual changes in waist circumference (in) from baseline to week 8.



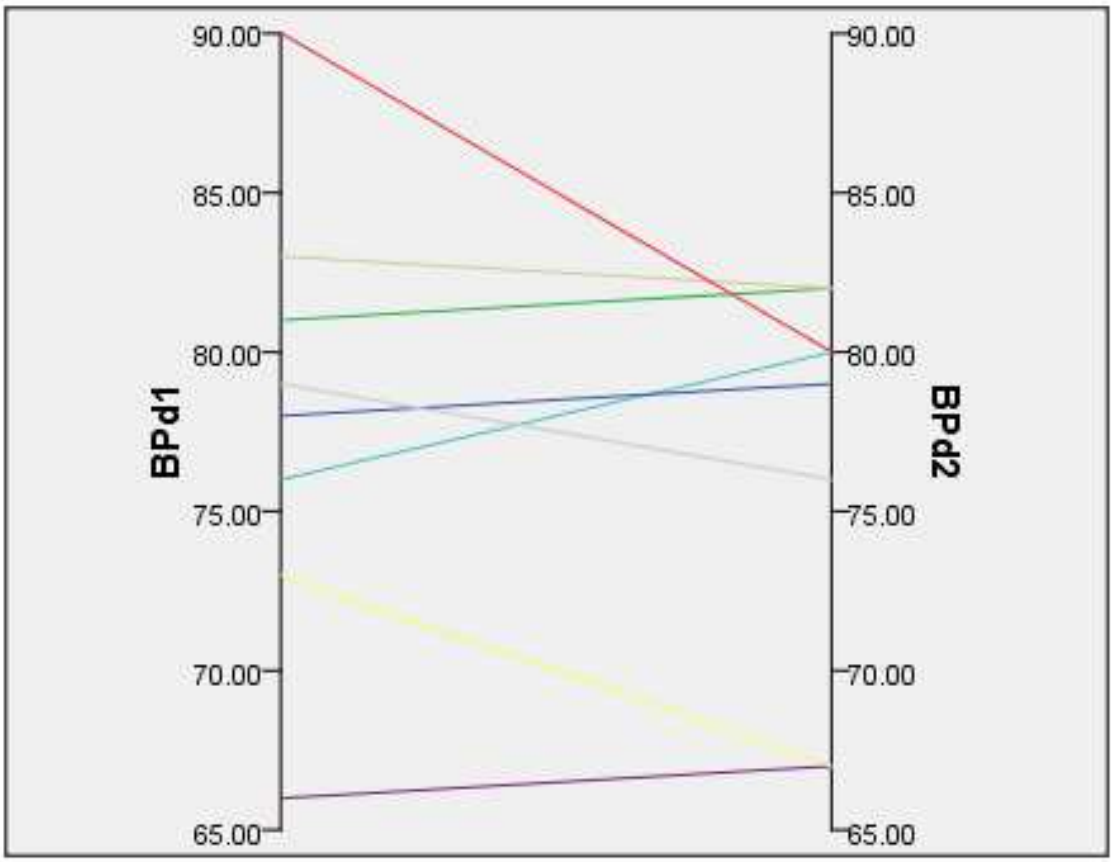
B. Individual changes in BMI (kg/m^2) from baseline to week 8.



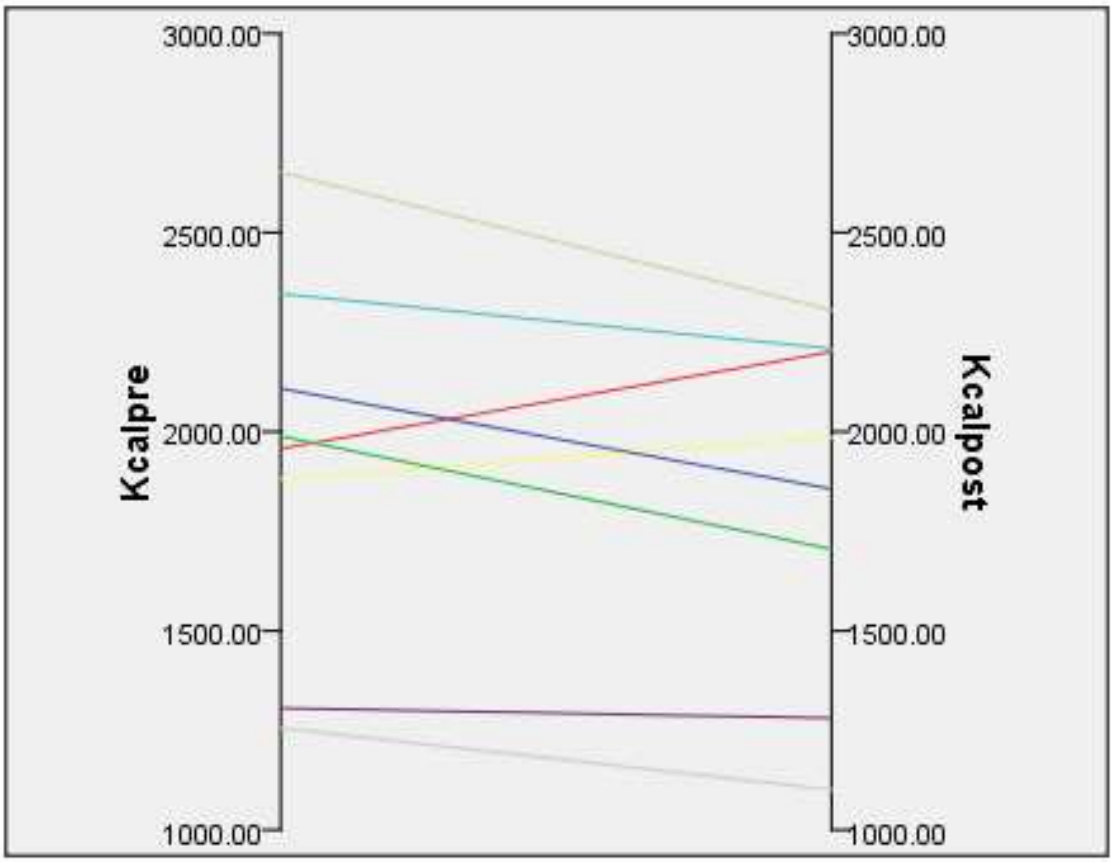
C. Individual changes in body fatness (%) from baseline to week 8.



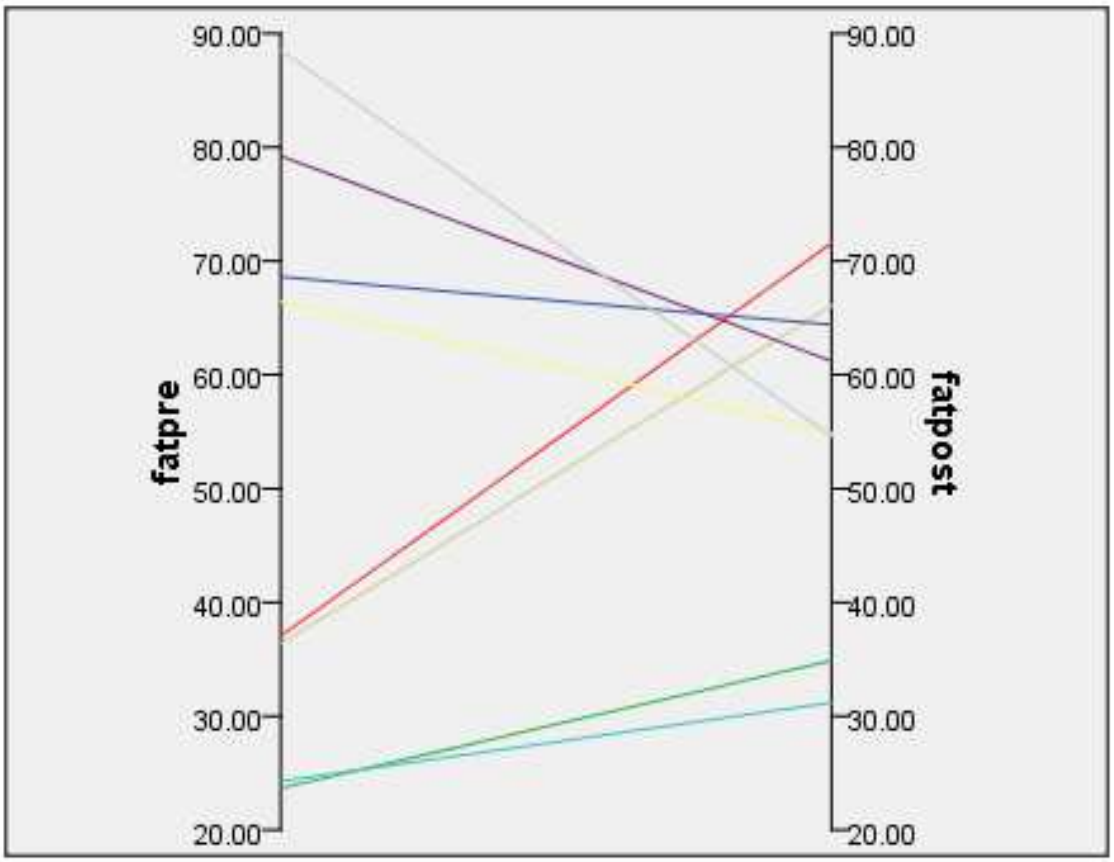
D. Individual changes in systolic blood pressure (mm Hg) from baseline to week 8.



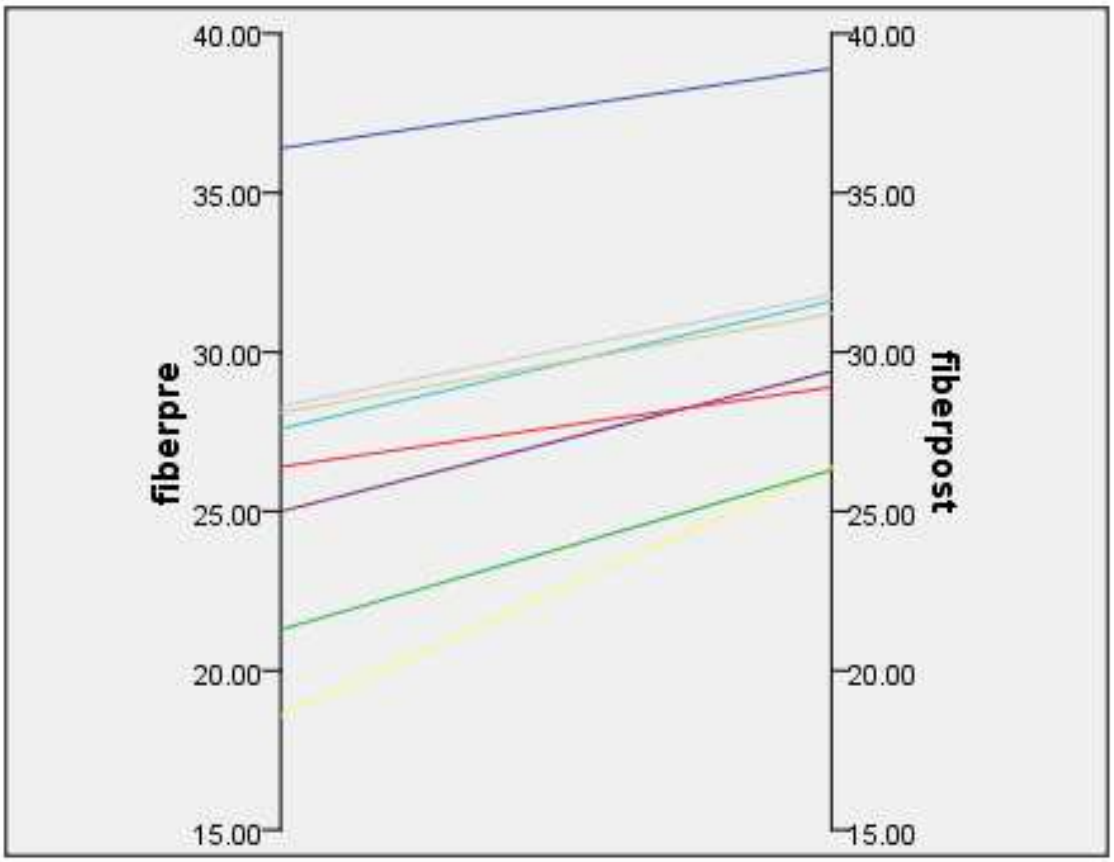
E. Individual changes in diastolic blood pressure (mm Hg) from baseline to week 8.



