

CHAPTER ONE

INTRODUCTION

Diabetes Mellitus (DM) and its associated health maladies have concerned physicians and investigators since medical research was first recorded (DeVries 2004). Diabetes compromises the quality of human life by contributing to numerous life-threatening and debilitating macrovascular complications such as hypertension, coronary artery disease, and stroke. DM is also associated with the pathological development of microvascular complications such as neuropathy, retinopathy, glaucoma, and cataracts (Powers 2001).

The prevalence of diabetes in the United States (U.S.) increased by sixty-one percent between the years 1990 and 2001 (Mokdad *et al.*, 2003). During the eight-year span between 1990 and 1998, adults between the ages of thirty and thirty-nine had a seventy-six percent increase in diagnosed diabetes (Mokdad *et al.*, 2001). This increase represents a change in disease demographics, because diabetes was formally associated with an aging population (Geiss 1999). Based on prevalence data from the National Health Interview Survey and population demographic projections from the U.S. Bureau of Statistics, Boyle *et al.*, (2001) estimated that 29 million Americans will be diagnosed with diabetes by the year 2050. Among those 29 million Americans, African-Americans are predicted to become the fastest growing ethnic population with diabetes (Boyle *et al.*, 2001). The percentage of African-American males and females diagnosed with diabetes is expected to increase by 363% and 217%, respectively between the years 2000 and 2050 (Boyle *et al.*, 2001).

Diabetes Mellitus represents a group of metabolic disorders characterized by hyperglycemia (ADA 2004). Two main categories with various subtypes currently identified are type 1 DM and type 2 DM. Type 1 DM occurs in less than 10 % of people diagnosed with diabetes (McCarthy & Menzel 2001) and is believed to be the result of an autoimmune response targeting β -cells of the pancreas resulting in insulin deficiency and hyperglycemia (Powers 2001). Type 2 DM is one subtype of diabetes which has been associated with obesity linked to physical inactivity and unhealthy dietary practices (Harris 1998). A person can be in the pre-diabetic state for as long as ten years without exhibiting any signs or symptoms of diabetes (Valsania & Mycosis, 1994; ADA 2004). Although no overt signs or symptoms may be seen, these pre-diabetic patients will have started developing macrovascular and microvascular complications (Powers 2001).

According to the American Diabetes Association persons with either impaired glucose tolerance (IGT) or impaired fasting glucose (IFG) are classified as having pre-diabetes (ADA 2002). The overt clinical signs of unexplained weight loss, frequent hunger, thirst, and/or urination that usually occur before the clinical diagnosis of type 2 DM are not apparent during the pre-diabetes phase. Although blood glucose levels are elevated, they are not high enough to be classified as type 2 DM. **Table 1** summarizes the diagnosis and classification of DM established by the American Diabetes Association (2004) based on fasting plasma glucose (FPG) and Oral Glucose Tolerance Test (OGTT).

Table 1. Classification of Normal, Pre-diabetic and Type 2 Diabetes Levels.

(Modified with data from the American Diabetes Association 2004).

Plasma Glucose Levels (mg/dL)		
<u>Patient Classification</u>	<u>FPG</u>	<u>*OGTT</u>
Normal	< 100	< 140
Pre-Diabetic (IFG/IGT)	100 to 125	140 to 199
Type 2 DM	≥ 126	≥ 200

FPG = Fasting Plasma Glucose; IFG = Impaired Fasting Glucose;
IGT = Impaired Glucose Tolerance; OGTT = Oral Glucose Tolerance Test.
*OGTT levels are evaluated two-hours after ingesting 75 grams anhydrous glucose dissolved in water, also referred to as an oral glucose tolerance test (American Diabetes Association 2004).

The risk factors associated with pre-diabetes are being overweight (body mass index greater than 25 kilograms per meter squared), sedentary lifestyle practices, unhealthy dietary practices, and being of African-American, Native American or Latino American descent (ADA 2003). Modifiable westernized lifestyle practices, including decreased physical activity levels, consumption of larger quantities of food as well as increased intakes of highly processed foods low in resistant starch, have been linked to increased obesity and pre-diabetes prevalence and the epidemic of type 2 DM (Laakso 2005).

Starch is the primary human dietary energy source (WHO, 1985; Langkilde *et al.*, 1998) and a main dietary component used in processed food preparation. A large amount of highly processed foods have been typified as easily digestible and absorbable in the small intestine. However, there remains a portion of starch that is not digested in the small intestine (Anderson *et al.*, 1981; Englyst *et al.*, 1982; Topping & Clifton 2001). This undigested fraction of starch is referred to as resistant starch. Resistant starch as defined by Asp, comprises “the sum of starch and products of starch degradation not absorbed in the small intestines” (Asp 1992) the majority of which is undigested upon entering the large bowel (Topping & Clifton, 2001). Health benefits such as reduced glycemic response and increased short chain fatty acid concentration have been attributed to starches and starchy foods that are either relatively or absolutely resistant to digestion in the small intestine (Jenkins *et al.*, 1998; Salmeron *et al.*, 1997; Kritchevsky 1995).

Four types of resistant starch (RS), each with separate properties have been identified (Englyst *et al.*, 1992; Skrabanja & Kreft 1998; Topping & Clifton 2001). RS1 is physically inaccessible to digestive enzymes and is found in partially milled grains,

seeds, and legumes (Englyst *et al.*, 1992). RS2 are native starch granules that contain crystalline structure that prevents the alpha-amylase from degrading them (Lineback 1999; Niba 2002). Commercially available, Hi-maize amyloseTM 260 is an RS2 and was utilized in the current study for several reasons. It retains its granular conformation when exposed to processing conditions used in many food preparations (Niba and Hoffman 2003). It has also been reported to contain more than 60% total dietary fiber after being analyzed by the Association of Analytical Chemists (AOAC) approved enzymatic gravimetric method for total dietary fiber (AOAC 991.43, 1994). In addition it is easily incorporated into a food matrix. RS3 is formed by retrogradation of starch as a result of food processing. RS4 has been chemically modified with chemical reagents such as ethers and esters in order to disrupt the digestibility of digestive enzymes (Englyst *et al.*, 1992; Skrabanja & Kreft 1998; Topping & Clifton 2001; Niba 2002).

The majority of earlier studies examined the efficacy of acute dosages of resistant starch consumption to lower postprandial blood glucose and insulin responses in normal weight and obese persons with or without hyperglycemia, insulin resistance, hyperinsulinemia, and or type 2 DM (Higgins 2004). These studies led researchers to investigate the possible link between the effects of increased consumption of readily digestible starch and the pervasiveness of obesity and type 2 DM (Brown 2004). Results from these studies have indicated no change (de Roos *et al.*, 1995; Jenkins *et al.*, 1998), to little change (van Amelsvort & Westrate 1992; Westrate & van Amelsvoort 1993) to decreased postprandial glucose and increased insulin levels (Raben 1994; Behall & Howe, 1995; Behall *et al.*, 1989; Skrabanja *et al.*, 2001) after acute consumption of resistant starch. Various sources of resistant starch, which have different physiological

properties, were utilized in each of these studies. The type of starch, amylose as opposed to amylopectin (Behall *et al.*, 1989; van Amelsvoort & Westrate, 1992), the structure of the amylose (Vonk *et al.*, 2000), the amount and type of fiber (insoluble versus soluble) as well as food processing techniques used, i.e. cooking, recipes, storage, autoclaving, soaking (Bjorck *et al.*, 1994; Niba 2003; Niba and Hoffman 2003) impact the effect of readily digestible and slowly digestible starchy foods on postprandial blood glucose and insulin levels (Behall & Hallfrisch 2002).

JUSTIFICATION

Although results from studies examining the effects of resistant starch intake have varied, current literature does indicate that increased dietary intakes of resistant starch can lower blood glucose and blood insulin responses in healthy individuals as well as in overweight people and persons with hyperinsulinemia and type 2 DM (Park *et al.*, 2004; Hoebler *et al.*, 1999; Giacco *et al.*, 1998; Behall and Howe 1995). In addition, high-risk populations with high susceptibility rates to diabetes and insulin-related conditions have not been closely examined. African-Americans are a vulnerable population group with disproportionately elevated rates of type 2 DM. To date however, there is a dearth of published research studies on the effect of resistant starch on African-Americans who are at increased risk for type 2 DM. There are also minimal available data on this high-risk population using resistant starch added to a food matrix such as common dietary items like bread to test the effects on fasting plasma glucose, fructosamine, hemoglobin A1c, insulin, glucagon-like peptide-1, C-reactive protein, and short chain fatty acid levels, including serum acetate, propionate, and butyrate.

Resistant starch is a promising food ingredient that has the potential to reduce the risk factors involved in the development of type 2 DM as well as delay and/or prevent the onset of the disease. The current study assessed glucose homeostasis by monitoring changes in clinical blood values associated with the onset of type 2 diabetes. Therefore, the major objective of this study was to determine if daily consumption of approximately twelve grams of High-maize™ 260 resistant starch (RS2) added to bread helped to improve glucose homeostasis in African-American subjects with risk factors for type 2 diabetes. Information garnered from this study will be a vital foundation for further

research on the effects of new dietary ingredients such as resistant starch in African-Americans at increased risk for chronic diseases such as type 2 DM and other associated diseases. Consumption of resistant starch in bread products may also provide a viable alternative for reducing the risk factors for type 2 diabetes. Numerous trials (Pan *et al.*, 1997; Eriksson *et al.*, 1999; Tuomilehto *et al.*, 2001; Diabetes Prevention Research Group 2002) have evaluated the effectiveness of dietary, pharmacological, and exercise interventions in persons at increased risk for type 2 DM. The potentially successful lifestyle changes suggested from these later studies have yet to be adopted as permanent lifestyle choices for the prevention of diabetes.

OBJECTIVES

The goal of this study was to assess the effects of resistant starch in a food matrix on parameters for pre-diabetes in a susceptible, high-risk population.

This goal will be attained by examining the following specific objectives:

1. To determine if there was a difference in fasting plasma glucose, fructosamine, and hemoglobin A1c levels in a select group of African-Americans at increased risk for type 2 diabetes due to the consumption of Hi-maize™ RS 260 baked in bread and eaten for six-weeks.
2. To determine if there was a difference in insulin levels in a select group of African-Americans at increased risk for type 2 diabetes due to the consumption of Hi-maize™ RS 260 baked in bread and eaten for six-weeks.
3. To determine if there was a difference in the Homeostasis Model Assessment for Insulin Resistance (HOMA-IR) and Homeostasis Model Assessment for Beta Cell Function (HOMA-Beta) in a select group of African-Americans at increased risk for type 2 diabetes due to the consumption of Hi-maize™ RS 260 baked in bread and eaten for six-weeks.
4. To determine if there was a difference in C-reactive protein levels in a select group of African-Americans at increased risk for type 2 diabetes due to the consumption of Hi-maize™ RS 260 in bread and eaten for six-weeks.
5. To determine if there was a difference in glucagon-like peptide-1 levels in a select group of African-Americans at increased risk for type 2 diabetes due to the consumption of Hi-maize™ RS 260 in bread and eaten for six-weeks.

6. To determine if there was a difference in serum acetate, butyrate, or propionate levels in a select group of African-Americans at increased risk for type 2 diabetes due to the consumption of Hi-maize™ RS 260 in bread and eaten for six-weeks.
7. To determine if there was a difference in body mass index, waist circumference, or blood pressure in a select group of African-Americans at increased risk for type 2 diabetes due to the consumption of Hi-maize™ RS 260 in bread and eaten for six-weeks.

NULL HYPOTHESES

The following null hypotheses were tested:

H₀₁: There is no significant difference in fasting plasma blood glucose levels due to the presence or absence of Hi-maize™ RS 260 in subjects' diet.

H₀₂: There is no significant difference in fructosamine levels due to the presence or absence of Hi-maize™ RS 260 in subjects' diet.

H₀₃: There is no significant difference in hemoglobin A1c levels due to the presence or absence of Hi-maize™ RS 260 in subjects' diet.

H₀₄: There is no significant difference in insulin levels due to the presence or absence of Hi-maize™ RS 260 in subjects' diet.

H₀₅: There is no significant difference in the Homeostasis Model Assessment for Insulin Resistance (HOMA-IR) and Homeostasis Model Assessment for Beta Cell Function (HOMA-Beta) due to the presence or absence of Hi-maize™ RS 260 in subjects' diet.

H₀₆: There is no significant difference in C-reactive protein levels due to the presence or absence of Hi-maize™ RS 260 in subjects' diet.

H₀₇: There is no significant difference in glucagon-like peptide-1 levels due to the presence or absence of Hi-maize™ RS 260 in subjects' diet.

H₀₈: There is no significant difference in serum acetate, butyrate, or propionate levels due to the presence or absence of Hi-maize™ RS 260 in subjects' diet.

H₀₉: There is no significant difference in weight, body mass index, waist circumference, or blood pressure levels due to the presence or absence of Hi-maize™ RS 260 in subjects' diet.

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CHAPTER TWO

LITERATURE REVIEW

Broad Definition of Diabetes Mellitus

Diabetes Mellitus (DM) is a group of metabolic disorders characterized by hyperglycemia (ADA 2004). Depending on the etiology of diabetes, hyperglycemia results from either a defect in the secretion of the hormone insulin or from defects in target tissue uptake of the hormone, and in some cases from both (ADA 2004). Chronic hyperglycemia leads to changes in several organ systems that can result in heart disease, stroke, hypertension, renal disease, visual acuity loss, limb amputation, and dental disease (ADA 2002; McCarthy & Menzel 2001). Environmental factors, genetic predisposition, as well as personal dietary and exercise practices are all components of the complex interactions that result in diabetes (Powers 2001). Figure 2.1 illustrates the effects of these processes that result in the disease. (Groff and Groppier 2000).