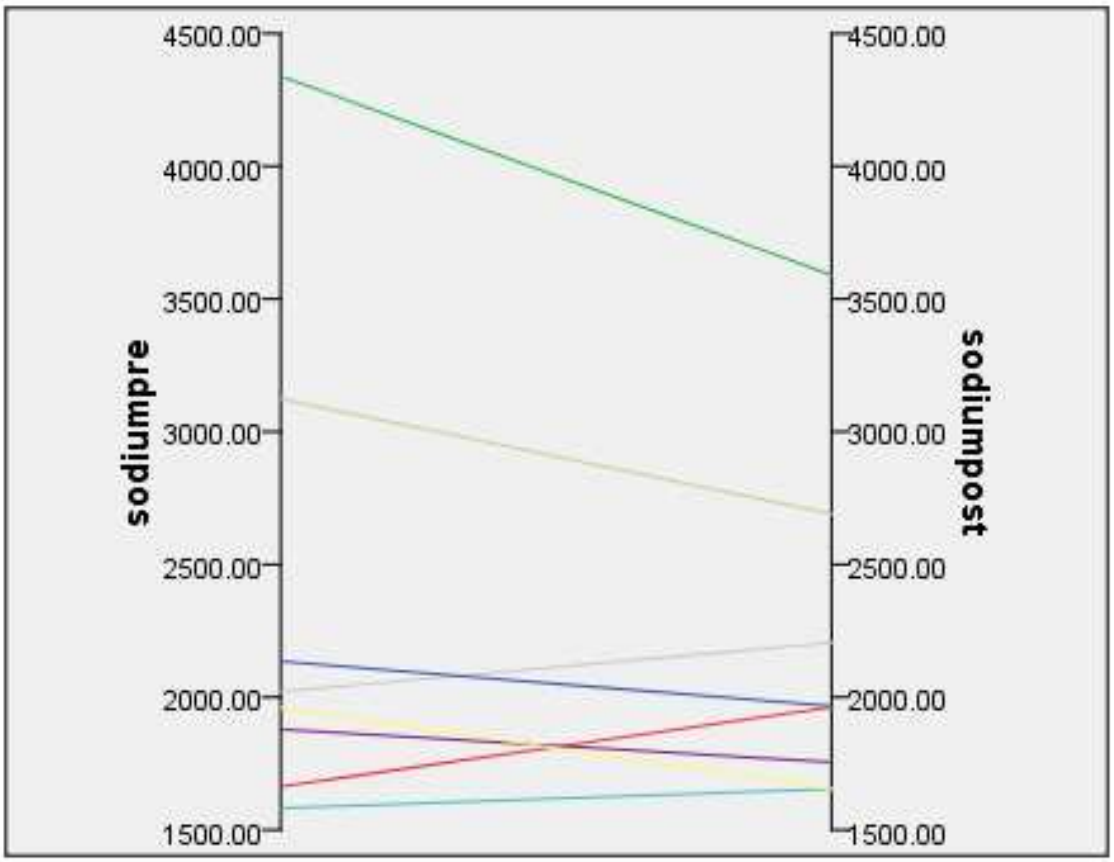
F. Individual changes in total energy (kcal) intake from baseline to week 8.



F. Individual changes in total fat intake (g) from baseline to week 8.



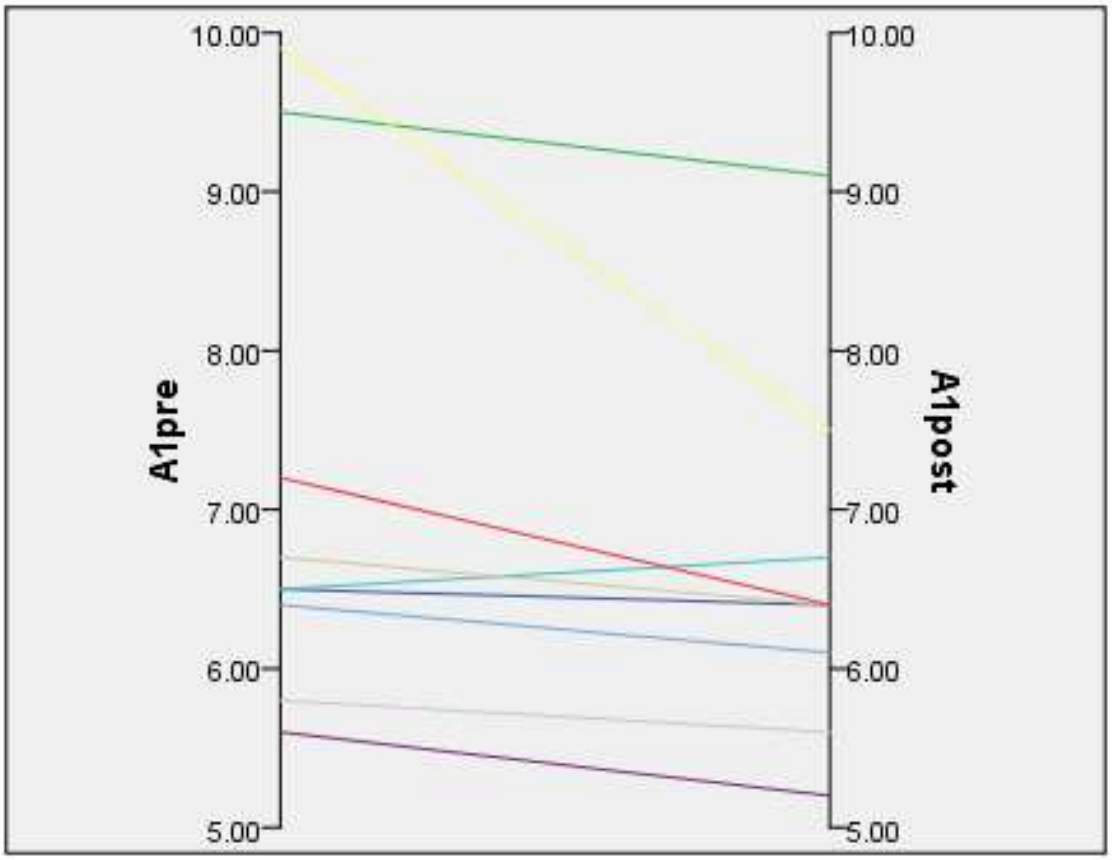
G. Individual changes in total fiber (g) intake from baseline to week 8.



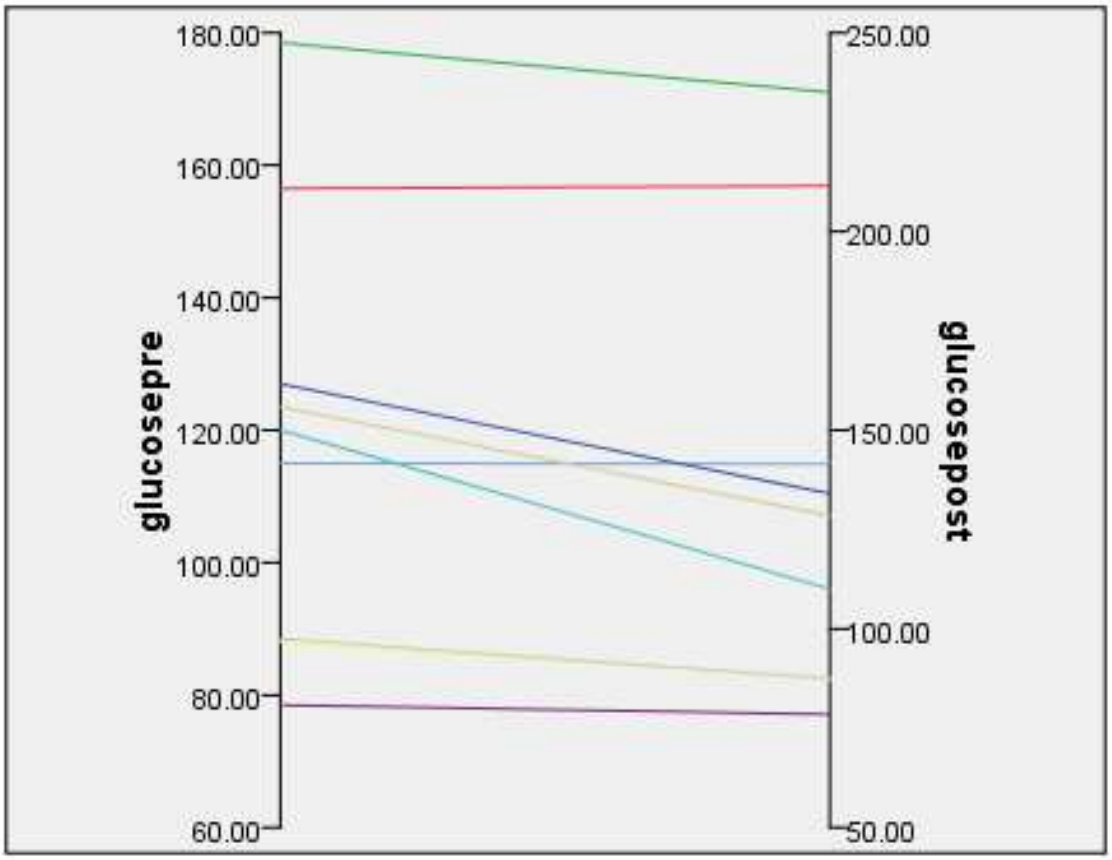
H. Individual changes in sodium (mg) intake from baseline to week 8.