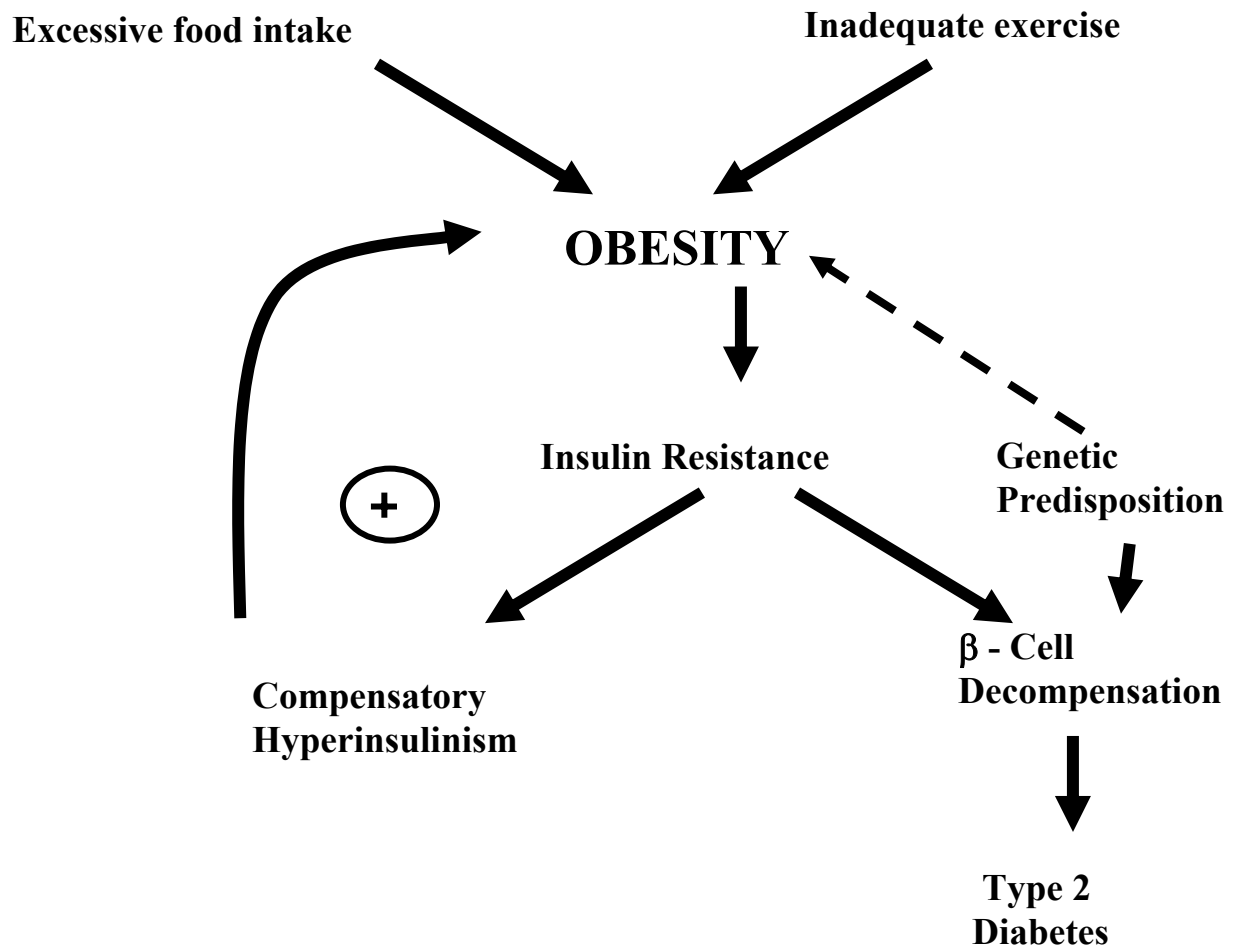


Figure 2.1 Advanced Nutrition and Human Metabolism Third Edition James L. Groff and Sareen S. Groppier 2000 Wadsworth Publishers Chapter 8. Integration and Regulation of Metabolism and the Impact of Exercise and Sport (used with permission).

Categories of Diabetes Mellitus (DM)

Two categories of Diabetes Mellitus (DM) are type 1 DM, previously referred to as Insulin Dependent Diabetes Mellitus (IDDM) and type 2 DM formerly known as Non-Insulin Dependent Diabetes Mellitus (NIDDM) or adult onset diabetes mellitus (ADA 2004). The former names are presently obsolete since type 1 DM also occurs in adults and type 2 DM is now being diagnosed in children (Alberti *et al.*, 2004).

Type 1 DM is further categorized as type 1A DM or type 1B DM. Type 1A DM is an autoimmune response that destroys the pancreatic beta cells responsible for making insulin, resulting in insulin deficiency (Powers 2001). Although type 1B DM lacks the immunologic markers that indicate an autoimmune destruction of pancreatic beta cells, insulin deficiency and hyperglycemia are still indicated (Powers 2001). Less than ten percent of all individuals with Diabetes Mellitus have one of these two forms of type 1 DM (McCarthy & Menzel 2001), whereas, the overwhelming majority of all diagnosed cases of Diabetes Mellitus are type 2 DM (ADA 2002, 2004).

Type 2 Diabetes Mellitus (DM)

Type 2 DM as described by Powers (2001) is a “heterogeneous group of disorders usually characterized by variable degrees of insulin resistance, impaired insulin secretion” or a combination of both (Sheard *et al.*, 2004). It is manifested by increased hepatic glucose production, reduced peripheral uptake (De Fronzo 1997). In some instances the β -cells of the pancreas are not capable of secreting enough insulin to compensate for the cells and tissues inability to absorb this glucose lowering hormone (Kahn 1994). These defects in insulin action and or insulin secretion often lead to the

development of glucose intolerance (De Fronzo and Ferrannini 1991; ADA 2003, 2004). Under these circumstances insulin resistance increases and insulin secretion gradually decreases as the body moves from glucose intolerance to type 2 diabetes (Tripathy *et al.*, 2000). Since impairment of insulin secretion and defects in insulin action often coexist in individuals with type 2 diabetes, it remains unclear whether the impairments that cause hyperglycemia are acting separately or simultaneously (ADA 2002).

Pathogenic Premises of Type 2 Diabetes Mellitus

Several hypotheses have been proposed in an endeavor to explain the pathogenesis of type 2 diabetes. These pathogenic components are multifactorial and thought to result from the effects of genetics, maternal nutrition on fetal growth and development, beta-cell over stimulation, as well as environmental sources (Bell and Hockaday 1996). The evidence for genetic influence is based on monozygotic twin studies where it was shown that if a monozygotic twin develops type 2 diabetes the other twin has between 60 and 85 percent probability of developing clinical type 2 diabetes (Bell and Hockaday 1996). Research findings indicate this probability holds true even when the twins are reared separately, even in totally different social environments (Medici F *et al.*, 1999).

As recently as 2004, the American Diabetes Association reported that the genetics of type 2 diabetes remains complex and has not been clearly elucidated (ADA 2004). Instead of one major gene locus being the major causative factor in the disease (Muller-Weiland *et al.*, 2003), this genetic complexity is believed to result from changes in

several gene loci leading to type 2 diabetes. In fact, there have been several hypotheses that promote genetic influence on the prevalence of type 2 diabetes in adulthood.

For one, during the early 1960's, Neel proposed the "thrifty" genotype hypothesis. This hypothesis suggested that insulin resistance and beta cell dysfunction associated with type 2 diabetes were actually adaptations that evolved to conserve energy during periods of famine (Neel, 1962). During the late 1990's Lev-Ran added another facet to Neel's original hypothesis to include the impact of environmental factors, since changes in gene frequency and genetic pool did not explain the increased prevalence and incidence rates which have resulted in the recent type 2 diabetes epidemic. Lev-Ran based his hypothesis upon the evidence that the increase in type 2 diabetes has occurred within some populations in one or two generations (Lev-Ran 1999). As westernized societies have become increasingly well-fed, and people have become less physically active, has the once protective role of the "thrifty" genotype proven instead to be harmful?

Studies conducted by Hales in 1992 and McCance in 1997 postulated that there may be a relationship between low birth-weight babies and an increased risk of type 2 DM during adulthood. (Hales *et al.*, 1992; McCance *et al.*, 1997). Boyko explored this belief and recently conducted an analysis of five studies that investigated diabetes incidence in adults as a result of low infant birth-weight. His findings indicated low infant birth-weight was actually linked to very few cases of type 2 DM (Boyko EJ, 2000). As a result, the total impact maternal nutrition plays during various disease processes such as obesity and type 2 diabetes continues to be investigated.

A final study to be mentioned is one that hypothesized that the over-stimulation of pancreatic beta cells (impaired insulin secretion) is believed to occur during the development of obesity (Bell & Hockaday, 1996). In many instances, continuously over-stimulated beta cells eventually lose their ability to secrete enough insulin to maintain normoglycemia. As a matter of fact the capacity of beta cells to continue to secrete enough insulin in response to peripheral insulin resistance determines whether or not an individual progresses to type 2 DM (Weyer *et al.*, 1999). Therefore, in order to diagnose type 2 DM, both reduced insulin action and defective insulin secretion have to occur (Muller-Wieland *et al.*, 2003).

There also exist several types of diabetes caused by genetic defects, other diseases, drugs, infections, uncommon forms of immune-mediated diabetes, and other genetic syndromes. One example is Maturity Onset Diabetes of the Young (MODY) which was first introduced by Tattersall and Fajans (1975) to describe type 2 diabetes in patients younger than 25 years. This classification was named before it was known that type 2 diabetes could also occur in children (Glaser 1997) and prior to the discovery that autosomal dominant diabetes could exist in individuals older than 25 (Doria *et al.* 1999). Maturity Onset Diabetes of the Young is a monogenetic defect of the β -cell and it takes several forms. This autosomal, dominant, inherited trait is a result of impaired insulin secretion with few to no defects in insulin action. However, defects on six gene loci located on different chromosomes have currently been found. Although Glucokinase (GCK1) was the first MODY gene to be identified (Froguel *et al.* 1992; Hattersley *et al.* 1992; Vionnet *et al.* 1992), hepatocyte nuclear factor (HNF)-1 α is the most common form of MODY (ADA 2004). HNF-1 α has a mutation on chromosome 12 and is a

severe form of MODY which requires insulin therapy (Yamagata et. al. 1996). While prevalence estimates to date have not been fully evaluated, autosomal dominant diabetes is believed to affect only a small (one to three) percent of all persons with diagnosed diabetes (Alessandro D, 2005).

Other known but rare types of diabetes, namely, Type A insulin resistance, Leprechaunism, Rabson-Mendenhall syndrome, and Lipoatrophic diabetes are all associated with severe insulin resistance, but are even less common than MODY (Alessandro D, 2005). The pancreas which is the site of the insulin-producing Islet of Langerhans is often involved in the onset of diabetes mellitus. “Pancreatitis, trauma, infection, pancreatectomy, and pancreatic carcinoma” are diseases of the pancreas that can lead to diabetes as a result of damage to this exocrine organ (ADA 2004). A more complete list of other types of diabetes can be found in Table 2.1 “The Etiological Classification of Diabetes Mellitus” (ADA 2006).

The more common Type 2 DM is often associated with increased caloric intake, a sedentary lifestyle, obesity, excess morbidity, mortality, and substantial health care costs (Harris 1998). In addition, type 2 DM is more prevalent in certain ethnicities such as African-Americans, Hispanic Americans, and Native Americans than it is in Caucasians (Haffner 1998). Type 2 DM is usually preceded by an asymptomatic stage of the disease now known as pre-diabetes (ADA 2002).

Table 2.1. Etiological classification of Diabetes Mellitus (ADA 2006)

Type 1 diabetes (β -cell destruction, usually leading to absolute insulin deficiency)

- A. Immune mediated
- B. Idiopathic
- II. Type 2 diabetes (may range from predominately insulin resistance with relative insulin deficiency to a predominately secretory defect with insulin resistance)
- III. Other specific types
 - A. Genetic defects of β -cell function
 1. Chromosome 12, HNF-1 α (MODY3)
 2. Chromosome 7, glucokinase (MODY2)
 3. Chromosome 20, HNF-4 α (MODY1)
 4. Chromosome 13, insulin promoter factor-1 (IPF-1; MODY4)
 5. Chromosome 17, HNF-1 β (MODY5)
 6. Chromosome 2, *NeuroD1* (MODY6)
 7. Mitochondrial DNA
 8. Others
 - B. Genetic defects in insulin action
 1. Type A insulin resistance
 2. Leprechaunism
 3. Rabson-Mendenhall syndrome
 4. Lipoatrophic diabetes
 5. Others
 - C. Diseases of the exocrine pancreas
 1. Pancreatitis
 2. Trauma/pancreatectomy
 3. Neoplasia
 4. Cystic fibrosis
 5. Hemochromatosis
 6. Fibrocalculous pancreatopathy
 7. Others
 - D. Endocrinopathies
 1. Acromegaly
 2. Cushing's syndrome
 3. Glucagonoma
 4. Pheochromocytoma
 5. Hyperthyroidism
 6. Somatosttinoma
 7. Aldosteronoma
 8. Others

Table 2.1 (continued) Etiological classification of Diabetes Mellitus

- A. Drug- or chemical-induced
 1. Vacor
 2. Pentamidine
 3. Nicotinic Acid
 4. Glucocorticoids
 5. Thyroid hormone
 6. Diazoxide
 7. β -adrenergic agonists
 8. Thiazides
 9. Dilantin
 10. α -Interferon
 11. Others

- B. Infections
 1. Congenital rubella
 2. Cytomegalovirus
 3. Others

- C. Uncommon forms of immune-mediated diabetes
 1. “Stiff-man” syndrome
 2. Anti-insulin receptor antibodies
 3. Others

- D. Other Genetic syndromes sometimes associated with diabetes
 1. Down’s syndrome
 2. Klinefelter’s syndrome
 3. Turner’s syndrome
 4. Wolfram’s syndrome
 5. Friedreich’s syndrome
 6. Huntington’s chorea
 7. Laurence-Moon-Biedl syndrome
 8. Myotonic dystrophy
 9. Porphyria
 10. Prader-Willi syndrome
 11. Others

II. Gestational diabetes mellitus (GDM)

Patients with any form of diabetes may require insulin treatment at some stage of their disease. Such use of insulin does not, of itself, classify the patient.

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Pre-Diabetes

Recently representatives from the United States Department of Health and Human Services (HHS) and the American Diabetes Association (ADA) formed a panel including doctors and diabetes experts from the Centers for Disease Control and Prevention (CDC) and the HHS' National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) to introduce the term "pre-diabetes". This new terminology was implemented in order to increase public awareness of diabetes, provide a clearer explanation of hyperglycemia as well as to emphasize the importance of early detection and treatment.

Pre-diabetes affects more than 17 million Americans, dramatically increases the risk for developing type 2 DM and increases the risk of cardiovascular disease by fifty percent. The majority of Americans with pre-diabetes are unaware that they have the disease (ADA 2002).

During the pre-diabetic state, postprandial blood glucose levels are slightly elevated, while fasting blood glucose levels tend to remain very close to the normal range. Type 2 diabetes does not develop in a few individuals with pre-diabetes whose pancreatic beta cells compensate for insulin resistance by increasing insulin secretion. However, in most individuals with pre-diabetes, after prolonged periods of hyperglycemia pancreatic beta cells are unable to compensate and eventually type 2 diabetes with its associated factors develops (Kahn 2003).

Factors associated with increased risk of pre-diabetes include obesity; body mass index ≥ 25 kilograms per meter squared; waist circumference ≥ 102 centimeters (40 inches) in males; waist circumference ≥ 88 centimeters (38 inches) in females; age (> 45 years); family history of diabetes; selected racial and ethnic groups; history of impaired

glucose tolerance (IGT) or impaired fasting glucose (IFG); gestational diabetes; lipid abnormalities; hypertension; and exercising fewer than three times per week (ADA 2002). If uncorrected these risk factors can lead to type 2 diabetes and the numerous debilitating life-threatening problems associated with the disease.

Long-Term Complications Associated with Type 2 Diabetes Mellitus

The complications caused by chronic hyperglycemia and associated with type 2 DM affect various organ systems which often result in cardiovascular disease include heart disease, stroke, hypertension, as well as renal disease, visual acuity loss, limb amputation, dyslipidaemia, microvascular and macrovascular diseases, in addition to dental diseases (ADA 2002, McCarthy and Menzel 2001). Persons with undiagnosed type 2 diabetes are at increased risk for cardiovascular disease, coronary heart disease, stroke, and or peripheral vascular disease (Laakso 2003). These diseases which result in part from microvascular and macrovascular complications caused by type 2 diabetes are disproportionately greater in high-risk ethnic populations such as African-Americans, Native Americans and Hispanic Americans (Davidson 2003). The diabetic complication of end stage renal disease (ESRD) is 2.6 to 5.6 times greater in African-Americans, six to seven times higher in Hispanic Americans, and six times greater in Native Americans when compared to the American Caucasian population (American Diabetes Association 2003; Davidson 2003). Heart disease is one of the leading causes of diabetes-related deaths and is two to four times greater in people with diabetes in these high-risk ethnic populations (Davidson 2003). Although the majority of cases of type 2 diabetes are preventable, overall, it was the fifth leading cause of death in the U.S. during 2000

(Anderson, 2002). While these statistics are indeed alarming, the statistics that follow are even more startling.

Diabetes Statistics

The worldwide prevalence of diabetes has drastically increased during the past forty years (Winer and Sowers 2004) and in 1985, approximately 30 million people had diabetes worldwide (Bloomgarden 1998). A mere ten years later 135 million people were diagnosed with diabetes worldwide. It is projected that 300 million people (5.4 percent of the world population) will have diabetes by 2025 (King *et al.*, 1998). These alarming statistics include both type 1 and type 2 diabetes although 90% of diabetes cases are type 2 (Winer & Sowers 2004).

The incidence of type 1 diabetes is also increasing. Onkamo *et al.*, (1999) performed a data analysis using thirty-seven studies conducted in twenty seven countries. Each of these studies had been carried out for a minimum of eight years. Their findings revealed that incidence rates for type 1 diabetes are increasing globally by three percent annually (Onkamo *et al.*, 1999).

By 2025, India, China, and the United States are the countries projected to have the largest number of people with diabetes (King *et al.*, 1998). They are projected to have 37 million and 21 million with diagnosed diabetes, with prevalence rates of 3.4 and 8.9 percent, respectively by the year 2025 (King *et al.*, 1998). India has been predicted to have more than 57 million people with diabetes with a prevalence rate of six percent, by 2025. Using revised epidemiological data from African and Middle Eastern countries, Wild *et al.*, (2004) estimated the prevalence of global diabetes to reach 366 million by the

year 2030. One factor that attributes to this increase in prevalence is the rising percentage of people that will reach 65 years of age and older (Wild *et al.*, 2004)

According to the National Institute of Health/National Institute of Diabetes, Digestive and Kidney Disorders (NIH/NIDDK), 17 million Americans have been diagnosed with diabetes and an alarming 5.9 million Americans are unaware they have diabetes (National Diabetes Statistics, 2003). Boyle *et al.*, 2001 applied population projections from the United Nations and estimated that more than 21 million Americans over the age of 20 will have diabetes by the year 2025 and 29 million Americans over the age of 20 are projected to have the disease by the year 2050 (Boyle *et al.*, 2001).

As daunting as these statistics are, it is important to note these data do not take differences in racial composition into account. According to population projections from the U.S. Census Bureau, African-Americans will represent more than 14.7 percent of the U.S. population by 2050, an increase of almost two percent from the year 2000 (Day 1996). The populations of other races, excluding Caucasians, are projected to increase by more than five percent. During this same time frame the Caucasian population is expected to decrease by 7.3 percent (Day 1996). Since current diabetes prevalence rates are the highest in the U.S. in minority populations such as African-Americans, Hispanic Americans, American Indians, Alaska Natives, Asian Americans, and Pacific Islanders; and the populations of many of these ethnicities are expected to increase, it is safe to deduce that diabetes will also increase in these ethnicities unless significant dietary and lifestyle changes occur.

Presently, an estimated 2.8 million African-Americans (more than 11% of this population) have been diagnosed with diabetes, even while one-third of the total African-

American population is currently undiagnosed (US Bureau of the Census, 2000).

African-Americans are also twice as likely to have diabetes as non-Hispanic Caucasians of similar age groups (National Diabetes Statistics 2003).

Normal Regulation of Blood Glucose

The body's inability to utilize ingested food, including carbohydrates, proteins, and lipids in order to regulate blood glucose levels often results in marked hyperglycemia, which results in diabetes. In order to understand this complex disease it is important to first understand how the human body regulates blood glucose levels under normal conditions.

The human body prefers homeostatic environments and therefore requires blood glucose levels to be maintained in a narrow range. The pancreas produces the hormones insulin and glucagon, which act in a counter regulatory manner to help control blood glucose levels. The pancreatic beta cells normally secrete insulin, even at low levels. In response to ingested food, blood glucose levels rise, triggering the increased secretion of insulin (Bell & Hockaday, 1996). Insulin acts to lower blood glucose levels. As blood glucose levels reach a normal range, the quantity of insulin secretion decreases. In response to insulin, red blood cells, fat cells, and muscle cells help to absorb glucose from the bloodstream, also assisting in maintaining normal blood glucose levels.

Glucagon has a counter regulatory effect on blood glucose levels in response to the hormone insulin in peripheral tissues, primarily the liver. Glucagon is an amino acid peptide hormone secreted by the alpha cells of the islets of Langerhans. It causes the liver to release stored glucose (known as glycogen) from the body's cells into the

bloodstream. This action of glucagon prevents hypoglycemia (low blood glucose levels) between meals and during exercise. Glucagon also causes the muscle cells to make new glucose molecules out of protein derived from amino acids. This process is known as gluconeogenesis (Muller-Wieland *et al*, 2003). The insulin to glucagon ratio determines the rate of gluconeogenesis and glycogenolysis. To state it simply the insulin to glucagon ratio determines the speed new glucose molecules are made from amino acids derived from protein as well as the rate the storage form of glucose known as glycogen, is broken down. **Figure 2.2** illustrates the functions of the counter regulatory hormones insulin and glucagon. Persons with type 2 diabetes often display disordered control of glucagon and insulin secretion, which can be altered by the amount of dietary carbohydrate, fat, and protein they ingest.

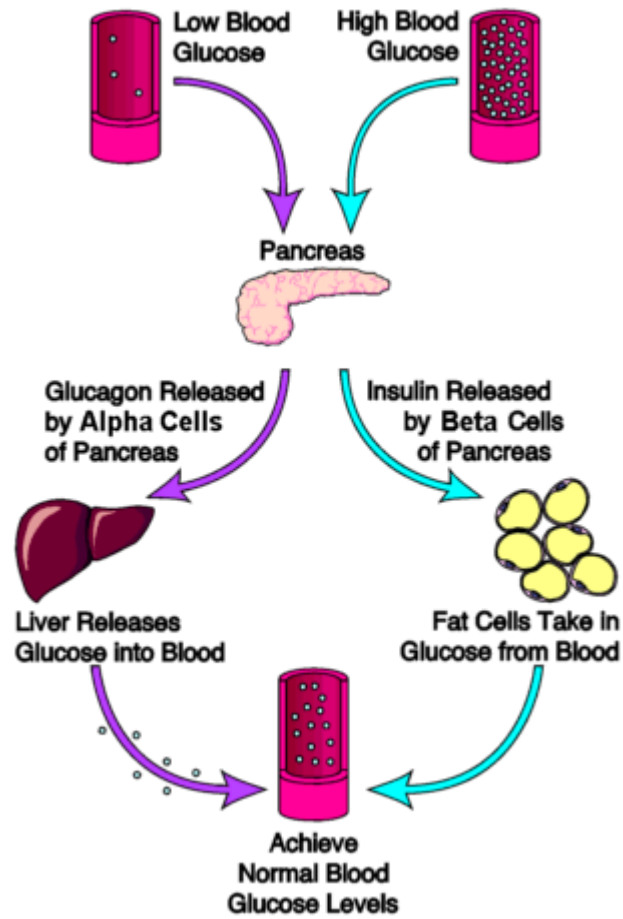


Figure 2.2 The Important Roles of Insulin and Glucagon

www.endocrineweb.com accessed 1/1/2004

Homeostasis is vital to the human body. The counterregulatory hormones insulin and glucagon help blood glucose levels remain within normal values. Low blood glucose levels stimulate the alpha cells of the pancreas to release glucagon, which causes the liver to release glucose into the blood stream to maintain normal blood glucose levels. Insulin is constantly secreted at low levels under normal conditions. When blood glucose levels are higher than normal several processes occur: 1.) insulin is released by the pancreatic beta cells; 2.) fat cells absorb glucose within the blood; and 3.) blood glucose levels are returned to normal.

Carbohydrates

Carbohydrates are organic compounds that contain the elements carbon, hydrogen, and oxygen. Most carbohydrates are formed from photosynthetic plants and are the major energy source in the human diet. These plant carbohydrates vary in absorption rate and digestibility (Ettinger 2004).

Carbohydrates are related to the etiology of type 2 diabetes as a result of their digestibility, metabolism and absorption. The major classes of carbohydrates are monosaccharides, oligosaccharides, and polysaccharides (Ettinger 2004). Structurally, monosaccharides are the simplest form of carbohydrate (also referred to as simple sugars) because they cannot be hydrolyzed into smaller components. Glucose, fructose, and galactose are all monosaccharides, of which glucose is the most abundant in nature and nutritionally the most important. This six-carbon sugar glucose is the primary source of energy in humans.

Oligosaccharides range from two to ten monosaccharide units and are attached through glycosidic linkages. The disaccharides maltose, lactose, and sucrose are the most common oligosaccharides and the main energy suppliers. Maltose consists of two glucose units formed as a result of the partial hydrolysis of the polysaccharide, starch. Maltose can be found in beer and malted liquors. Lactose is made up of galactose and glucose, and is found naturally in milk and milk products. The cane and beet sugar sucrose is composed of glucose and fructose and is the most frequently used natural sweetener (Davidson and Mills 2000).

Polysaccharides are macromolecules composed of many monosaccharide units. Starch consists of amylose, amylopectin, and modified starches and is one of the two subgroups of polysaccharides. Cellulose, hemicellulose, pectins, and hydrocolloids are the non-starch polysaccharide subgroups (Ettinger 2004).

Fats and proteins are also important energy sources in the human diet. The American Diabetes Association (ADA) recommends consuming 60 to 70 percent of total daily energy intake from carbohydrates and monounsaturated fat, and 15 to 20 percent from protein (provided kidney function is normal) (ADA 2004). According to data obtained from the Centers for Disease Control and Prevention, the percentage of total caloric intake among the U.S. population is obtained from consuming these three main components of the human diet as 50 percent from carbohydrates, 15 percent from protein, and 35 percent from fat (CDC 2004).

Following ingestion these macronutrients are digested, absorbed, and or stored for energy production, growth, and maintenance (Feldman 1988). The major portion of

macronutrient absorption takes place in the small intestine following enzymatic degradation (Davidson and Mills 2000). **Figure 2.3** illustrates the conversion of these macronutrients in the small intestine and their transportation to the major organs (heart, adipocytes, muscle, liver, kidney, blood cells, and brain) involved in their metabolic transformation and utilization (Davidson and Mills 2000).

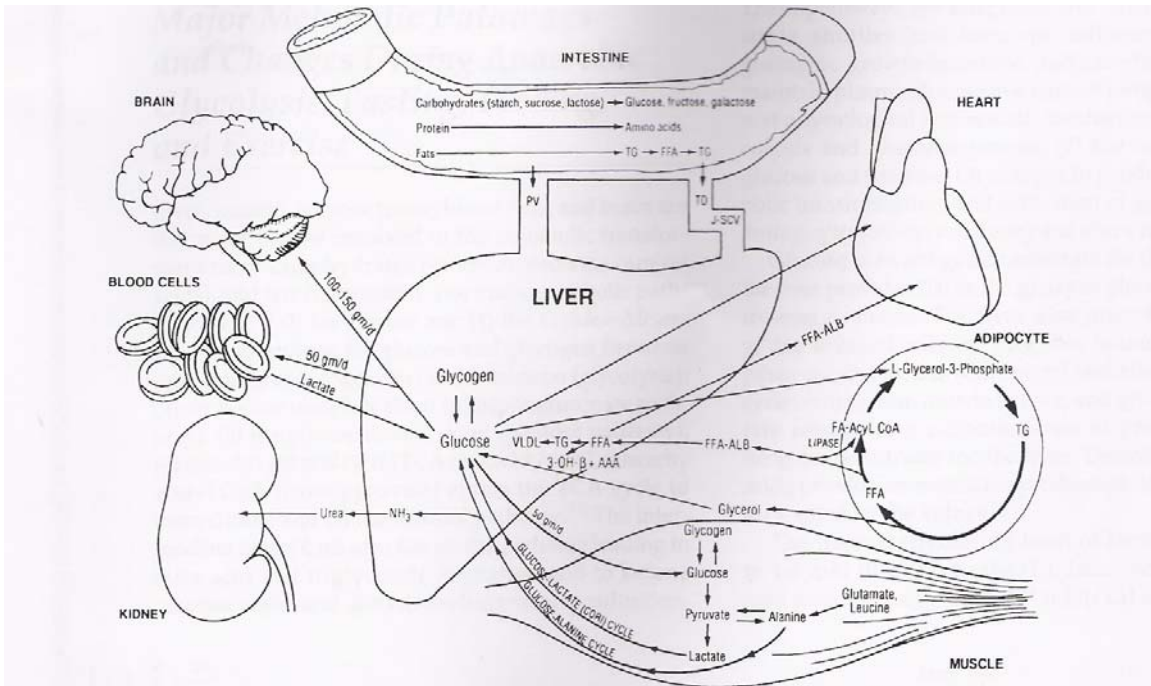


Figure 7-1. This figure illustrates the flow of substrates to and from the major organs involved their metabolic transformations and utilization. *Abbreviations:* PV, portal vein; TD, thoracic duct; J-SCV, jugular and subclavian veins; CO₂, carbon dioxide; NH₃, ammonia; FFA, free fatty acid; TG, triglyceride; FA Acyl Co A, fatty acid acyl CoA; Alb., albumin; β-OHB, beta-hydroxybutyric acid; AAA, acetoacetic acid.

Figure 2.3 Davidson and Mills (2000)

(Used with permission)

As pictured in Figure 2.3, the enzyme, alpha-amylase which is secreted by the salivary glands and the pancreas (Davidson and Mills 2000) is responsible for converting the polysaccharide starch into the disaccharides maltose and isomaltose. The disaccharide enzymes, maltase, lactase, and sucrase, which are located on the intestinal brush border, convert maltose and isomaltose to the monosaccharide glucose, lactose to the monosaccharides glucose and galactose, and sucrose to the monosaccharides glucose and fructose (Davidson and Mills 2000). Glucose and galactose enter the portal circulation following a meal. Whereas, fructose enters the portal circulation after partial conversion to glucose by the intestinal cells (Davidson and Mills 2000).

Dietary proteins are hydrolyzed to form amino acids in the gastric and pancreatic juices by the protein-degrading (proteolytic) enzymes pepsin, located in the stomach, trypsin, chymotrypsin, carboxypeptidase, elastase, and the peptidases, aminooligopeptidase and dipeptidase which are located in small intestines (Davidson and Mills 2000). Twenty amino acids are required for protein synthesis of which ten cannot be synthesized by the body and therefore must be derived from food sources (Davidson and Mills 2000). The carbon chain of 18 of the amino acids can be converted by gluconeogenesis to form glucose (Davidson and Mills 2000). Gluconeogenesis, the process of producing glucose from noncarbohydrate sources occurs when dietary carbohydrate intake and blood glucose levels decline. Amino acids, lactate, pyruvate, and glycerol are all important noncarbohydrate sources (Groff and Gropper 2000).

Intestinal lipases hydrolyze triglycerides that have passed through the liver and adipose tissue producing free fatty acids, monoglycerides, and glycerol. Storage triglycerides are made as a result of free fatty acids combining with glycerol-3-phosphate.

Glucose and excess amino acids can also be converted to plasma free fatty acids and triglycerides (Davidson and Mills 2000). Plasma free fatty acids are believed to increase beta cell failure, and raise plasma glucose levels. This hypothesis proposed by Wolever (2000) displays the role of plasma free fatty acids, pancreatic beta cell function, and insulin resistance in the development of type 2 diabetes as a result of insulin resistance and is illustrated in **Figure 2.4**.