APPENDIX M

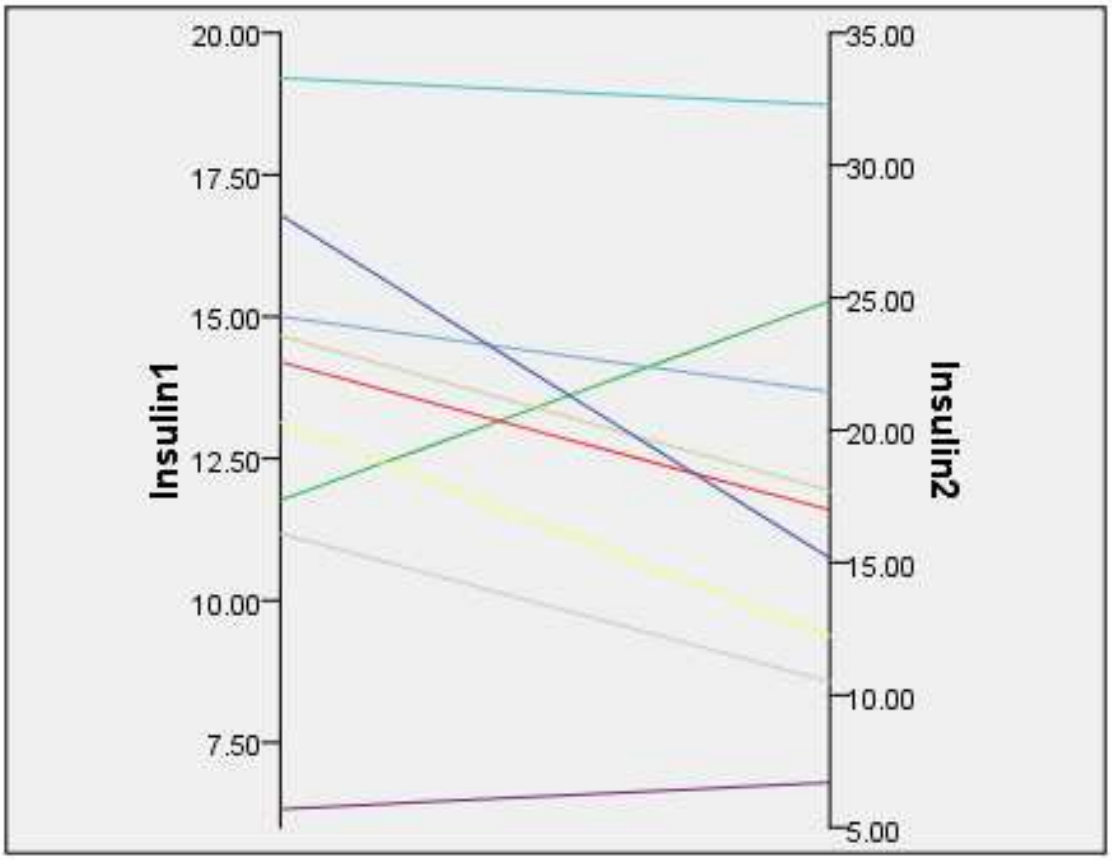
INDIVIDUAL CHANGES IN MEASUREMENTS OF GLUCOSE REGULATION FOR FLAXSEED GROUP



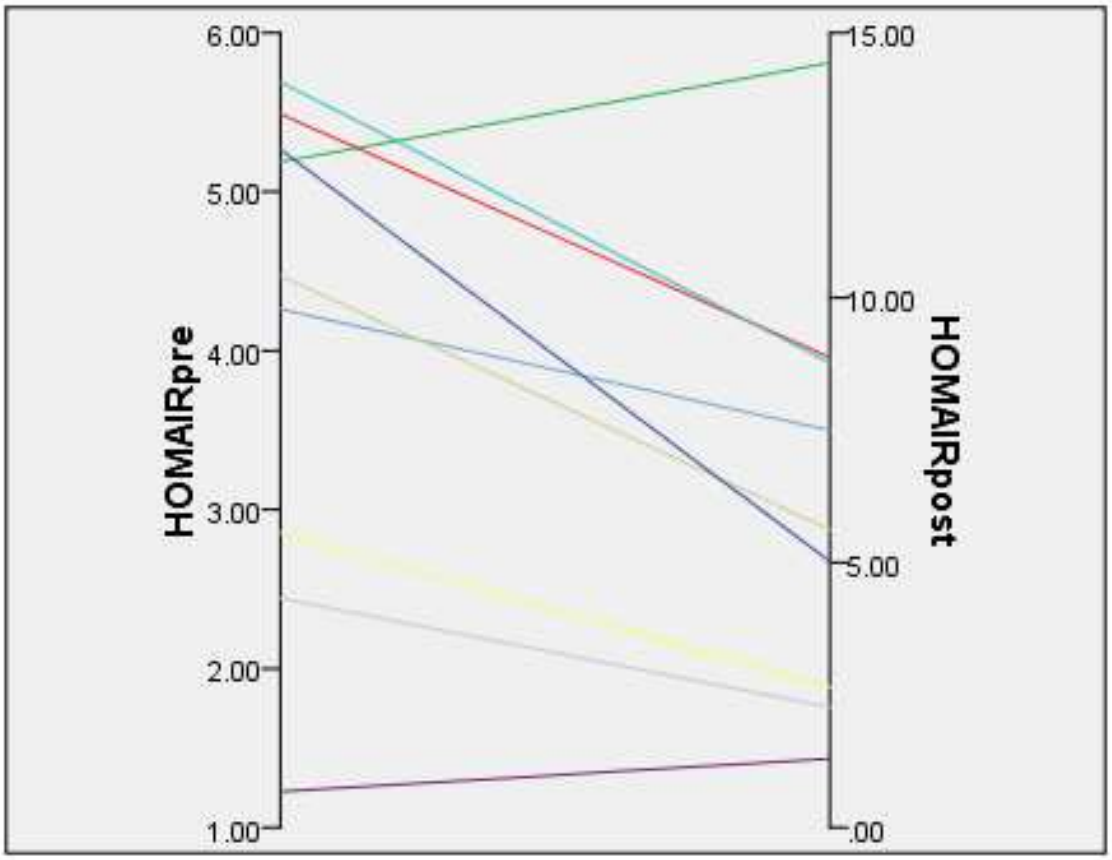
A. Individual changes in HbA1c (%) from baseline to week 8.



B. Individual changes in fasting plasma glucose (mg/dL) from baseline to week 8.



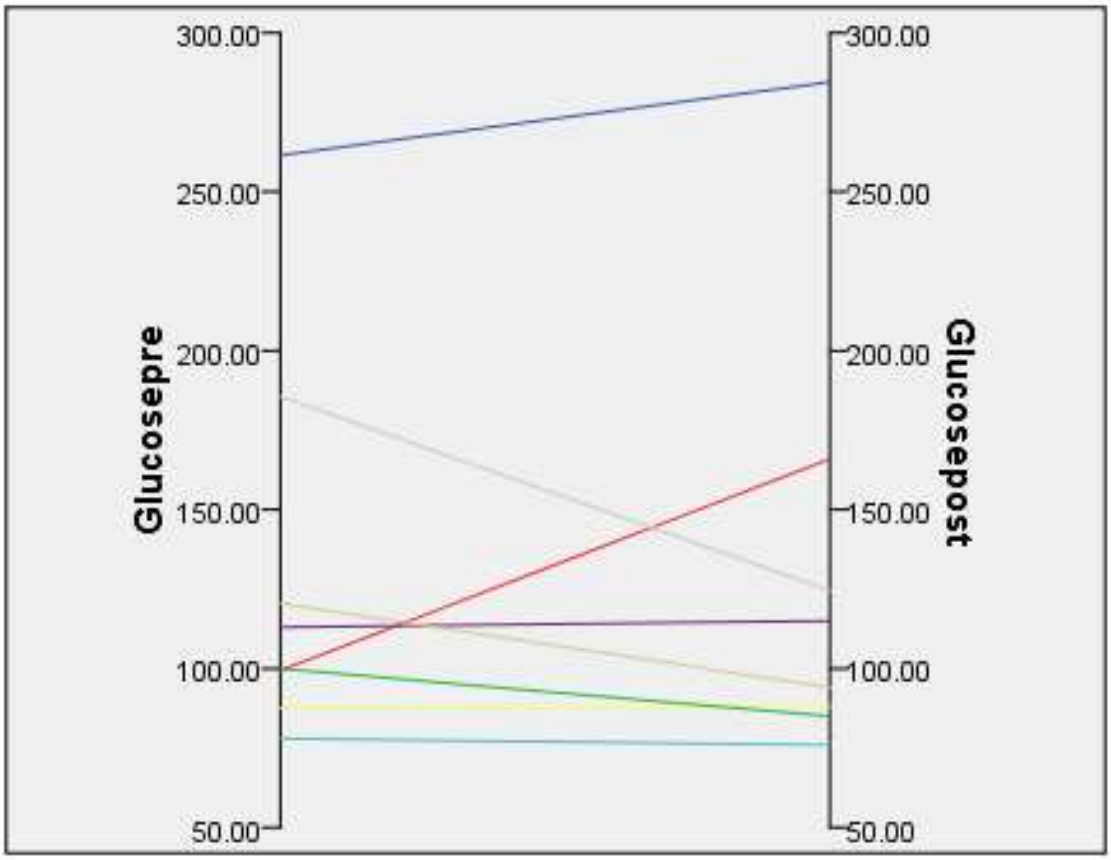
C. Individual changes in fasting insulin (mg/dL) from baseline to week 8.



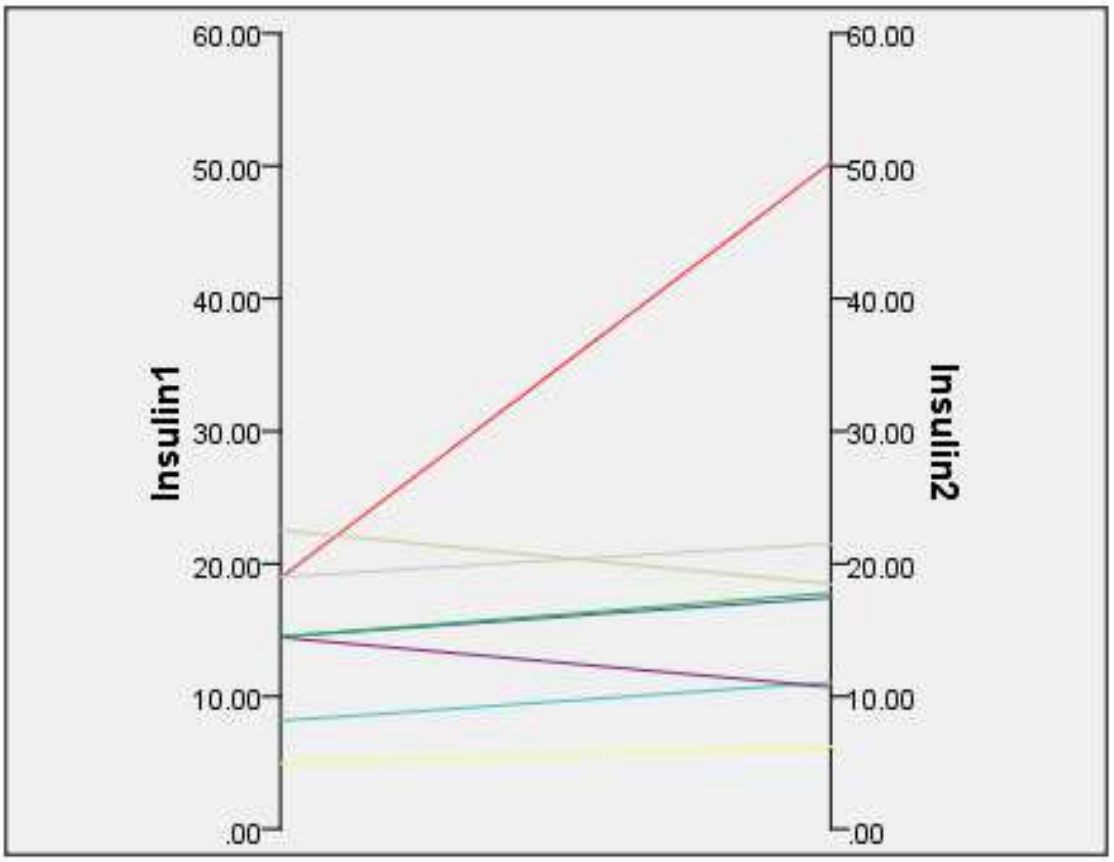
D. Individual changes in HOMA-IR from baseline to week 8.