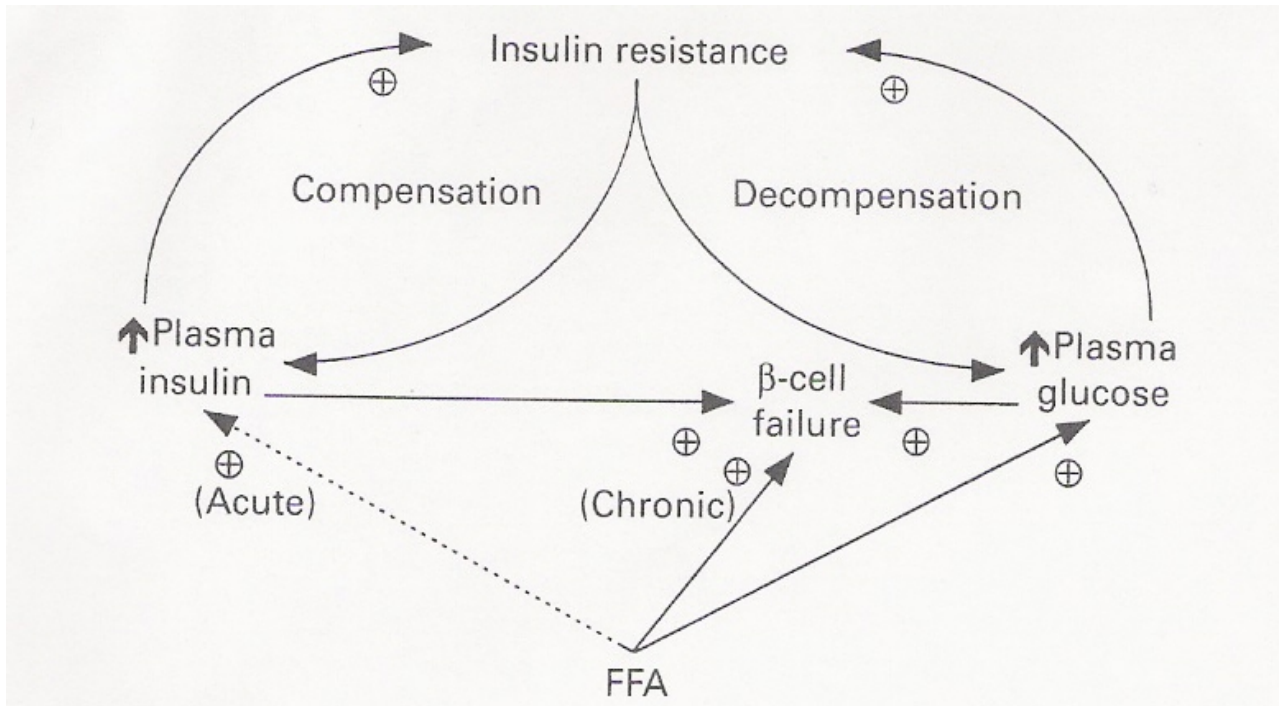


Figure 2.4 (Wolever 2000) “Proposed model for the role of insulin resistance, pancreatic Beta-cell function and plasma free fatty acids in the development of diabetes.” (Used with permission)

There are several metabolic pathways needed for either glucose formation or degradation. These include the Embden-Meyerhof-Parnas pathway, the pentose phosphate shunt, the glucuronic acid shunt, the tricarboxylic acid cycle and the sorbitol pathway (Feldman 1988).

These pathways are integrated and balanced by substrate shuttles and hormone influences (insulin, glucagon, growth hormone, and catecholamines) to maintain plasma glucose in a narrow range by the normal physiological homeostatic mechanisms of: (1) glycolysis and gluconeogenesis; (2) the interaction of glucose and fructose; (3) changes in production, metabolic transformation, and utilization of glucose during fasting, after feeding, and during and after exercise (Davidson and Mills 2000).

The major organs involved in the metabolic transformations of glucose, amino and fatty acids are the liver, muscle, adipose tissue, blood cells, and brain. The brain requires glucose and the liver provides a total of approximately 250 grams of glucose per day, 100 to 150 grams per day for the brain, and 50 grams per day for the blood cells and muscle.

The primary changes in carbohydrate, protein, and fat metabolism that occur in diabetes are: (1) hyperglycemia due to increased glucose production by the liver, (2) increased gluconeogenesis, (3) increased lipolysis, (4) preferential use of free fatty acids as an energy source, and (5) increased ketone production (Davidson and Mills 2000).

Protein and fats are catabolized at an increased rate, which can result in a negative nitrogen balance, hyperlipidemia, and ketosis. Glucose uptake is inefficient in peripheral tissues due to the down-regulation of insulin receptors, inadequate insulin production, as well as a postreceptor defect in cellular insulin action (Davidson and Mills 2000).

Starch

The polysaccharide, starch is the most important reserve polysaccharide of many higher plants. Starch is located in the granules of the plant tissues leaves, stems, roots, seeds, fruit, and pollen. Wheat, corn, rice, and barley starch are all common food starches that are derived from the seeds of plants while potato and cassava starch are obtained from plant roots (Lineback 1999).

Starch is made up of the two polymers amylose and amylopectin, which are composed entirely of glucose units. Amylose is mainly a linear polyglucan (1000 or less glucose units) with alpha-D- (1-4)-glucosidic linkages. However, it does contain some branching at the alpha-D- (1-6)-glucosidic linkages. Amylose can form strong intermolecular hydrogen bonds, resulting in gel formation when cooled (Lineback 1999).

Amylopectin is made up of approximately 4000 glucose units with highly branched alpha-D- (1-6)-glucosidic linkages (Zobel 1988). It has clusters that contain 20-25 chains of 12-16 glucose units (Hizukuri 1986), which are responsible for the crystalline regions of the starch granules. **Figure 2.5** illustrates the difference in the structure of amylose and amylopectin.

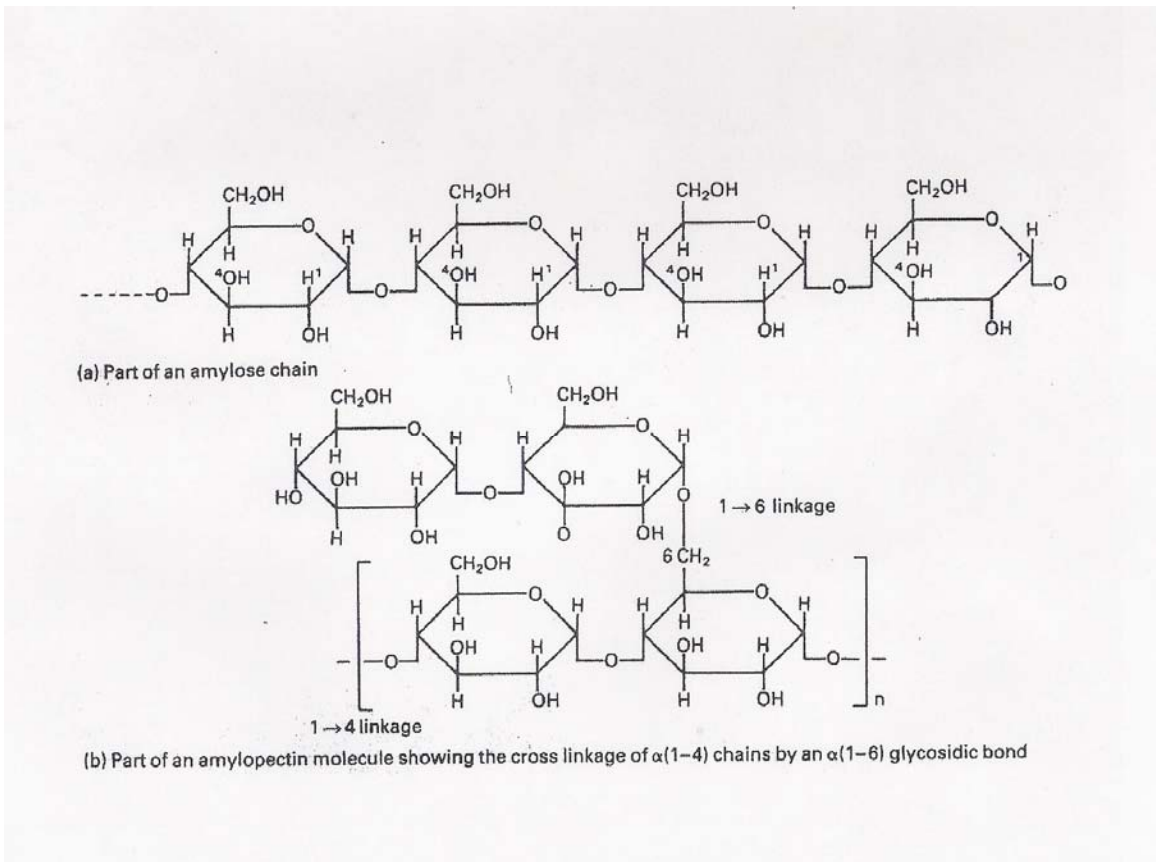


Figure 2.5 Chemical Structure of Amylose and Amylopectin

(Ihekeronye and Ngoddy, 1985)

The most common cereal starches contain 20-30% amylose. Waxy starches (rice, sorghum) are comprised of 100% amylopectin. High-amylose starches such as maize and barley that contain 50-70% amylose are available. Waxy and high-amylose starches are currently being used in several food products since their properties are different from normal starch granules (Lineback 1999).

Native starch granules hydrate and expand in aqueous solutions. Additional heating causes more expansion and the loss of the starch granules, crystalline x-ray diffraction pattern, and birefringence. Still further heating causes this expansion process to continue resulting in the leaching of amylose and amylopectin from the granules thus producing a gelatinous suspension when cooled (Lineback 1999). This disruption of starch granules by heating in copious amounts of water is known as gelatinization (Haralampu 2000). Depending on the source of starch as well as its amylose content, it typically hydrates between 40 to 120 degrees Celsius. In order to disrupt the starch granules of high amylose cornstarch, gelatinization temperatures of 154-171 degrees Celsius, must be reached. Once starches have gelatinized and begun cooling, over time a slower re-association process known as retrogradation occurs as a result of the realignment of the linear amylose and short amylopectin chains (Lineback 1999; Haralampu 2000). Since amyloses branches are shorter than amylopectin, retrogradation occurs faster and more extensively in amylose than amylopectin (Lineback 1999).

Although starch granules are digested by alpha-amylase the rate of enzyme degradation can be altered if the starch molecules are ungelatinized, retrograded, or chemically modified (Haralampu 2000). It is currently known that all starch is not digested and absorbed in the small intestine, but is instead passed to the large intestine

and after fermentation yields short-chain fatty acids (SCFA). This minimally absorbed fraction is known as resistant starch (Englyst *et al.*, 1992; Lineback 1999).

Resistant Starch

The definition of resistant starch (RS) was defined by EURESTA (European Food-Linked Agro-Industrial Research (FLAIR)- – Concerted Action on Resistant Starch) in 1993, where RS was defined as “the sum of starch and products of starch degradation not absorbed in the small intestine of healthy individuals” (EURESTA, 1993). RS can be found in many fruits and vegetables, partially milled grains, seeds, legumes, green bananas, raw potatoes, bread crusts, cereals, and cooked and cooled pastas (Englyst *et al.*, 1992). Recently RS has become available as an ingredient that can be used in commercial food products (Brown, 2004).

Four types of resistant starch with separate properties have been characterized. They are identified by using the abbreviation RS, which stands for Resistant Starch, and categorized by using the numbers one through four.

RS1 is a starch that is physically inaccessible to digestive enzymes because it is located within cell walls such as those found in whole and partially milled grains, seeds and legumes (Englyst *et al.*, 1992). Nevertheless, food processing and milling practices can affect the quantity of RS1 (Lineback 1999; Niba 2002). In addition to decreasing the RS1 content milling can destroy the RS1 content of grains, seeds, and legumes (Lineback 1999).

RS2 is described as native resistant starch granules because its crystalline structure prevents the alpha-amylase enzyme from degrading it (Brown 2004). RS2 is found naturally in green bananas and raw potatoes (Englyst *et al.*, 1992). Tuber (root)

starches are more resistant to digestion than cereal or the legume starches of peas and beans due to differences in their x-ray diffraction pattern (Gallant 1992). Commercially available Hi-maizeTM 260 is derived from RS2 and was obtained for this study from National Starch and Chemical Company, Bridgewater, New Jersey. Hi-maizeTM 260 retains its granular shape and much of its resistant starch and dietary fiber content following many food processing techniques (Brown 2004). Brown (1995) observed an increase in measurable resistant starch and dietary fiber quantity that corresponded with increased amylose content of starch granules. Hi-maizeTM 260 has also been found to contain more than 60% dietary fiber by the Association of Analytical Chemists (AOAC) approved enzymatic gravimetric method for measuring total dietary fiber (AOAC 991.43, 1994).

RS3 is formed by retrogradation of starch molecules after the starch has been altered by cooking above its gelatinization temperature (Brown 2004). These starch molecules reassociate during cooling, which provides some resistance to amylase digestion. This procedure results in non-granular starch that is found in processed foods such as cooked and cooled potatoes and cornflakes (Englyst *et al.*, 1992). Retrogradation can appreciably reduce the amount of RS3 available for consumption. Commercially available Hi-maizeTM 240 RS is classified as RS3 and is also manufactured by National Starch and Chemical Company, Bridgewater, New Jersey.

RS4 refers to chemically modified starch and is not currently classified as a food ingredient (Brown 1996). It is noteworthy to mention that all four classifications of RS can be affected by food processing (Thompson 2000). Baking for extended periods of time (Rabe and Seivert 1992) and autoclaving (Skrabanja and Kreft 1998; Niba and

Hoffman 2003) have been shown to increase the resistant starch content. While, canning and microwave heating have been shown to decrease resistant starch levels (Periago *et al.*, 1996; Meance *et al.*, 1999; Marconi *et al.*, 2000).

Resistant starch can be labeled as dietary fiber on food labels as measured by the Association of Analytical Chemists (AOAC) approved enzymatic-gravimetric method for total dietary fiber (AOAC 991.43, 1994). The high-amylose starch (Hi-maizeTM 260, RS2) utilized in this study is resistant to intestinal enzymes and has been described as having properties similar to fiber (Behall and Hallfrisch, 2002).

Dietary fiber definitions vary worldwide (Goldring, 2004) with differences based on the origin of the fiber, the physiological response as a result of fiber consumption, or the analytical methodology used to measure the amount of fiber. Several definitions maintain that fiber must contain carbohydrate (U.S. Institute of Medicine, 2002). In order to be defined as dietary fiber in the United States (U.S.) the material must contain plant cell remnants, polysaccharides, lignin, and associated substances that are resistant to hydrolysis by human digestive enzymes (Cho *et al.*, 1997). During 2002, the United States Institute of Medicine further proposed two categories of dietary fiber, termed dietary fiber and functional fiber. In order to be categorized as dietary fiber the substance in question must comprise nondigestible carbohydrates and lignin that are whole and natural plant components. Functional fiber includes nondigestible carbohydrates that provide advantageous physiological responses (U.S. Institute of Medicine, 2002). If the definition of resistant starch was not a functional definition (EURESTA, 1996) under these categorizations, RS1 could be considered dietary fiber, while RS2 and RS3 could be considered functional fiber. The four classifications of resistant starch were developed

in an effort to connect the physiological effects with the manner in which the RS is obtained (Brown *et al.*, 1995). Table 2.2 highlight's some of the key differences between dietary fiber and resistant starch (Niba 2002).