APPENDIX N

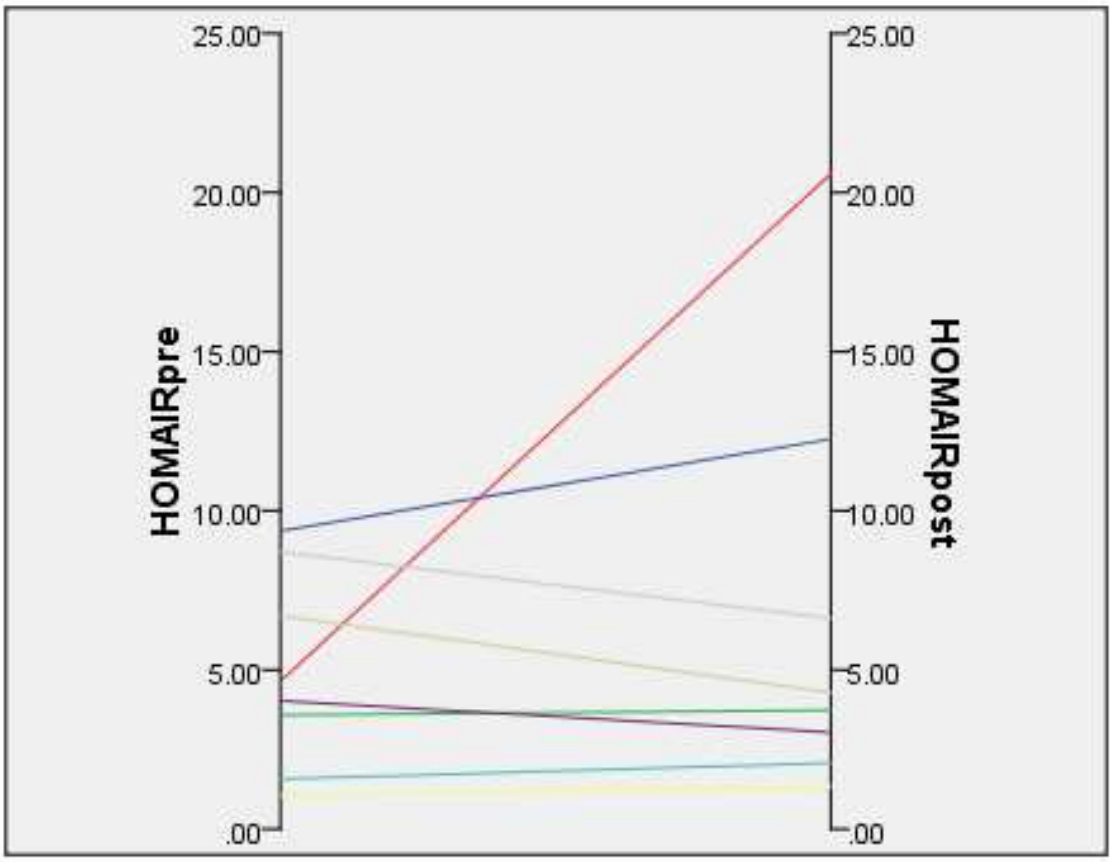
INDIVIDUAL CHANGES IN MARKERS OF GLUCOSE REGULATION FOR CONTROL GROUP



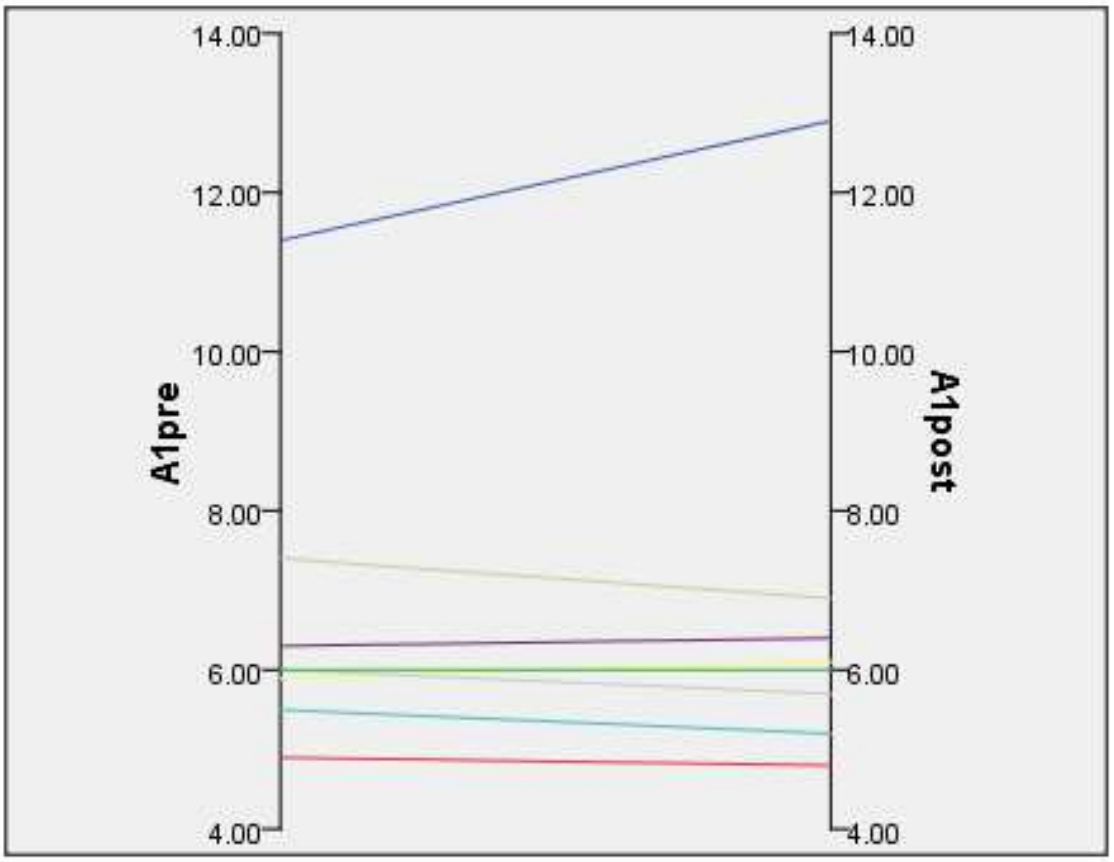
A. Individual changes in fasting plasma glucose (mg/dL) from baseline to week 8.



B. Individual changes in fasting insulin (mg/dL) from baseline to week 8.



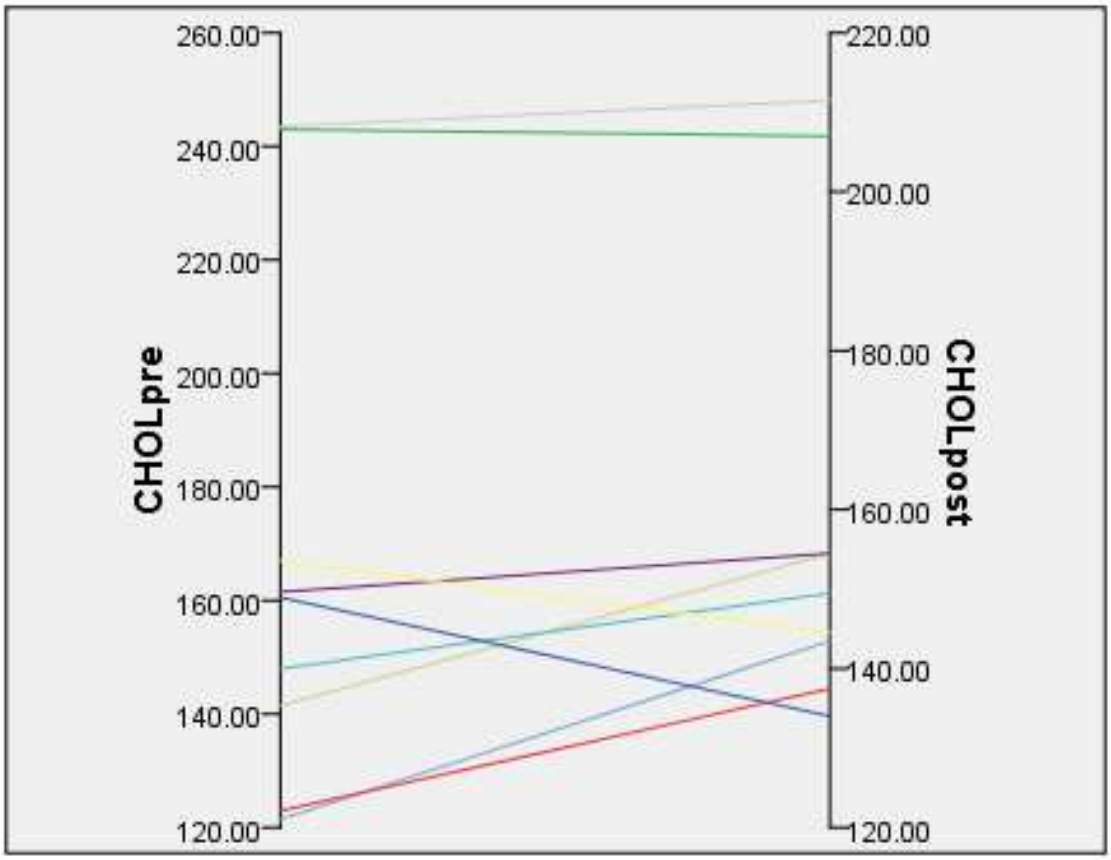
C. Individual changes in HOMA-IR from baseline to week 8.



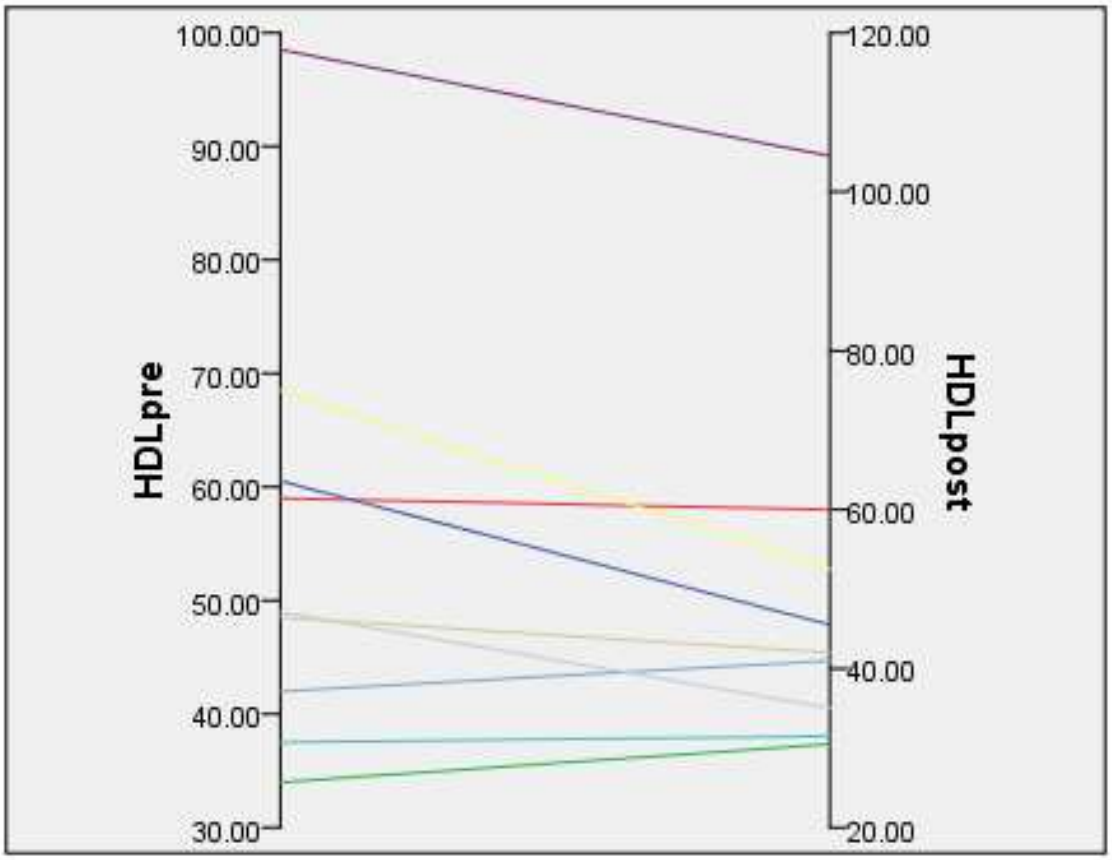
D. Individual changes in HbA1c (%) from baseline to week 8.

APPENDIX O

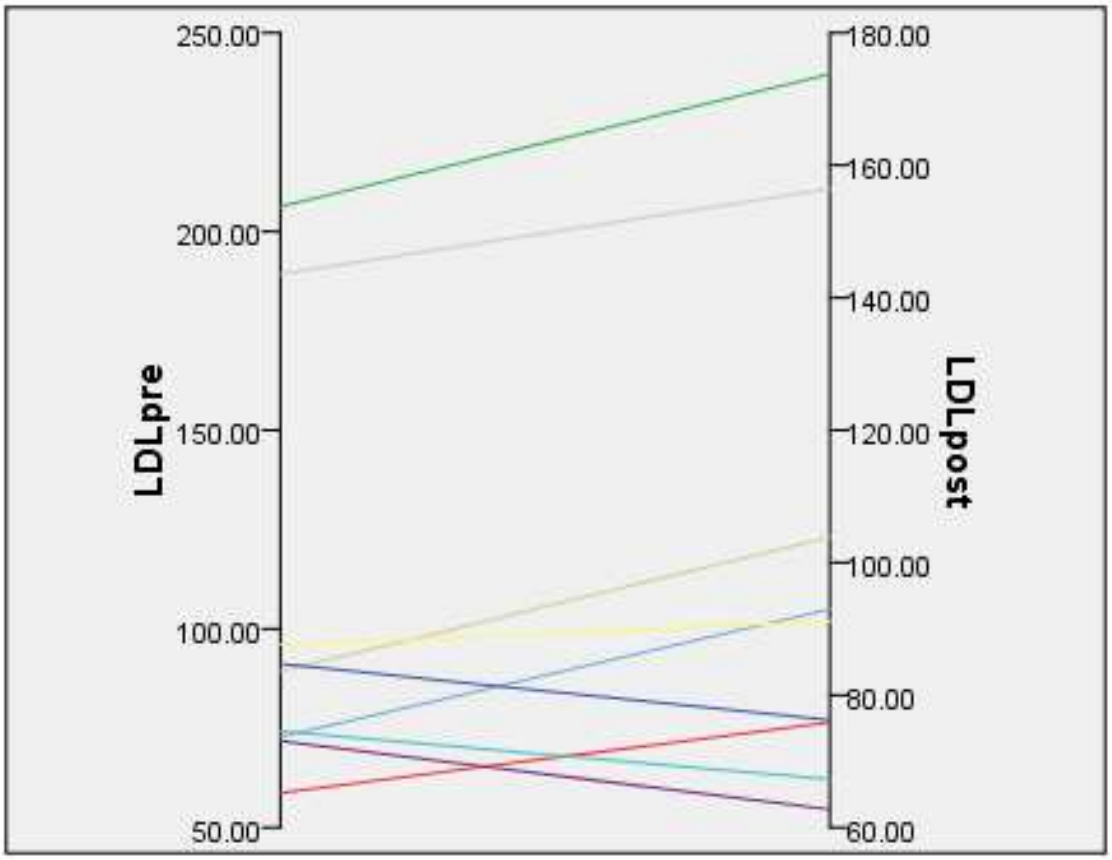
INDIVIDUAL CHANGES IN SERUM LIPID MEASUREMENTS FOR FLAXSEED GROUP



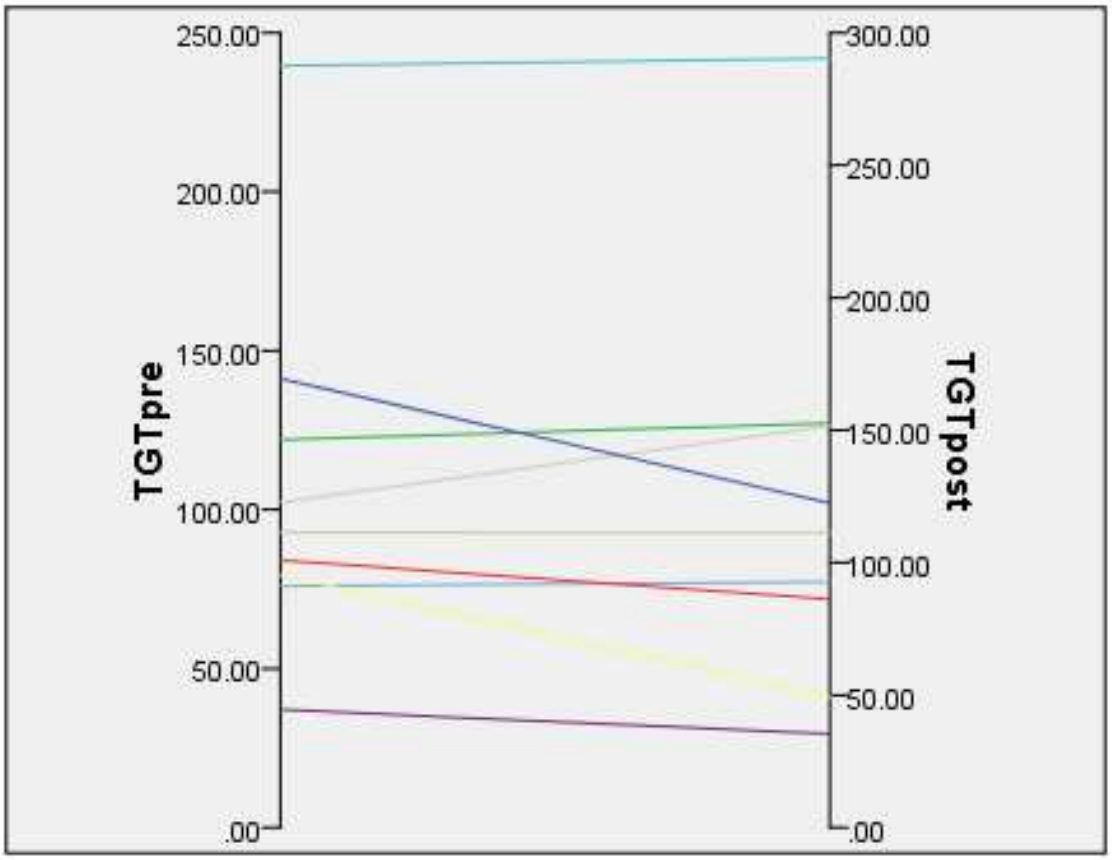
A. Individual changes in total cholesterol (mg/dL) from baseline to week 8.



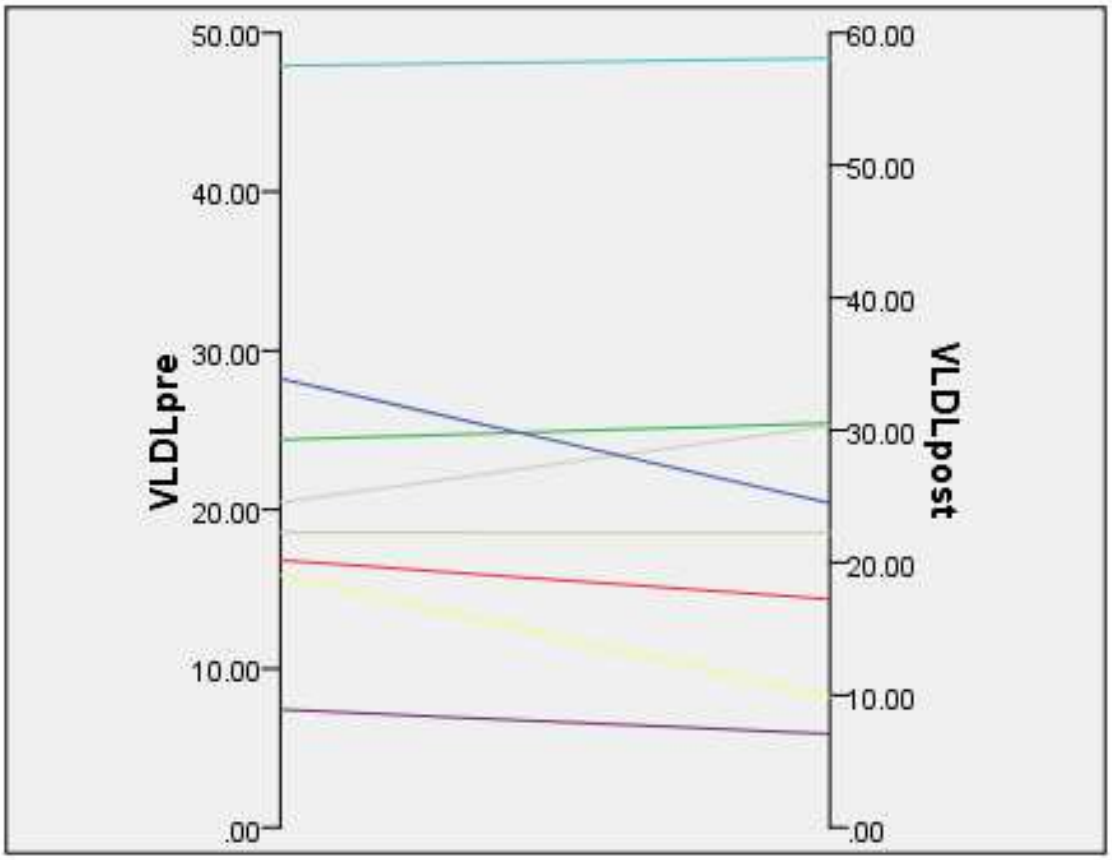
B. Individual changes in HDL-C (mg/dL) from baseline to week 8.