Table 2.2 Characteristics of Dietary Fiber and Resistant Starch (Niba 2002)

Table 2.2 Resistant starch and dietary fiber

Characteristic	Dietary fiber	Resistant starch
Composition	Plant cell wall components; cellulose; Lignins, mucilages, etc.	Four forms characterized: RS ₁ : entrapped, inaccessible starch; RS ₂ : native, ungelatinized granules; RS ₃ : retrograded amylose; RS ₄ : chemically modified starch
Digestibility in small intestine	None: complex linkages render non-susceptible to hydrolysis by digestive enzymes	Minimal; resistant to pancreatic amylases
Reported physiological benefits	Mainly bulking and intestinal transit Fermented to short chain fatty acids by colonic bacteria Bonds minerals and mutagens Colorectal cancer prevention	Fermented to short chain fatty acids by colonic bacteria Decrease in colon pH immune system boost Colorectal cancer prevention by Inducing apoptosis of tumor cells Induce chemopreventive enzymes Enhance cholic acid excretion Influence colonocyte metabolism
Processing effects	Restructuring and redistribution Increased palatability	Increased or decreased levels Amylose is retrograded to form resistant starch Techniques such as extrusion decrease levels, while autoclaving increases levels Affected by storage
Foods that contain significant levels	Whole grains – oats, barley, rye wheat, rice, corn, barley, sorghum, millet Legumes, most fibrous plant foods and grasses	High amylose maize, high amylose rice, firm banana, cooked/cooled potatoes and peas Legumes, such as mottled beans, chickpeas, faba beans, lentils, Haricot beans, red kidney beans; cooked and cooled pasta Retrograded stale foods

Table reproduced and used by permission Niba, 2002 “Resistant starch: a potential functional food ingredient”.

Sources: Eggum *et al.* (1993); Johnson and Gee (1996); Lintas and Capelloni (1992); Marsono and Topping (1993); Periago *et al.* (1996); Rabe and Seivert (1992); Slavin *et al.* (1999); Taylor *et al.* (1999); Topping and Clifton (2001)

Resistant Starch has also been classified as a low glycemic index (GI) food source. This term was first introduced by Jenkins and colleagues in the early 1980s (Jenkins *et al.*, 1981) and is used to categorize carbohydrate foods based on their effect on postprandial glycemia (Bjorck I *et al.*, 2000). Glycemic Index “is a relative in vivo measure of the plasma glucose response to a standardized amount of carbohydrate” (Gross *et al* 2004). Carbohydrate foods that are rapidly absorbed, release glucose in the bloodstream at a fast rate, and are referred to as high GI. Those carbohydrates that are more slowly released and therefore more slowly absorbed are classified as low GI foods (Jenkins *et al.*, 1981; Englyst 1999). Fifty grams of white bread is usually the standard carbohydrate reference amount and GIs range from less than 20% to approximately 120% (Bjorck *et al.*, 2000). Glycemic index is influenced by the amount of carbohydrate ingested, the type of monosaccharide, the nature of starch and other food components, such as fat, protein, nutrients, and dietary fiber, which all affect the absorption rate, as well as, processing and food preparation methods (Jenkins *et al.*, 1981; Bjorck *et al.*, 2000).

Salmeron *et al.* (1997 a, b) conducted two prospective studies and found that dietary GI was positively associated with increased risk of type 2 diabetes in both men and women. Therefore it is plausible that low-GI diets may reduce type 2 diabetes. The addition of resistant starch to starchy foods that typically have a high-GI, such as breads and pastas can have a lowering effect on the GI. It is known that a high RS content adds to the total amount of indigestible carbohydrate since RS is highly resistant to digestion by pancreatic enzyme amylase and is not metabolized in the small intestine. Resistant Starch is passed, undigested, into the large intestine and enters the colon for fermentation

by bacteria that naturally reside there, producing short chain fatty acids, primarily butyrate, acetate, and propionate (Topping and Clifton, 2001).

Muir *et al.* (1995) conducted a study to measure the effects on the colonic fermentation of a diet high in RS ($59.1 \pm 4.7\text{g}$) compared to a diet low in RS ($5.2 \pm 0.4\text{g}$) RS. Breath hydrogen and serum acetate were used as markers of colonic fermentation. Eight healthy subjects, whose ages ranged from twenty-four to forty years, were randomly assigned to consume a high-RS or low-RS diet by eating three specialized meals in one day. Each dietary feeding period was separated by one week. The RS in the high-RS diet contained 13 g RS from uncooked ground wheat (RS1 physically trapped starch), 13g RS from uncooked green banana flour (RS2 ungelatinized starch), and 33 g RS from high-amylose bread (RS3, retrograded amylose). Non-starch polysaccharides (NSPs) and macronutrient composition were identical for each participant for both diets.

Bowel function questionnaires were given to each participant in order to track gastrointestinal symptoms (Muir *et al.*, 1995). Participants reported gastrointestinal symptoms, in particular, flatulence, abdominal distension and cramping to be significantly higher ($P < 0.05$) after consuming the high-RS diet. “Breath hydrogen and average total serum acetate levels were significantly higher during the high-RS diet compared to the low-RS diet: 34.1 ± 4.7 and 23.9 ± 3.9 ppm ($P < 0.001$) and 169.1 ± 12.8 and 118 ± 6.6 umol/L ($P < 0.01$), respectively.” The short-chain fatty acids propionate and butyrate were detected in the serum samples. Although butyrate increased with the high-RS diet, it was not statistically significant (Muir *et al.* 1995). This study showed that RS is fermented anaerobically by colonic microflora resulting in the production of hydrogen and acetate.

Jenkins and colleagues (1998) conducted a sixteen-week, random crossover design study consisting of four two-week feeding periods, separated by two-week washout periods in between treatments. This study was designed to determine the effects of RS2 and RS3 on blood lipids, fecal short chain fatty acid production, and fecal bulking. Fasting serum blood glucose levels were not evaluated. Twelve healthy men and twelve pre-menopausal women were fed muffins and cereals containing 30 ± 2 g high-amylose granular resistant cornstarch (RS2), high-amylose non-granular, dispersed retrograded cornstarch (RS3), in order to compare the effects of 30 ± 2 g low-fiber and high-fiber wheat bran muffin and cereal supplement controls.

The participant's ages ranged between 22 to 55 years with body mass indices ranges between 19.4 and 34.2 kg/m² (kilograms per meter squared). Baseline weights and 12-hour fasting blood samples were acquired at the beginning and end of each of the four two-week treatment periods. A baseline seven-day diet history was also obtained before the study began and at the end of each treatment period. Twelve-hour expired air and four-day fecal collections were also taken at the end of each treatment period. Glycemic indices were assessed for 50 grams of available carbohydrates from the cereal and muffin supplements, in a separate group of people, following an overnight fast (Jenkins *et al.*, 1998).

The wheat bran supplement fecal output was significantly greater (258 ± 22 g/day) when compared with RS2 (187 ± 24 g/day); RS3 (182 ± 23 g/day) and low-fiber control (163 ± 23 g/day) $P < 0.010$ supplements. Increases were seen in both forms of RS when compared with low-fiber control supplements (RS2, $24 \pm 9\%$, $p = 0.017$) and (RS3, $29 \pm 14\%$, $p = 0.046$). No statistically significant differences were seen between

treatments in the mean concentrations of breath hydrogen, methane, or serum lipids and glycemic indices. However, their research findings did indicate that Hi-maizeTM RS 240 and Hi-maizeTM RS 260 increased butyrate concentrations thereby improving colonic health ($31 \pm 14\%$, $p = 0.035$) (Jenkins *et al.*, 1998). This study and others help validate why resistant starch is currently accepted as functional food ingredient that improves colonic health, thereby reducing colon cancer risk (Cassidy *et al.*, 1994; Ahmed *et al.*, 2000; Topping and Clifton, 2001). Butyrate has been found to inhibit cancer cell division, lead to cell death in colon tumor cell lines, and to inhibit proliferation of colonic mucosal cells and hydrogen peroxide (Barnard and Warwick 1993; Van Munster *et al.* 1994; Taylor *et al.* 1999 in Niba 2002).

Behall and colleagues examined whether mineral loss was associated with increased high-amylose RS intake as the major source of dietary carbohydrate (Behall *et al.*, 2002). They investigated the effects of a diet containing 30 grams of RS on apparent mineral retention of calcium, chromium, magnesium, and zinc in ten control and 14 hyperinsulinemic free-living men, between 28 and 58 years of age, weighing between 55 and 115 kilograms. Participants in both the control and treatment groups of the study consumed 55% of their total carbohydrate energy from bread, muffins, cookies, corn flake cereal, and cheese puffs. These primary carbohydrate sources were fed in a crossover pattern for two fourteen-week periods and consisted of either high amylose cornstarch (containing 70% amylose and 30% amylopectin, AM) or standard cornstarch (containing 70% amylopectin, 30% amylose, AP). Starch sources contained no wheat products and were eaten with meat, fat, low starch vegetables and fruits, during the first ten weeks of each starch replacement period. Subjects were provided with pre-weighed

seven-day controlled diets designed to meet the Recommended Dietary Allowance (NRC 1989) based on the sex and ages of each participant. The controlled diets contained no wheat products and consisted of 34 % of the total energy from fat, 15% from protein, and 51% from carbohydrate, including the same five products from the starch replacement period, along with vegetables and fruits. A dietitian taught each participant how to keep dietary intake records and each participant kept dietary records for seven days at baseline and twice during each starch replacement period. Urine, feces, and duplicate foods were collected during week twelve for seven days.

Urinary chromium losses did not differ or alter by diet in the hyperinsulinemic or control subjects following 24-hour collections or measurement after glucose tolerance tests. Apparent retentions of calcium and magnesium were not significantly different. Apparent copper retention was significantly affected more by the AP diet than the AM diet ($P < 0.02$). While the AM diet had a greater effect on apparent zinc retention ($P < 0.018$) and iron retention ($P < 0.09$) than the AP diet did. Overall results indicated a high-amylose diet containing 30 grams of RS could be consumed long-term without adversely affecting mineral retention and the retention of some mineral such as calcium, magnesium, iron, zinc, and chromium could actually be improved (Behall *et al.*, 2002).

Giacco and colleagues (1998) carried out a randomized placebo controlled study to evaluate the potential of smaller amounts of RS (16 g) on lowering postprandial blood glucose and lipid responses in persons with type 2 DM. They further investigated the effect of RS on these same blood and lipid parameters following a second meal. This study was conducted in six men and four women, mean age 50 ± 2 years, body mass index 27 ± 0.9 , who were previously diagnosed with type 2 DM for a period of five to

seven years. Type 2 DM was treated with diet alone in seven of the participants and the sulfonylurea drug (an oral hypoglycemic, glucose lowering drug) was used in combination with diet in order to control the effects of hyperglycemia in three of the participants.

The participants consumed in random order, two cheese cake biscuits containing either 16 grams (Hylon VII high-amylose starch, high RS) or one gram of RS (high-amylopectin starch, low RS). A small amount of water was used in the amylopectin biscuits in order to limit starch gelatinization and to obtain similar low glycemic indexes for both starch type. Both test meals also contained low fiber contents and were similar in nutrient composition (Giacco *et al.*, 1998). A two-day low fiber diet, to minimize possible interference with RS effects, preceded the test meals. After an overnight fast (12 hours), participants were admitted to the Clinical Research Center. Blood samples were obtained from an intravenous catheter and collected at zero minutes, thirty minutes, one, two, three, and four hours after the test meals were consumed. In order to assess their second research objective (to examine effect of RS on a subsequent meal), participants were given the same meal four hours after each test meal was consumed. Blood samples were obtained in the same manner and at the same timed intervals as before (Giacco *et al.*, 1998).

Lower blood glucose responses occurred following the 16 grams, Hylon VII high-amylose starch test meal when compared to the one gram of high-amylopectin; low RS test meal ($P < 0.03$). The triglyceride response was also lower following ingestion of 16 grams of Hylon VII high-amylose RS ($P < 0.03$). Subsequent meals containing either high or low amounts of RS were not effective in lowering postprandial blood glucose,

insulin or triglyceride responses. This study did show that high amylose RS did improve postprandial blood glucose and triglyceride levels in men and women with type 2 diabetes. Study limitations included the short length of time (four hours) the participants were given the resistant starch feedings, and the fact that some of the effect of the RS could have been caused by differences in the available carbohydrate of the two test meals. Overall the researcher's two study goals were accomplished which included evaluating the metabolic response of patients with type 2 DM after a meal enriched with RS and assessing the effects of RS consumption on the same metabolic responses after a subsequent meal was eaten (Giacco *et al.*, 1998).

Robertson and colleagues (2003) examined the acute effects of RS on postprandial plasma glucose, insulin, and plasma triacylglycerol levels of ten healthy subjects. Sixty grams of high-amylose maize Novelose 260 RS (currently distributed as Hi-maizeTM RS 260) was added to meals containing 33% fat and less than two grams of fiber. The starch was ingested during a 24- hour period (Robertson *et al.*, 2003).

Participants consumed either a high or low-resistant starch diet for 24 hours the day before the study in order to standardize nutritional status. Each participant was given a basal diet containing the same amount of energy from fat, protein, carbohydrate, and fiber as the study diet contained in order to control for the effects of these nutrients. Findings indicated that acute ingestion of 60 g of Novelose 260 RS for a maximum of 24 hours significantly lowered postprandial plasma glucose ($p = 0.037$) and insulin ($p = 0.038$) levels. No effect was seen on plasma triacylglycerol levels (Roberston *et al.*, 2003). This study contradicts the results find by Jenkins *et al.*,(2000) that RS has no effect on postprandial glycemia (Jenkins *et al.*, 2000).

Delaying the Onset of Type 2 Diabetes

During the late seventies and early eighties, two small studies were conducted in England to examine whether diet or drug therapy reduced the incidence of type 2 diabetes. The researchers were unable to detect a significant effect from either diet or drug therapy on the reduction of the incidence of type 2 diabetes (Jarrett *et al.*, 1984; Keen *et al.*, 1979). However, these results were contradictory to the findings by Sartor *et al.* (1980) where type 2 diabetes was prevented in subjects with impaired glucose tolerance (IGT) after receiving the antidiabetic drug tolbutamide and after making dietary changes (Sartor *et al.*, 1980). Tolbutamide is a first generation sulfonylurea that lowers blood glucose levels by 30-60 mg/dL and hemoglobin A1c (HBA1c) levels by 1.0-2.5% (Pfeiffer 1984).

Before obesity became an epidemic, it was known that diet, exercise, and genetic predisposition were major factors in the development of type 2 DM. In order to investigate whether the synergistic effects of diet and exercise were effective in reducing the incidence of type 2 diabetes researchers conducted the non-randomized, six-year Malmo feasibility study with 41 persons with newly diagnosed type 2 diabetes and 181 persons with impaired glucose tolerance (IGT) The incidence of diabetes was reduced without antidiabetic therapy after adherence to diet and exercise programs after an average follow-up of six years (Eriksson & Lindgarde 1991). The moderate weight reductions and dietary changes resulted in normalized glucose tolerance in more than 50% of the subjects with IGT and in remission of type 2 DM in more than 50% of those with the disease (Eriksson and Lindgarde, 1991). These studies and others helped lay the foundation for similar larger randomized controlled studies.