C. Individual changes in LDL-C (mg/dL) from baseline to week 8.



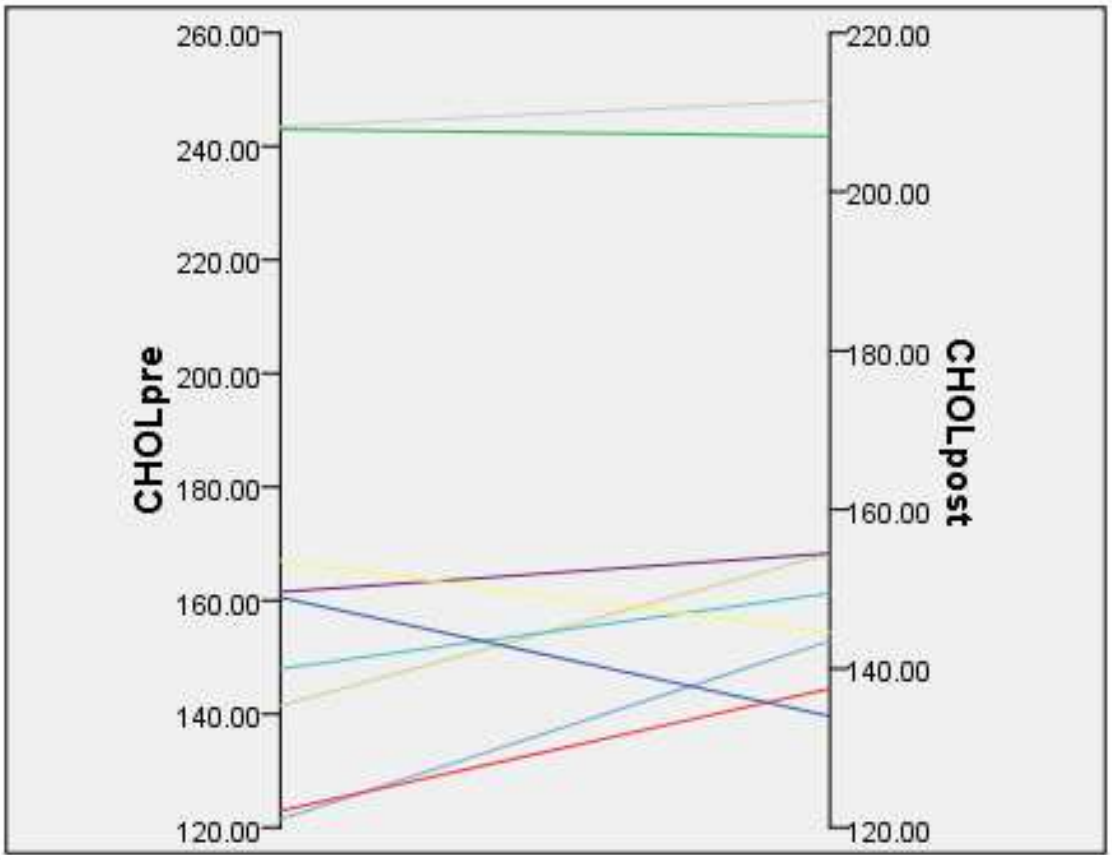
D. Individual changes in total triglycerides (mg/dL) from baseline to week 8.



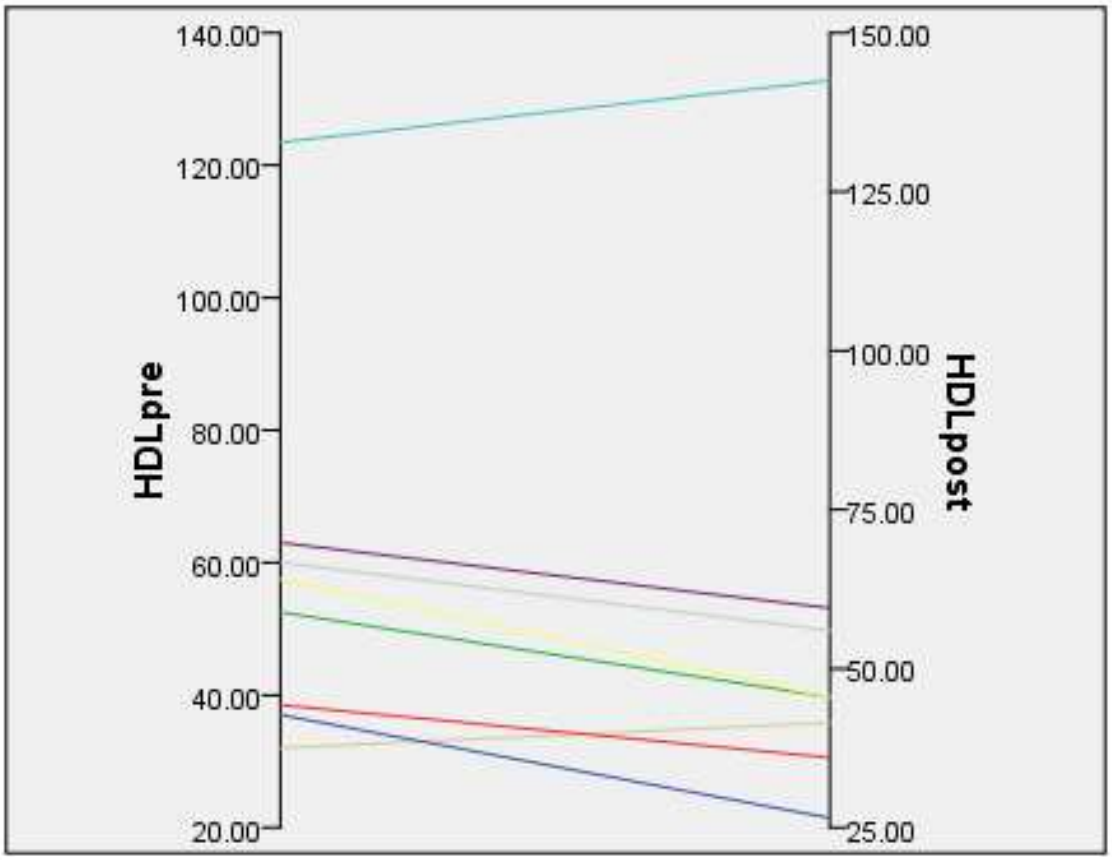
E. Individual changes in calculated VLDL (mg/dL) from baseline to week 8.

APPENDIX P

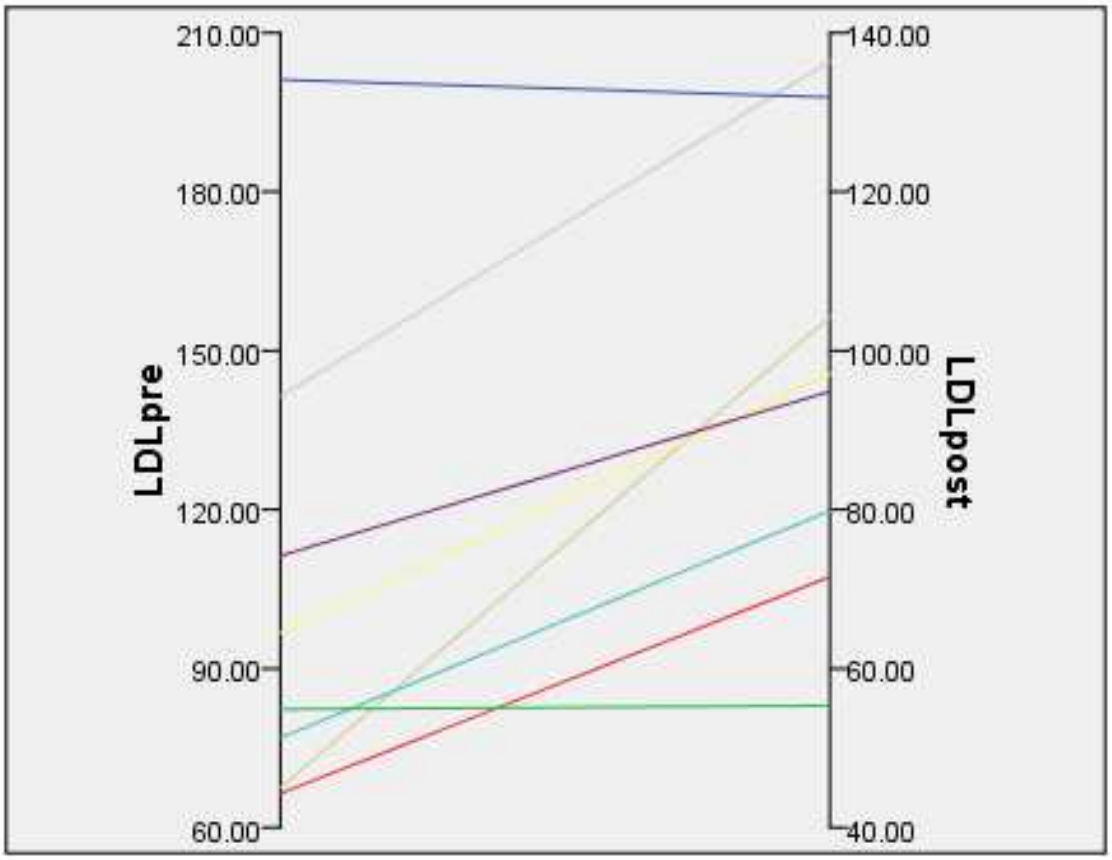
INDIVIDUAL CHANGES IN SERUM LIPIDS MEASUREMENTS FOR CONTROL GROUP



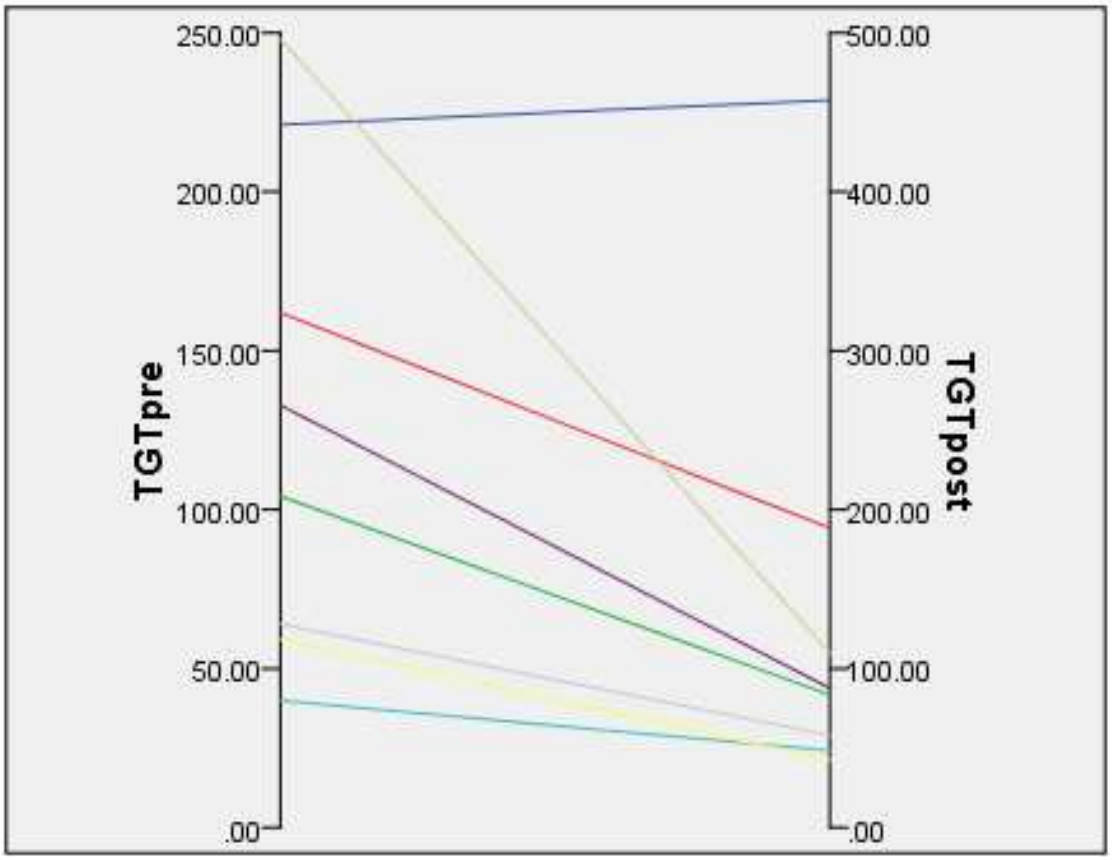
A. individual changes in total cholesterol (mg/dL) from baseline to week 8.