After screening more than 110,000 men in Da Qing, China, Pan *et al.*, (1997) began conducting the well known “Da Qing IGT and Diabetes Study” with 577 men and women with IGT. Their mean ages and BMI were 45 years and 25.6 kg/m², respectively. This study was designed to detect if interventions of diet, exercise, or the combination of diet and exercise could reduce the risk of developing type 2 diabetes (Pan *et al.*, 1997). During the six-year follow-up period a “proportional hazards analysis adjusted for differences in baseline BMI and fasting glucose” indicated a 46% reduction in the risk of developing type 2 diabetes in the exercise intervention group (P<0.0005). This was followed by a 42% (P<0.005) reduction in the diet and exercise intervention group. A 31% (P<0.03) reduction in risk was reported in the group that received dietary intervention exclusively (Pan *et al.*, 1997). When comparing the “Da Qing IGT and Diabetes Study” with other similar studies it is important to take into consideration that the health care clinics were actually randomized in this study instead of the participants. This appears to be the first controlled trial that demonstrated a significant reduction in the incidence of type 2 diabetes in persons with IGT due to dietary changes and/or increased physical activity (Pan *et al.*, 1997).

Since it has been established that persons with pre-diabetes are at increased risk of developing type 2 diabetes (The DECODE Study Group, 1999), there have been additional, recent well-designed, randomized, controlled trials examining dietary and life-style modifications as well as pharmacological agents to prevent or delay the onset of diabetes in this target population. Tuomilehto and others (2001) looked at the Finnish population which has a 35 % cumulative incidence of diabetes. Tuomilehto *et al.* (2001) conducted a study in this population and randomly assigned 552 middle-aged (mean age

55 years), obese (mean BMI 31 kg/m²), pre-diabetic men and women with impaired glucose tolerance to an intervention or control group. The intervention group was provided detailed advice of how to reduce their body weight by five percent or more; maintain dietary fat intake to less than 30% of total energy; increase their fiber intake to at least 15 grams per 1000 kilocalories; and to exercise moderately for a minimum of thirty minutes per day. Dietary advice was tailored to each subject based on three-day food records that were completed four times annually. The control group was given a two-paged leaflet about diet and exercise without any specific individualized dietary or exercise programs. They completed three-day food diaries at baseline and annual visits, without the guidance of nutritionists (Tuomilehto 2001).

Success in achieving the intervention goals were estimated on the basis of the food records and exercise questionnaires collected at the exam at the end of the first year. The effect of the intervention was assessed after one year. Forty-three percent of the intervention group achieved weight reduction by more than five percent compared to 13% in the control group. Forty-seven percent achieved recommended dietary fat intake of less than 30 % of total calories compared to 26% of the control group. The exercise goals of more than four hours per week were achieved by 86% in the intervention group and 71% in the control group. A strong inverse correlation was found between the success in achieving intervention goals and diabetes incidence. Diabetes did not develop in either the intervention or control group subjects who had achieved four of the five goals (Tuomilehto 2001). After an average follow-up of 3.2 years, the incidence of diabetes was 58% lower in the intervention group and most pronounced among individuals who made comprehensive lifestyle changes. However, the incidence of

diabetes was close to the 35% estimated for this Finnish population in those subjects who failed to make any changes. Tuomilehto and colleagues demonstrated that type 2 diabetes could be prevented with lifestyle changes (Tuomilehto 2001).

While the findings of this Finnish study were very important, conducting a study of this magnitude in a heterogeneous population of various ethnicities at increased risk of type 2 diabetes was still warranted. The following year the Diabetes Prevention Program (DPP) published findings from such a trial (Diabetes Prevention Research Group 2002). The DPP enrolled 3,234 slightly younger (mean age 51 years) and more obese (mean BMI 34 kg/m²) nondiabetic men and women with similar glucose intolerance as the Finnish study subjects had. Each subject was assigned to one of three treatment groups, (1) standard lifestyle recommendation plus placebo twice daily, (2) standard lifestyle recommendations plus metformin (also known as Glucophage, an oral blood glucose lowering agent) twice daily, or (3) an intensive program of lifestyle modification with goals of at least seven percent weight loss and two and one-half hours of physical activity each week. African-Americans, American Indians, Asians, Latino Americans, and Pacific Islanders made up 45 % of the subjects, a stark contrast to the single ethnicity that was evaluated in the Finnish study.

After an average follow-up of 2.8 years, the incidence of diabetes was 58% lower in the lifestyle intervention group, 31% lower in the metformin group, compared to 11% lower in control subjects receiving placebo. In the lifestyle intervention group, 51% achieved the weight loss goal of seven percent or more by the end of the twenty-four weeks and 75% met the goal of a minimum of 150 minutes of physical activity weekly. Eleven percent of people with pre-diabetes in all groups developed type 2 diabetes each

year during the average three years of follow-up. This study demonstrated the applicability of reducing diabetes incidence with lifestyle interventions in culturally diverse populations in the United States (DPP 2002).

Both the Tuomilehto study and the Diabetes Prevention Program required substantial efforts to achieve modest changes in weight and exercise sufficient enough to reduce the progression from impaired glucose tolerance (IGT) to diabetes. These efforts included several sessions with nutritionists and incentives such as free membership to exercise clubs (Tuomilehto 2001), free exercise tapes or equipment, free low-calorie foods, home encouragement and counseling visits (DPP 2002). Even with the success that both of these two studies achieved, some participant's still regained weight, which increases their risk for type 2 diabetes. Therefore it still remains to be proven whether these successful findings can be implemented in communities or if the lifestyle changes made during the study period will be adopted longer term.

Pharmacological Agents and the Delay/Prevention of Type 2 Diabetes

Pharmacotherapeutic agents such as metformin (DPP 2002), acarbose, which significantly decreases the postprandial rise in plasma glucose by inhibiting alpha glucosidase activity (Chiasson 1996, 2002), and troglitazone, which may affect the progression of glucose intolerance and was also recently banned from commercial sale in the U.S. (Buchanan *et al* 2002) have all been successful in reducing the progression of IGT to diabetes. However, intervention using pharmacological agents with persons who do not have diagnosed illness raises a plethora of economic, ethical and practical issues

(Simpson, 2003). Yet it is obvious that the development of preventative therapeutic protocols aimed at individuals with pre-diabetes are still warranted.

The overwhelming amounts and frequency of ingestion of processed food has dramatically impacted Western diets. This diet of processed food, which is low in raw fruits and vegetables and high in refined carbohydrates have resulted in a significant decline in health within these populations. In view of the fact that the obesity and type 2 DM are occurring at epidemic rates in the U. S., increased consumption of refined carbohydrates has continued to be linked to the obesity and the type 2 DM pandemic (Mokdad *et al.*, 1999; Frost 2000; Liu *et al.*, 2000; Jenkins 1987).

It is well known that many of 21st century carbohydrates are made from processed whole grains that are milled into white flour, thereby increasing calories at the expense of reducing fiber by 80% and dietary protein by approximately 30% (Durtschi 2001). This refining process removes many of the main ingredients, which results in a starchy carbohydrate of low nutrient value with a high glycemic index (GI) (Brand-Miller 1999). Refined carbohydrates and high GI have been implicated in increasing the risk of insulin resistance (Liu and Manson 2001; Jenkins 1987; Brand-Miller 1994), which contributes to pre-diabetes. The discovery of cornstarch and its subsequent ability to be converted to glucose and used as refined corn sugar after the removal of fiber, germ, and protein components led to the commercialization of high-fructose corn syrup (HFCS). HFCS's are used as sweeteners and currently make-up more than 56% of the U.S. nutritive sweetener market (Gross *et al.*, 2004).

Gross and colleagues conducted an ecological correlation study using univariate analysis and found a significant correlation between the percentage of carbohydrates from the refined carbohydrate, corn syrup, and the prevalence of type 2 diabetes in the U. S. between 1935 and 1997 ($r = 0.85$, $P < 0.001$) (Gross *et al.*, 2004; Jenkins *et al.*, 1981; CDC 1999). The researchers obtained type 2 DM prevalence estimate data for 1935 and annually beginning in 1957 to 1997 from the National Health Interview Surveys (NHIS) maintained by the Centers for Disease Control and Prevention's Diabetes Surveillance System (CDC 1999; CDC 1997). Gross and colleagues found proportional increased consumption of corn syrup and decreased consumption of dietary fiber resulted in increased obesity and type 2 diabetes prevalence (Gross *et al.*, 2004). The authors stated that randomized trials are needed to determine the complete effect of interventions that lower the risk of type 2 DM by substituting whole-grain, high-fiber foods, and low-GI foods for the HFCS that are currently prevalent in the American diet.

Low levels of resistant starch in diets continue to be linked to diet-related chronic diseases such as, colorectal cancer and type 2 DM and the beneficial effects of resistant (RS) in the management of diabetes are currently being explored (Brennan and Tudorica 2003; Robertson *et al.*, 2003; Giacco *et al.*, 1998). As expressed by Niba (2002), "as the substantial contribution of resistance starch to food nutritional quality and disease prevention becomes more obvious, the challenge of incorporating it in the diet and increasing its intake remains" (Niba 2002). Therefore methods of increasing dietary intakes of resistant starch in order to improve health, quality of life, and decrease medical costs are needed.

Resistant Starch is a safe, efficacious, natural food ingredient that can be easily incorporated into food products. Type 2 diabetes is a chronic disease that is prevalent in several minority populations. It compromises the quality of human life, causes numerous life-threatening complications and is often preceded by a decade of reversible, easily detectable pre-diabetes. Numerous trials have evaluated the effectiveness of dietary, pharmacological, and exercise interventions in persons at increased risk of type 2 diabetes, although it still remains to be proven whether these successful lifestyle changes will continue to be implemented long term.

Biochemical Parameters

Currently one unique quantitative biological marker that separates all people with diabetes from all people without diabetes (ADA Committee Report 2004) is not available. This lack of a unique marker for diabetes has caused medical personnel and researchers alike to utilize hyperglycemia, the predominant metabolic abnormality associated with the disease, as an indicator of diabetes. Hyperglycemia can be measured by several biochemical markers such as the Fasting Plasma Glucose or Fasting Serum Glucose (FPG or FSG, respectively) or 2 Hour Plasma Glucose (2-Hr PG), Oral Glucose Tolerance designated by the letters OGTT (a combination of FPG and 2-hr PG), Hemoglobin A1c (HbA1c), and or Fructosamine levels.

Fasting Plasma Glucose

Blood glucose levels were among the first chemical procedures utilized in clinical laboratory medicine (Folin and Wu 1920). Glucose oxidization occurs under the influence of glucose oxidase yielding gluconic acid and hydrogen peroxide. The hydrogen peroxide formed reacts with phenol and 4-aminoantpyrine in the presence of peroxidase to form a red-violet quinone complex, Stanbio procedure No. 1070, Boerne, Texas. Glucose levels are determined by a spectrophotometer and the intensity of the color formed is proportional to the concentration of glucose. Fasting plasma glucose is indicative of blood glucose levels at a single point in time. Blood glucose levels vary throughout the day due to diet, exercise, and insulin levels in the blood.

Fructosamine

Glucose is able to bind to several structures including proteins in a non-enzymatic irreversible reaction, referred to as glycosylation (Mosca A *et al.*, 1987). The glycosylated proteins that result include glycohemoglobin, glycoalbumin, and glycosylated total protein (Day *et al.*, 1980). The term fructosamine refers to glycoalbumin and glycosylated total protein (Armbruster DA, 1987). The average life span of these proteins ranges between two to three weeks; therefore fructosamine levels are an indication of the average glucose concentration during this time and may serve as an index of intermediate glycemic control (Bortheiry *et al.*, 1994; Goldstein 1984; Baker *et al.*, 1984). Increased fructosamine levels are observed when glucose concentrations are continually elevated.

Ko and colleagues (1998) combined the use of fasting plasma glucose (FPG) concentrations with hemoglobin A1c (HbA1c) or fasting plasma glucose (FPG)

concentrations with fructosamine in order to evaluate if the paired values (FPG + HbA1c and FPG + fructosamine) improved the validity of FPG as a screening test for diabetes. The study was conducted with 2,877 Hong Kong Chinese men and women with various risk factors for glucose intolerance. These risk factors included obesity, family history of diabetes, impaired glucose tolerance or previous gestational diabetes. Each participant underwent one, two-hour plasma glucose 75-gram oral glucose tolerance test (OGTT), which is the World Health Organization (WHO) gold standard for diagnosing impaired glucose tolerance (WHO 1985). Findings indicated that approximately 80% of the subjects would have been identified without undergoing the invasive; time consuming, costly, poorly reproducible OGTT (Nelson 1998; Ko *et al.*, 1998). Paired values of FPG of 5.6 mmol/L (113mg/dL) and HbA1c of 5.5%, resulted in an optimal sensitivity of 83.8 % and specificity of 83.6% to predict a 2-hour plasma glucose that was less than 11.1 mmol/L (199.8 mg/dL).

Hemoglobin A1c

Hemoglobin is the substance in red blood cells that carries oxygen. Glycohemoglobin is a form of hemoglobin that has glucose attached to it. Hemoglobin A1c (HbA1c) measures the concentration of hemoglobin molecules, which are found in red blood cells that have glucose attached to them. Glycation of the hemoglobin molecule results in its linkage with excess glucose in the blood. The more glucose that is circulating in blood, as opposed to reaching target organs and tissues, the more glycated hemoglobin becomes. The average life span of a red blood cell is approximately 120 days. Red blood cell glycohemoglobin concentration is dependent on the average blood

glucose concentration over a period of eight to twelve weeks and is stable for the life of the red blood cell. Therefore, HbA1c is a measure of long-term glycemic control and is indicative of blood glucose levels during the past two to three months (Osei *et al.*, 2003). HbA1c levels have been used to predict the risk for the development of type 2 diabetes and/or the progression of microvascular diabetic complications, such as neuropathy, nephropathy, and retinopathy (DCCT 1993; UKPDS 1998; Alberti & Zimmet 1998).

Studies have compared the effectiveness of HbA1c and fasting plasma glucose as well as oral glucose tolerance as diagnostic tools for the classification and detection of type 2 diabetes in multiethnic high-risk populations (Perry *et al.*, 2001; Anand *et al.*, 2003; Jesudason *et al.*, 2003). Results from these studies indicated that fasting glucose and HbA1c measurements used together improve the identification of individuals who have diabetes (Anand *et al.*, 2003) as well as improving the ability to screen and detect individuals at increased risk (Perry *et al.*, 2001; Jesudason *et al.*, 2003). These two tests used in combination allow clinicians to streamline the use of the oral glucose tolerance test. While this is true, HbA1c is not currently universally accepted as a screening or diagnostic tool for diabetes. This is due to differences in the sensitivity (65%) and specificity (94%) of HbA1c (Peters *et al.*, 1996; Rohlfing *et al.*, 2000; Rohlfing *et al.*, 2002; Monnier L *et al.*, 2003) as well as the variability and poor standardization of assays (ADA 1997; Kilpatrick *et al.*, 1998 a,b). Osei and colleagues (2003) found that HbA1c was predictive of the Metabolic Syndrome and could be used as a convenient and practical screening tool for high-risk populations at risk for type 2 DM. The metabolic syndrome shares several characteristics with pre-diabetes such as obesity and increased fasting glucose levels (Ford *et al.*, 2002).