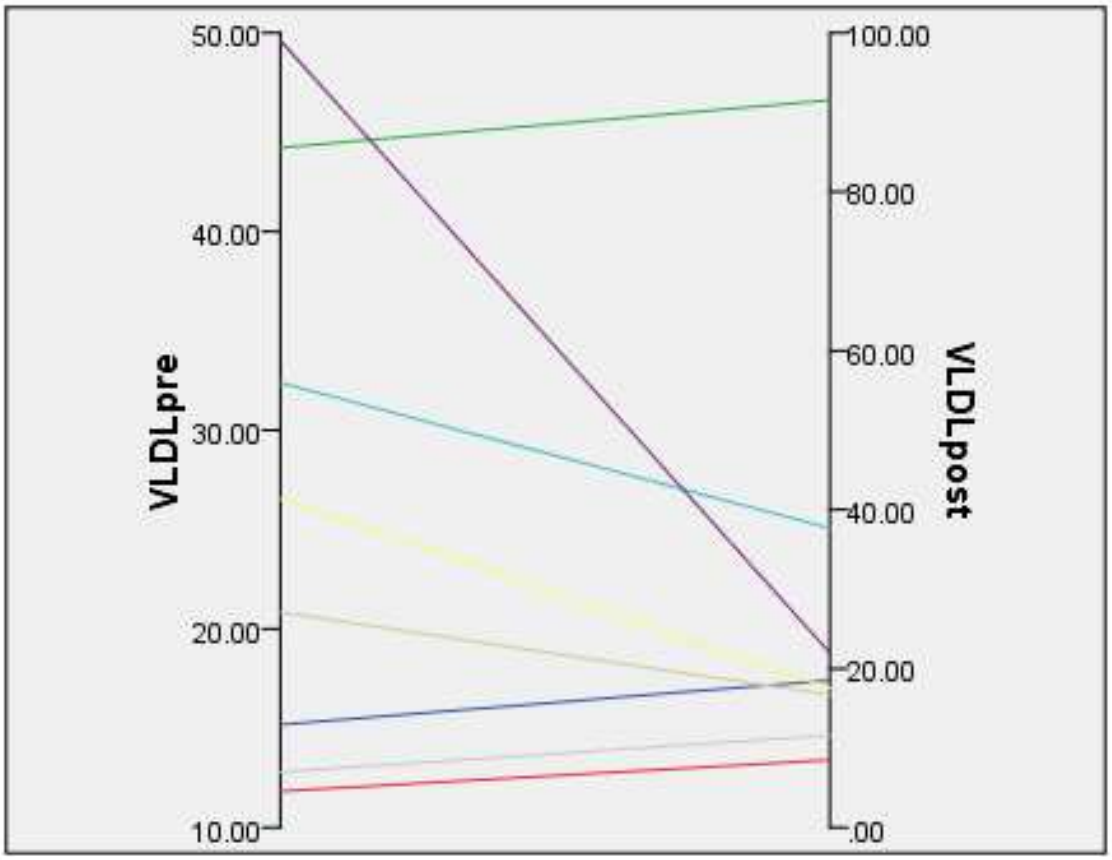
B. Individual changes in HDL-C (mg/dL) from baseline to week 8.



C. Individual changes in LDL-C (mg/dL) from baseline to week 8.



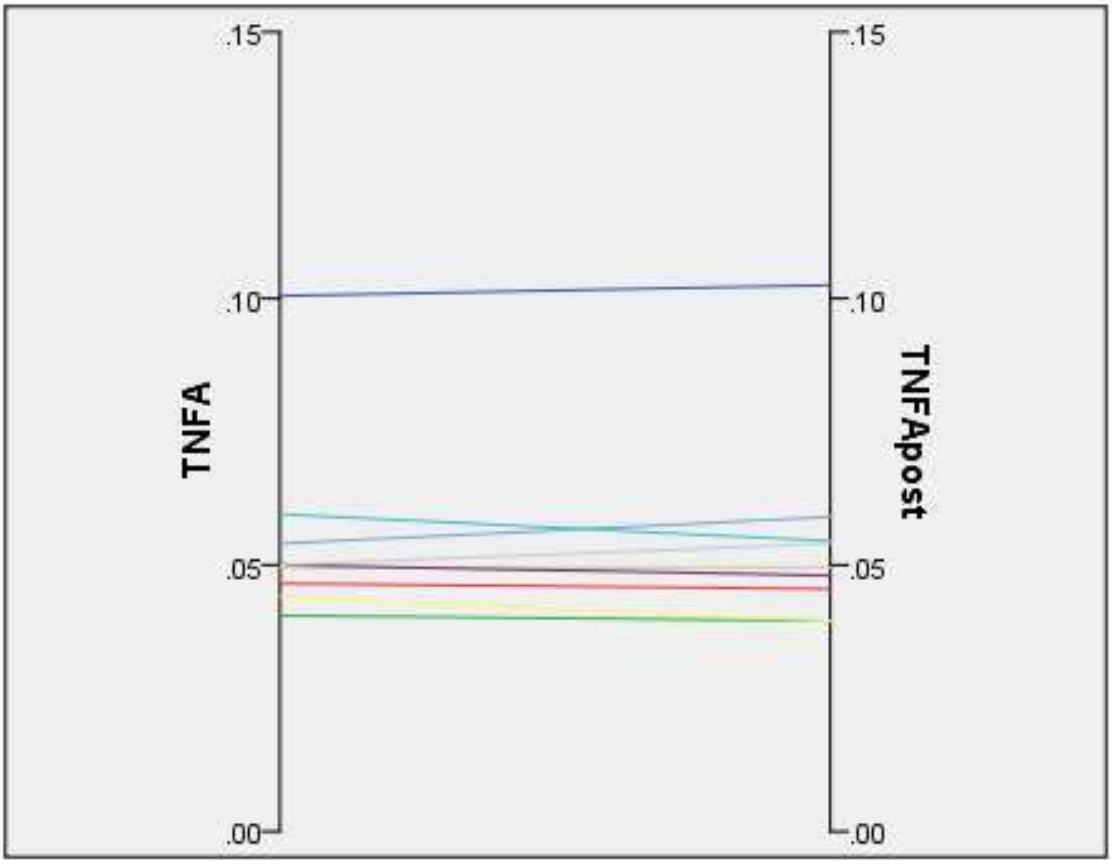
D. Individual changes in total triglycerides (mg/dL) from baseline to week 8.



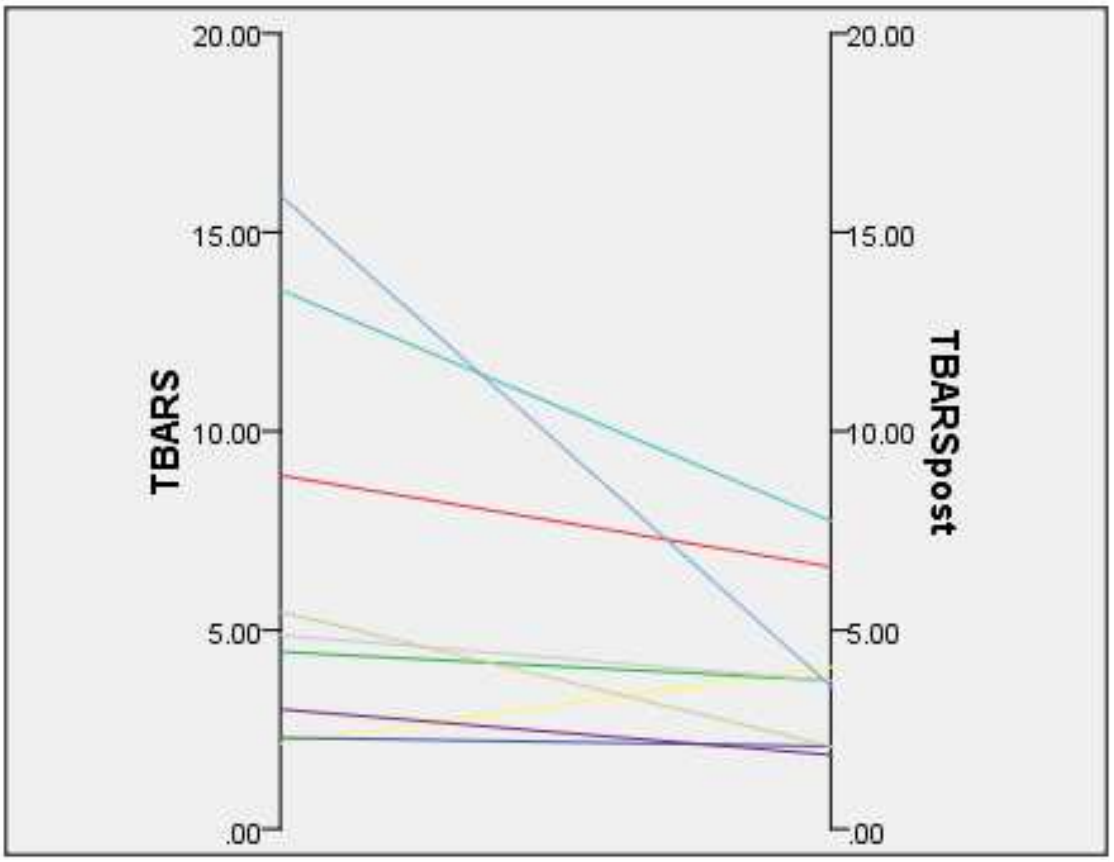
E. Individual changes in calculated VLDL (mg/dL) from baseline to week 8.

APPENDIX Q

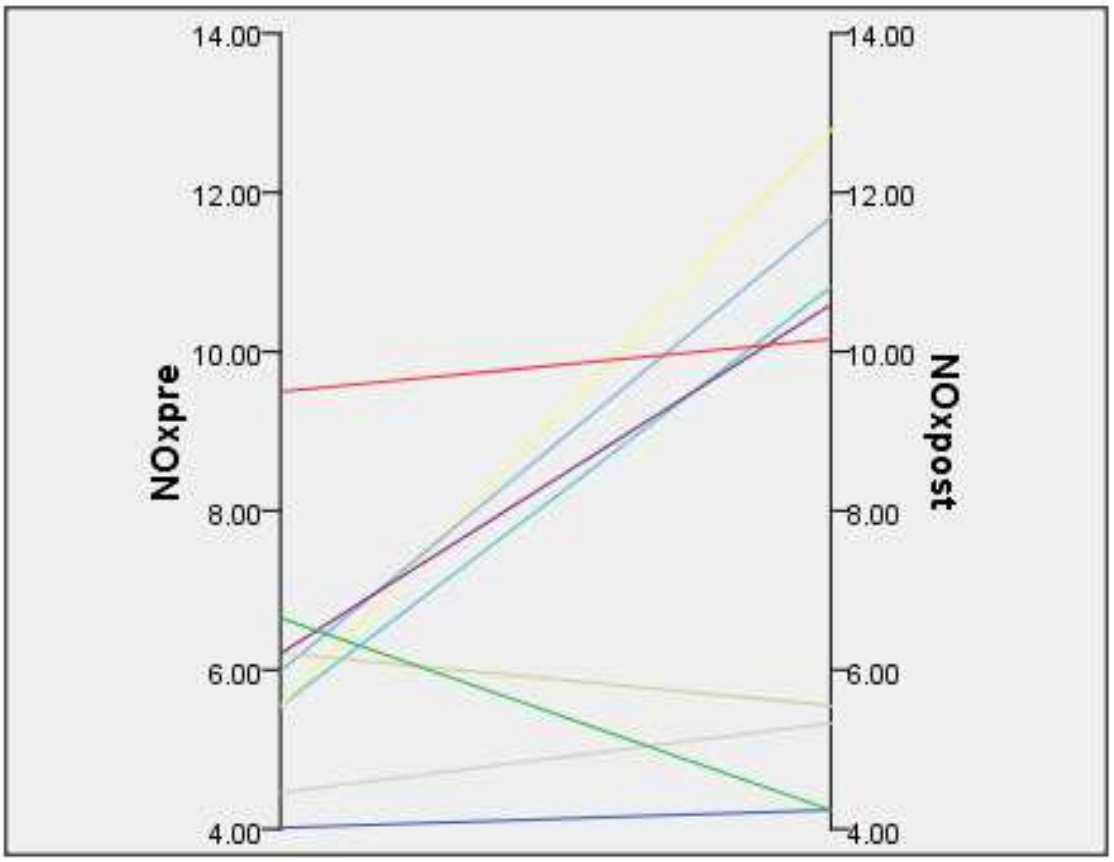
INDIVIDUAL CHANGES IN MARKERS OF INFLAMMATION FOR FLAXSEED GROUP



A. individual changes in plasma concentrations of TNF-alpha (pg/mL) from baseline to week 8.



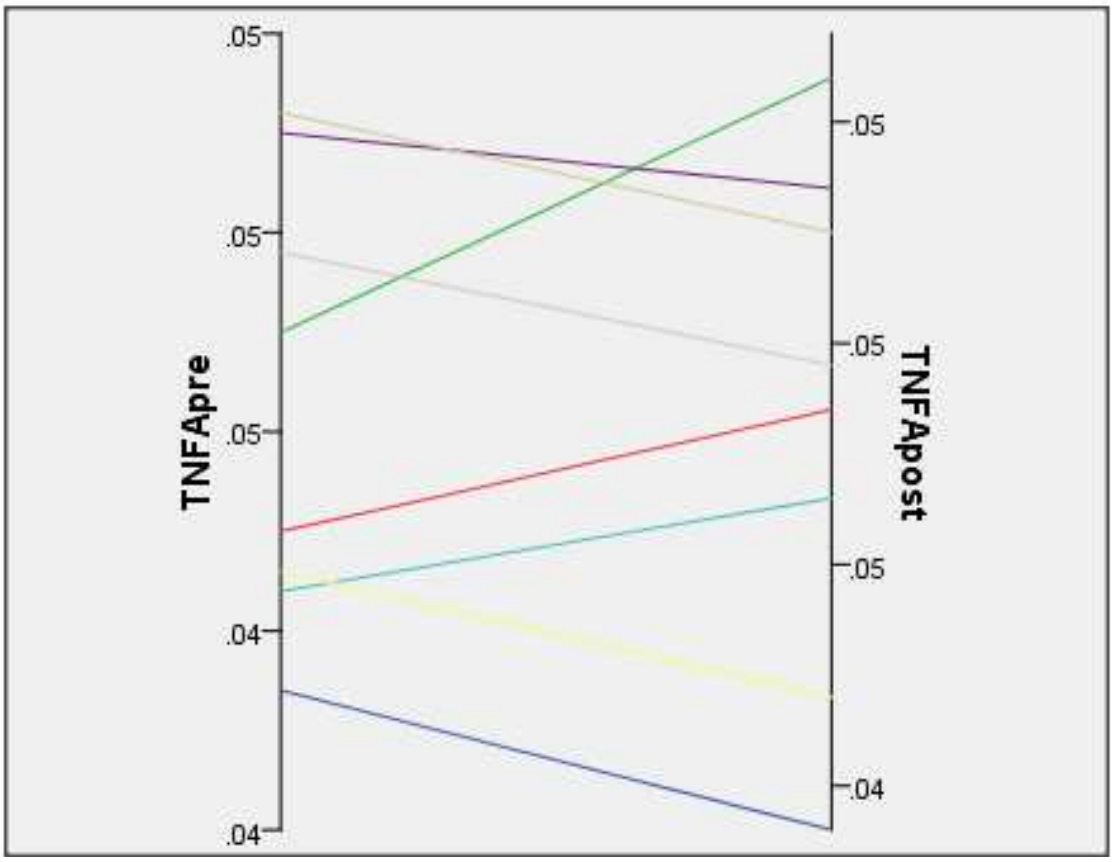
B. Individual changes in serum concentrations of TBARS (MDA nM/L) from baseline to week 8.



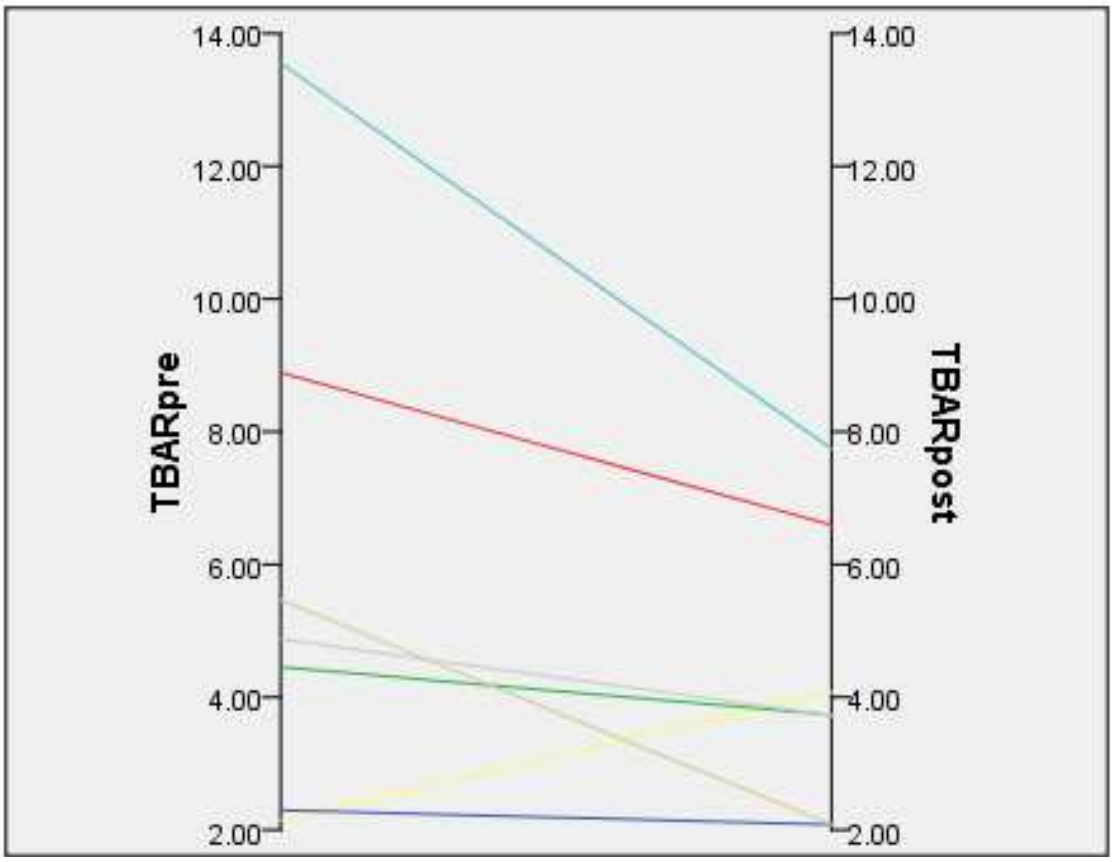
C. Individual changes in plasma concentrations of TBARS (nM/L) from baseline to week 8.

APPENDIX R

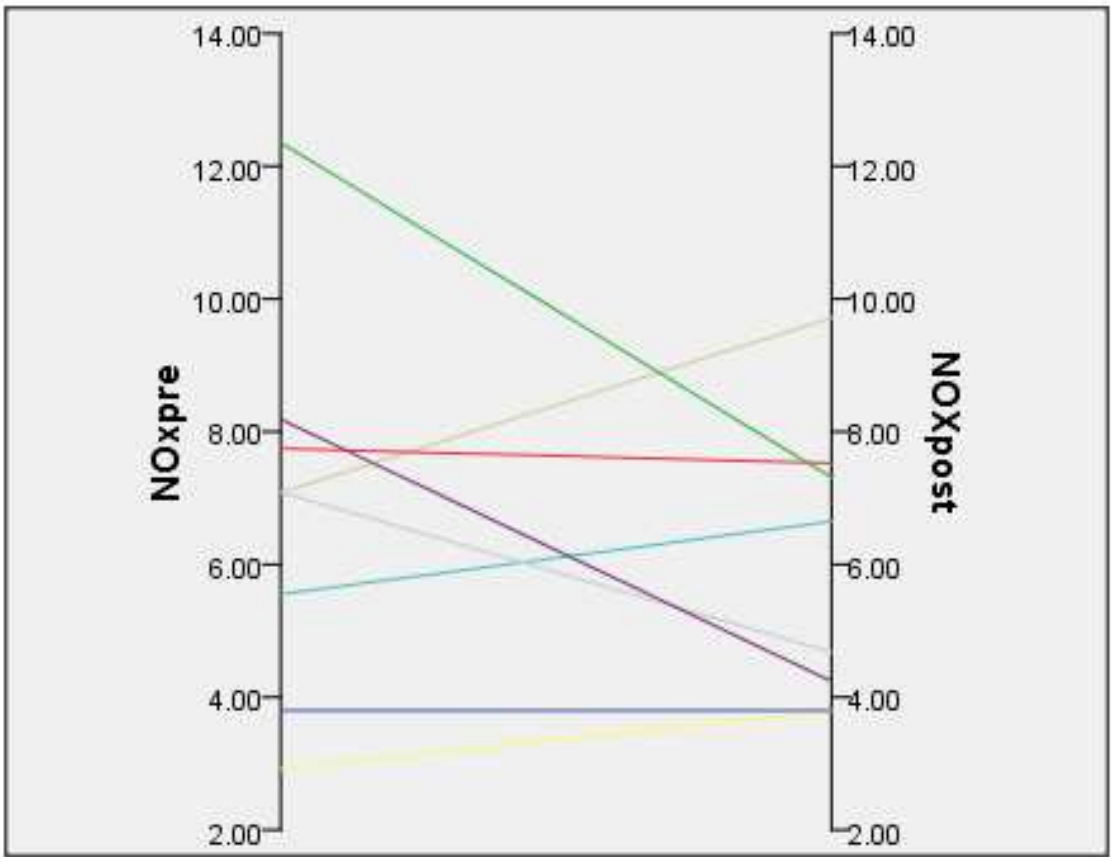
INDIVIDUAL CHANGES IN MARKERS OF INFLAMMATION FOR CONTROL GROUP



A. Individual changes in plasma TNF-alpha concentrations (pg/mL) from baseline to week 8.



B. Individual changes in serum TBARS concentrations (MDA nM/L) from baseline to week 8.



C. Individual changes in plasma NOx concentrations (nM/L) from baseline to week 8.