C-Reactive Protein

The most common form of C-Reactive protein (CRP) is the cyclic pentameric protein that consists of five identical non-glycosylated subunits (Pepys MB 1996; Kluff and deMaat 2001). This acute-phase plasma protein originates chiefly from hepatic biosynthesis, formed by way of the proinflammatory cytokine marker, interleukin-6 (IL-6) (Heinrich *et al.*, 1990; Pradhan *et al.*, 2001). Within the last fifteen years, a relationship between the systemic inflammatory markers CRP and IL-6 have been associated with levels of obesity, insulin resistance, hyperglycemia, as well as type 2 diabetes (Yudkin *et al.*, 1999; Pradhan *et al.*, 2001).

CRP is synthesized by the liver in response to trauma, tissue injury, microbial infection, and inflammation (Ford 1999; de Maat & Kluff 2001; Pepys MB 1996). Increases in CRP, even when levels are within the clinically normal range of less than eight milligrams per deciliter (Wallach 2001) have been used to predict the risk of cardiovascular disease as well as type 2 diabetes in individuals who appear healthy (Ridker PM *et al.*, 2000; Ford 1999; Freeman *et al.*, 2002; Pradhan *et al.*, 2001). Serum CRP concentrations of less than 1.5 milligrams per liter have been found in young, healthy men and women in the resting state (de Maat and Kluff, 2001). In the event of trauma, microbial infection, tissue injury, disease, inflammation these very small concentrations can be one thousand times greater than normally observed (de Maat and Kluff, 2001).

Pradhan *et al.*, 2001 measured C-reactive protein levels in 27, 628 healthy middle-aged women and reported that elevated baseline levels of CRP not only predicted the development, but also confirmed clinically diagnosed type 2 diabetes (Pradhan *et al.*,

2001). Freeman and colleagues evaluated 5,345 men and found that CRP predicted the development of type 2 diabetes in middle-aged men as well (Freeman *et al.*, 2002). These predictions were independent of body mass index (BMI) and glucose concentrations in men (Freeman *et al.*, 2002) and BMI, family history of diabetes, physical activity, and hormone replacement therapy in women (Pradhan *et al.*, 2001). Using data obtained from the Third National Health and Nutrition Examination Survey (NHANES III), Ford examined the relationship between CRP, BMI, and diabetes status among 16,573 NHANES III participants. His findings revealed elevated CRP levels in obese persons and persons with diabetes (Ford 1999). The association between obesity and increased CRP levels is believed to stem from the fact that adipose tissue is a site of IL-6 synthesis, and since CRP is associated with liver biosynthesis of IL-6 (Pradhan *et al.*, 2001), IL-6 levels can increase CRP gene expression (de Maat and Klufft 2001). However, increased measurements of CRP are not always associated with increased levels of IL-6. This is because CRP has a longer plasma half-life and is not affected by diurnal changes when compared to IL-6 (Pradhan *et al.*, 2001).

The primary objective for evaluation of CRP levels in our study was not intended to solely assess the effects of resistant starch intake. CRP levels were also evaluated in order to determine if participants that may not have shown other clinical indices of diabetes, may actually be at an earlier stage of the disease process, because CRP has been implicated in the pathogenesis of type 2 diabetes mellitus (Ford 1999, Pradhan *et al.*, 2001, Freeman *et al.*, 2002.)

Glucagon-Like Peptide-1 (GLP-1)

Glucagon-Like Peptide-1 (GLP-1) is an intestinal endocrine hormone, synthesized in the L cells of the small and large intestine (Drucker 2001). It exists primarily in two molecular forms GLP-1 (7-36) amide and GLP-1 (7-37). GLP-1 (7-36) is the major biologically active circulating form and was assayed in this research study (Linco Research, Inc. St. Charles, MO).

GLP-1 regulates nutrients such as glucose, fatty acids, and dietary fiber by way of gastric emptying and inhibiting food intake (Drucker 2001). These same nutrients can also stimulate the release of GLP-1 (Fehmann *et al.*, 1995). GLP-1 levels drastically increase after eating (MacDonald *et al.*, 2002). Following nutrient absorption, GLP-1 regulates blood glucose levels by stimulating glucose dependent insulin secretion, insulin biosynthesis, islet proliferation and neogenesis, and inhibiting glucagon secretion (Drucker 2001).

Dipeptidyl Peptidase (DPP-IV) is an amino peptidase present in serum, which inhibits the degradation of GLP-1 (MacDonald *et al.*, 2002). DPP-IV was used as an inhibitor and added to the test tubes of the blood samples as they were drawn from each participant in order to prevent degradation of GLP-1. Glucagon-like peptide 1 was measured in this study in order to assess the effect of resistant starch intake on this variable, since it has been found to reduce the absorption of nutrients from the gastrointestinal tract, resulting in more stable glucose levels, thereby decreasing the body's need for copious insulin levels following dietary intake (Perfetti *et al.*, 2000).

Insulin

The pancreatic beta cells normally secrete insulin in response to increases in blood glucose levels. Insulin is a physiological hormone that directly lowers plasma glucose and is necessary to maintain blood glucose levels in the normal range (Turner RC, 1993). Insulin binds to specific cell-surface receptors in insulin-sensitive tissues, including fat, muscle, liver, and brain. Insulin also binds to circulating red and white blood cells (Bell and Hockaday, 1996).

Insulin was one of the first hormones measured by radio-immunoassay, where the sensitive isotopic procedures are combined with specific immune methods (Bell and Hockaday, 1996). Presently non-radioactive quantification of human insulin in serum, plasma, and other biological material is available through the use of Human Insulin Enzyme-Linked Immuno Sorbent Assay (ELISA) procedures. ELISA detects and quantifies antigen (foreign macromolecules or microorganisms) and antibody (produced by specialized cells of the immune system and stimulated by antigen presence) reactions.

Osei and colleagues (2004) examined eighty-one African-Americans who were at increased risk for type 2 diabetes. Increased risk was defined as having a first-degree relative with type 2 diabetes. Findings from this study indicated that subjects who progressed to type 2 diabetes during the five to eight years of the follow-up, exhibited decreased glucose effectiveness, as well as decreased insulin secretion and action (Osei *et al.*, 2004). Glucose effectiveness as defined by Osei *et al.*, (2004) as “the ability of glucose to mediate its own glucose disposal as well as suppress basal hepatic glucose production at basal insulin level”.

Short-Chain Fatty Acids (SCFA)

Short-chain fatty acids (SCFA's) are formed by endogenous substrate fermentation of intestinal bacteria, resulting in the formation of acetate, propionate, and butyrate, carbon dioxide, methane, and hydrogen gases (Topping and Clifton, 2001) as well as exogenous carbohydrate, fat, and amino acid metabolism (Wolever *et al.*, 1997). Complex carbohydrates such as polysaccharides, nonstarch polysaccharides, oligosaccharides, and resistant starch serve as primary fermentation substrates (Topping and Clifton, 2001). The digestion of proteins, peptides, and glycoproteins also affects bowel function and metabolism; however, their contribution to SCFA production is smaller (Cummings and Macfarlane, 1991). Absorbed SCFA's are transported to the hepatocytes by way of the portal vein, while any unabsorbed SCFA's serve as metabolites for remaining body organs and tissues (Bergman 1990).

Short-chain fatty acid concentrations in human peripheral venous blood are decreased, because measurements do not include those within portal circulation (Topping and Clifton, 2001). Of the three major SCFA's produced by bacterial fermentation of carbohydrates, acetate has the highest quantifiable concentrations (Topping and Clifton 2001). Acetate is the central short chain fatty acid (SCFA) substrate for hepatic fatty acid and cholesterol synthesis (Nishina *et al.*, 1990), thus measurable concentrations are increased.

Human studies conducted by Wolever *et al.*, (2002) and animal studies conducted by Le Blay *et al.*, (1999) have shown that more than three months of dietary carbohydrate, resistant starch, and fiber dietary changes are necessary in order to demonstrate measurable changes in acetate and butyrate (SCFA) concentrations (Wolever 2002; Le

Blay *et al.*, 1999). SCFA's were measured in the study as an indicator of resistant starch fermentation and also to determine if twelve grams of RS improved colonic metabolism enough to modify disease risk as measured by clinical diabetes markers in this African-Americans population at increased risk for type 2 diabetes. Fermentation in the colon has previously been shown as a way in which RS enhances blood glucose metabolism (Giacco *et al.*, 1998).

Homeostasis Model Assessment (HOMA) Insulin Resistance (IR) and Beta-cell Function

The homeostasis model assessment (HOMA) is a non-invasive mathematical model based on fasting glucose and insulin concentrations (Beck-Nielsen *et al.*, 2005). HOMA has been widely studied and is acceptable as a surrogate model for measuring insulin resistance and beta-cell function (Bergman *et al.*, 1985; Wallace *et al.*, 2004; Radziuk 2000). Insulin resistance and beta cell dysfunction are chief causative factors associated with the development of type 2 DM (Beck-Nielsen *et al.*, 2005).

CHAPTER TWO

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CHAPTER THREE

MATERIALS AND METHODS

Study Design

A randomised, double blind, within-subject, crossover design feeding study was carried out in African-American males and females at increased risk for type 2 DM who reside in Southwest Virginia. This study was conducted to assess the effects of eighty-seven grams of Hi-maize™ 260 added to loaves of bread on each participant's fasting plasma glucose, fructosamine, hemoglobin A1c, C-reactive protein, Glucagon-like peptide-1, insulin, serum acetate, propionate, and butyrate levels (**Figure 3.2**). Hi-maize™ 260, previously known as Novelose 260, was supplied by the National Starch and Chemical Company in Bridgewater, New Jersey. See Appendix E for a complete listing of materials and equipment utilized in this study.

Brief Explanation of the Blood Indicators Used

Fasting plasma glucose measurements are used to identify persons with increased blood glucose levels. Measurement of circulating blood glucose levels were obtained after an overnight fast to minimize effects of food eaten (ADA 2002).

Fructosamine levels are indicators of blood glucose levels over the past two to three weeks (Bunn 1981; Jovanovic *et al.*, 1981; Goldstein *et al.*, 1981; Nathan *et al.*, 1984; Ko *et al.*, 1998).

Hemoglobin A1c levels are indicators of blood glucose levels for the previous two to three months (Osei *et al.*, 2003).

C-reactive protein (CRP) is an indicator of acute inflammation and is useful in diagnosing and monitoring infection and inflammatory diseases. High CRP levels have been found in patients diagnosed with type 2 DM (Pradhan *et al.*, 2001).

Glucagon-like peptide-1 (GLP-1) is a hormone that is released from gastrointestinal cells into the blood once food has been eaten. GLP-1 controls blood glucose levels and aids in insulin secretion (Drucker 2001).

Insulin is a hormone that lowers blood glucose levels.

Short Chain Fatty Acids (SCFAs) are produced in the colon by fermentation of carbohydrates such as Hi-maizeTM 260 (Topping and Clifton 2001).

Dietary intake status was assessed in order to determine the nutrient content of the foods the participants reported as typically consumed.

Homeostasis Model Assessment is an acceptable surrogate mathematical model for measuring insulin resistance and beta-cell function (Matthews *et al.*, 1985; Wallace *et al.*, 2004; Radziuk 2000; Kerskin *et al.*, 2005)

Initial Recruitment and Screening

Seventeen subjects were recruited from predominately African-American churches in the cities of Blacksburg, Radford and Montgomery and Pulaski counties of Virginia. A letter (Appendix A) was written to the pastors of these churches explaining the risk factors for pre-diabetes, along with a request, from the primary investigator to speak briefly with the congregation about the importance of having their risk evaluated for pre-diabetes. The primary investigator's contact information was included in the letter along with the study approval notification from the Institutional Review Board (IRB) at

Virginia Polytechnic Institute and State University (Appendix A). A simple pre-diabetes risk factor checklist was included in the letters distributed to the entire congregation to screen potential subjects and make people aware of their personal risk for type 2 DM.

Eight African-American males and nine African-American females, mean age 36.6 ± 1.55 years, who indicated they had a body mass index (BMI) of twenty-five or greater, according to a provided BMI chart (Clinical Guidelines on the Identification, Evaluation, and Treatment and Obesity in Adults), had a first or second degree relative with type 2 diabetes, were overweight, exercised thirty minutes per week or less, and answered yes to two or more of the statements listed in the “Simple Pre-Diabetes Risk Assessment Checklist” (Appendix A) were invited to participate in the study. People who were pregnant or had been previously diagnosed with type 1 or type 2 DM were excluded from the study. In addition any persons who were currently taking any medication to control blood glucose levels were not permitted to participate. Two of the female participants originally recruited were unable to complete the study. One had to undergo back surgery and the other was unable to tolerate the required blood samples.

The mean age at baseline for the fifteen participants was 36.6 ± 1.55 years. The mean Body Mass Index (BMI) measured in kg/m^2 was 37.1 ± 2.0 ($n=13$). These data are shown in Table 3.1.

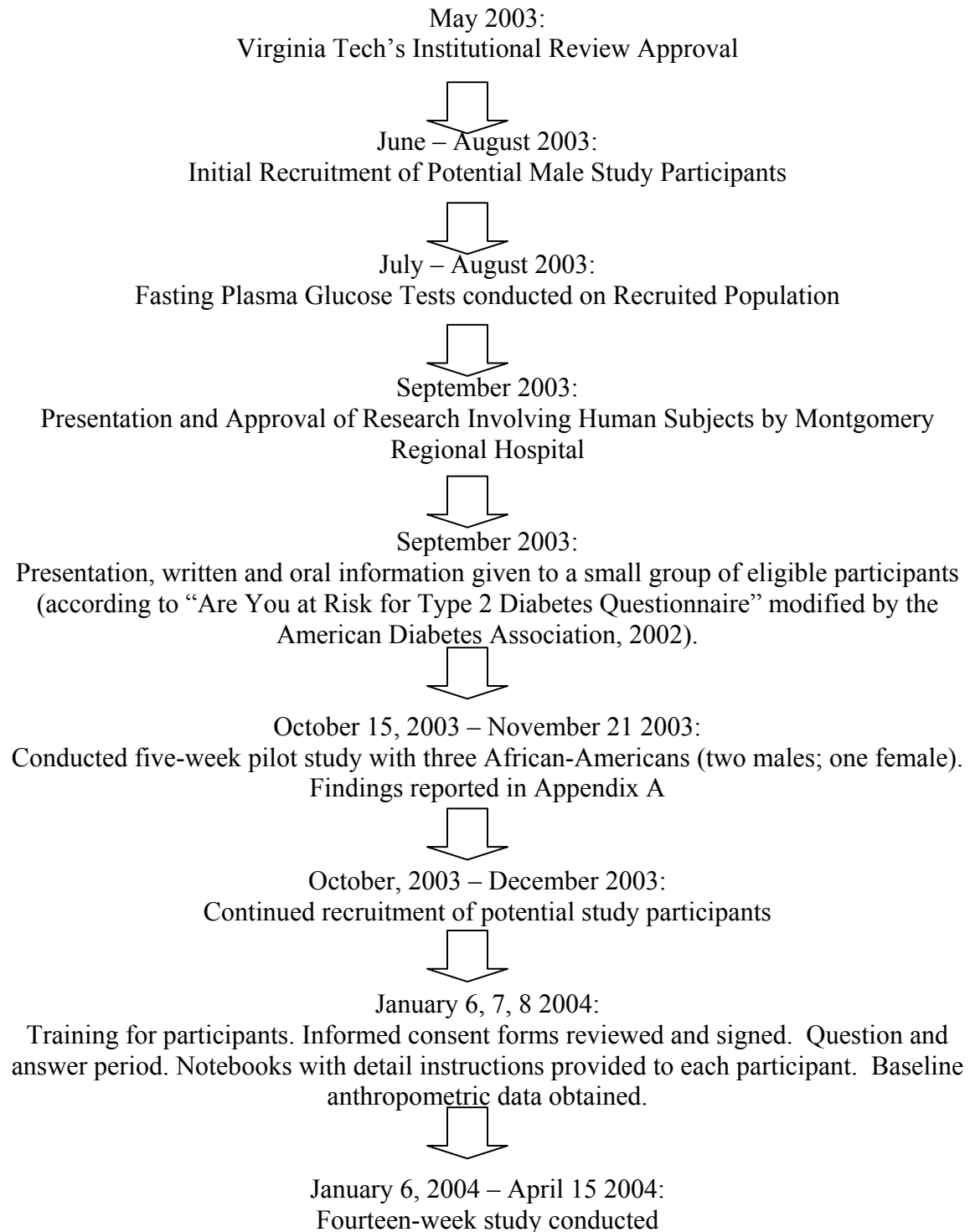
Table 3.1: Baseline Characteristics of the Participants

Variable	Baseline	Sample size
Age (y)	36.6 ± 1.55	(n=15)
Weight (lb)	256.0 ± 18.1	(n=13)
Height (in)	69.17 ± 1.2	(n=15)
BMI (kg/m ²)	37.1 ± 2.0	(n=13)
WC (in)	43.9 ± 2.1	(n=12)
SBP (mmHg)	119.7 ± 6.98	(n=9)
DBP (mmHg)	79.6 ± 2.3	(n=9)
FPG (mg/dL)	99.86 ± 2.63	(n=15)
Fructosamine (mmol/L)	2.48 ± 0.12	(n=15)
HbA1c (%)	6.9 ± 0.11	(n=15)
Insulin (μU/mL)	10.99 ± 1.43	(n=15)
CRP (mg/dL)	0.62 ± 0.16	(n=15)
GLP-1 (pM)	No baseline data to report	
HOMA-IR	3.51 ± 0.76	(n=13)
HOMA Beta cell (%)	43.26 ± 8.07	(n=13)

Data are shown as mean ± SEM. Age: years, BMI: Body Mass Index, WC: Waist Circumference, SBP: Systolic Blood Pressure, DBP: Diastolic Blood Pressure, FPG: Fasting Plasma Glucose, HbA1c: Hemoglobin A1c, CRP: C-Reactive Protein, GLP-1: Glucagon-Like Peptide-One, HOMA-IR: Homeostasis Model-Insulin Resistance, HOMA Beta cell: Homeostasis Model Beta Cell Function.

Figure 3.1

Study Timeline



Procedures

Each participant was randomly assigned to a six-week feeding treatment period of Hi-maize™ 260 resistant starch added to bread or a six-week feeding treatment period of control bread, which contained no added resistant starch. Nine hundred and sixty grams of Hi-maize™ 260 was added to the dough mixture, which was divided into eleven loaves. Each loaf contained approximately eighty-seven grams of Hi-maize™ 260 prior to baking. Each bread loaf contained twenty-one slices, excluding the ends of the bread loaves. Participants were asked to eat three slices of bread daily, including weekends during each six-week study period. Any slices of bread that were not eaten on one day were to be eaten the following day. Each slice of Hi-maize™ 260 bread, contained four point one grams of Hi-maize™ 260 prior to baking. Therefore each participant was asked to consume 12.39 grams of Hi-maize™ 260 prior to baking, during the six-week period he or she consumed the resistant starch bread.

A two-week washout period, where no resistant starch or control bread was eaten followed the first six-week bread feeding period in order to minimize carry-over effects. After the two-week washout period, participant's were crossed-over, meaning those that consumed the bread containing resistant starch during the initial six-week feeding period consumed the control bread, and those that consumed the control bread during the initial six-week feeding bread consumed the resistant starch bread. In this manner each subject served as his or her own control.

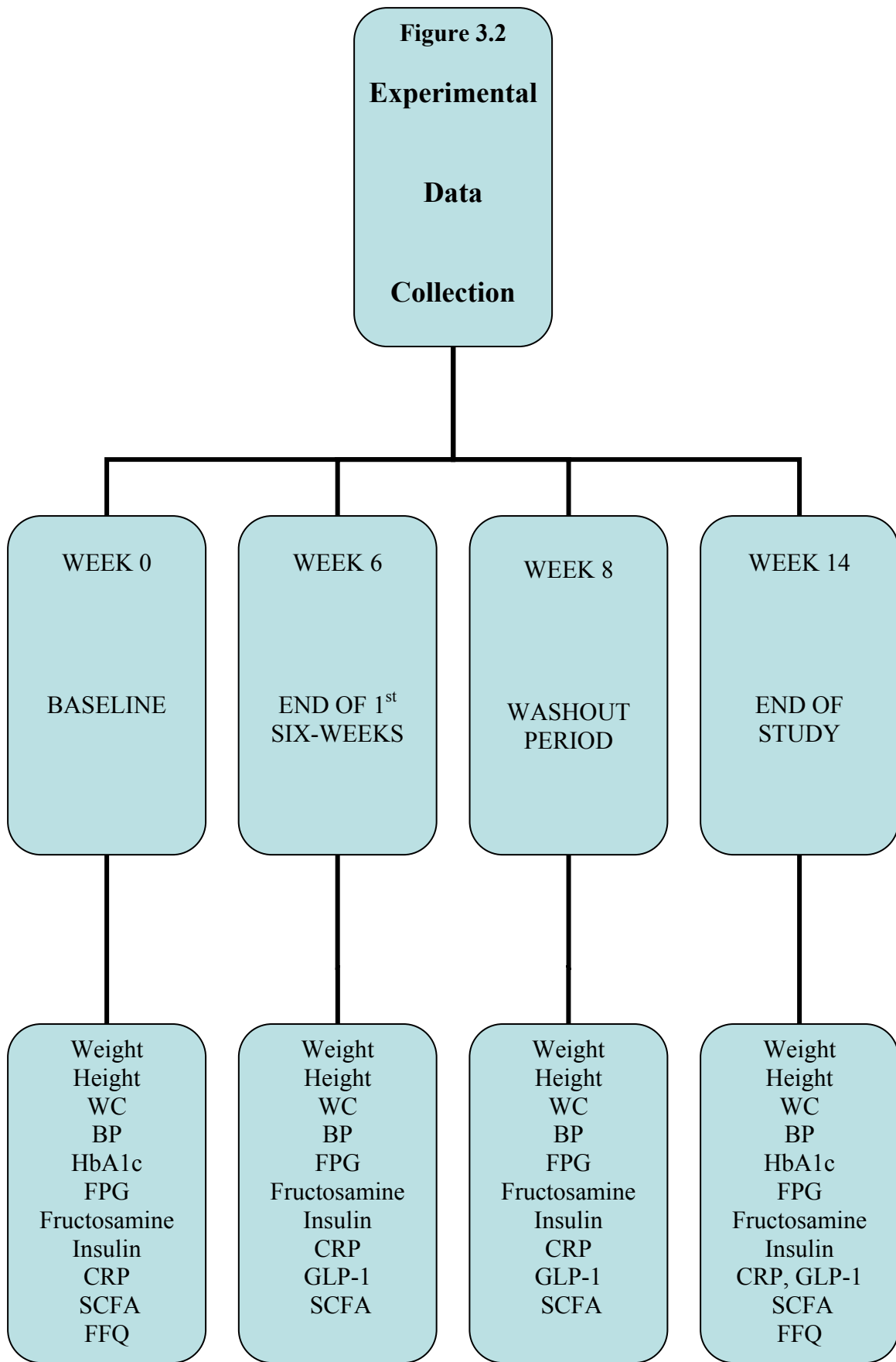
“Our Daily Bread Bakery” located in Blacksburg, Virginia used a modified recipe obtained from the National Starch and Chemical Company, Bridgewater, New Jersey to bake both the control and resistant starch bread. The recipe is located in (Appendix B).

The loaves of bread were baked fresh weekly and were sliced by the bakery. The primary investigator weighed, labeled, and hand delivered one loaf per week to each participant.

Blood samples were drawn at Montgomery Regional Hospital located in Blacksburg, Virginia from participants at the beginning of the study (baseline), after the first six-week feeding period, at the end of the two-week washout period, and at the end of the last six-week feeding period (see **Figure 3.2**). Blood was drawn from subjects after they underwent a ten to twelve hour overnight fast (only water and necessary medication's were allowed). Blood analyses were performed four times during the study period for fasting plasma glucose, fructosamine, C-reactive protein, insulin, and short chain fatty acid levels, including serum acetate, serum propionate, and serum butyrate. Hemoglobin A1c measurements were determined prior to the beginning of the study and at the end of the study. There was no baseline data available for glucagon-like peptide-1. However, glucagon-like peptide-1 measurements were taken at the end of the first six-week period, after the two-week washout period, and at the end of the last six-week period.

Anthropometric data (weight, height, blood pressure, waist circumference) were obtained four times during the study (at the beginning of the study (baseline), end of first six-week feeding period, end of two-week washout period and at the end of last six-week period). A bowel-symptom questionnaire was provided for each participant in the event they experienced gastrointestinal discomfort (Appendix C). A compliance survey sheet was also provided to gauge compliance (Appendix C). The subjects were asked not to

alter their normal physical-activity routines during the entire study period. Participants were asked to substitute the study bread for the bread they would normally eat.



Anthropometry

Heights and weights were measured in the fasting state with each participant wearing lightweight clothing, without shoes. Height was measured using a wall-mounted stadiometer (Heightronic™, Measurement Concepts, North Bend, WA). The participant's feet were placed together with their heels against the measuring board. Each subject stood erect, and looked straight ahead. The top of the ear and outer corner of the eye was in a line parallel to the floor. A horizontal bar or stadiometer was lowered to rest flat on top of the head and height was measured to the nearest 0.1 pounds (lb). (Mahan and Escott-Stump 1996). Weight was measured using a digital scale (Scaletronix, Wheaton, IL) and was recorded to the nearest 0.1 pound. (Mahan and Escott-Stump 1996). Body height and weight were used to calculate body mass index (BMI) measured in kilograms of weight divided by height in meters squared (kg/m^2).

Waist circumference

Waist circumference was measured to the nearest 0.01 centimeter with a measuring tape over the widest part of the gluteal region and the waist circumference was recorded by the primary investigator.

Blood Pressure Measurement

The primary investigator measured the participant's blood pressure in the Wallace Annex laboratory or Montgomery Regional Hospital using a sphygmomanometer. Blood pressure was recorded to the nearest 2 mmHg on the right arm, in supine position, after a ten-minute rest.

Blood Analysis

Trained phlebotomists at Montgomery Regional Hospital in Blacksburg, Virginia obtained five vials of blood during four separate visits by each study participant. Blood was drawn the week before the study began, at the end of the first six-week treatment period, at the end of the two-week washout period, and at the end of the second six-week treatment period. Blood was drawn from a vein (venipuncture). The puncture site was cleaned with antiseptic, and a tourniquet (an elastic band) was placed around the upper arm to apply pressure and restrict blood flow through the vein, allowing the veins below the tourniquet to distend (fill with blood). A needle was inserted into the vein, and the blood was collected in an airtight vial or a syringe. During the procedure, the tourniquet was removed to restore circulation. Once the blood was collected, the needle was removed, and the puncture site was covered to stop any bleeding.

Determination of Human C-Reactive Protein Human CRP ELISA Kit #1000, Alpha Diagnostic International, San Antonio, TX 28238

Blood was allowed to clot and the serum was separated by centrifugation at room temperature for C-reactive protein (CRP) measurements. The serum samples were stored at -20 °C until assayed.

C-reactive protein levels were determined using a C-Reactive Protein Enzyme-Linked Immunosorbent Assay (ELISA) KIT, #100, Alpha Diagnostic International, San Antonio, Texas. This human CRP ELISA kit is based on simultaneous binding of human CRP from serum samples to two antibodies. One antibody is immobilized on the microtiter

well plate, and the other antibody is conjugated to the enzyme horseradish peroxidase (HRP).

All reagents and serum samples were allowed to reach room temperature. Serum samples were diluted 1:100 (5 μ l sample in 500 μ l of diluent) using sample diluent (normal saline) provided in the kit. Ten μ l of each Standard (5, 10, 25, 50, 100 ng/mL) and diluted samples were added (using a multichannel pipet) to the microtiter anti-human CRP coated strip plate 96 well plate. Standards and patient samples were run in duplicate. One hundred microliters of antibody-enzyme conjugate was added to each well and mixed gently. The plate was covered and incubated for thirty minutes at room temperature.

The plate was aspirated and wells were washed five times with 300 μ l deionized water. The plate was tapped on paper towels between washings to ensure proper washing. One hundred μ l of HRP-substrate solution and 100 μ l of chromogenic substrate were mixed and added to each well. The plate was covered and incubated for ten minutes at room temperature. A blue color developed indicating the presence of CRP. The reaction was stopped after ten minutes by adding 50 μ l of 1 N sulfuric acid to each well. Wells were mixed gently until a yellow color developed. The absorbance was measured at 450 nm using an ELISA μ Quant Universal Microplate Spectrophotometer reader, Bio-Tek Instruments TM, Winooski, Vermont.

Mean CRP concentrations of standards were 6, 36, and 80 ng/mL. Intra assay percentage coefficients of variation for these means were 3, 2.1, and 4.5%, respectively. Mean CRP concentrations were 5.8, 35, and 88 ng/mL. Inter assay percentage coefficient

of variation for the means were 7, 2.7, and 5%, respectively (Alpha Diagnostic, San Antonio, Texas 78238).

Determination of Glucagon-Like Peptide-1 (Glucagon-Like Peptide-1 (Active) ELISA Kit 96-Well Plate (Cat. # EGLP-35K) Linco Research, Inc. St. Charles, MO, 63304.

Whole blood was collected by venipuncture in ice-cooled Vacutainer ethylenediaminetetraacetic acid (EDTA) plasma tubes from each participant after an overnight fast of ten to twelve hours. Ten microliters of dipeptidyl peptidase (DPP-IV) inhibitor per millimeter of blood was added less than thirty seconds after collection. DPP-IV is an amino peptidase present in serum, which causes degradation of GLP-1 and results in the formation of an inactive metabolite. DPP-IV inhibitor inhibits the degradation of this DPP-IV enzyme preventing GLP-1 degradation.

Blood specimens were either centrifuged immediately at 1000xg for ten minutes in a refrigerated centrifuge or tubes were placed on ice and centrifuged within one hour. Serum samples were stored at -70 °C until assay was performed. The GLP-1 Active ELISA assay was run in duplicate in a 200 microliter total volume.

Day One Glucagon Like-Peptide-1 Procedures

A ten X wash buffer concentration 10mM phosphate buffered saline (PBS) and tween 20 and sodium azide were diluted 1:10 with deionized water. Three hundred microliters of diluted wash buffer was added to each well and incubated at room temperature for five minutes. Wash buffer was decanted from each well and excess buffer was tapped onto absorbent paper towels.

Two hundred microliters of non-specific binding (NSB) assay buffer was added to the first two wells, according to manufactures well placement instructions. (Assay buffer contained 0.05M PBS, protease inhibitors, tween 20, 0.08% sodium azide (preservative) and 1% BSA). One hundred microliters of assay buffer was added to the remaining wells. One hundred microliters of standards (2 pM, 5 pM, 10 pM, 20 pM, 50 pM, 100 pM) were added in ascending order to each standard well. Standards contained GLP-1 (7-36 amide) in assay buffer. One hundred microliters of ELISA Quality Control 1 (EQC) and ELISA Quality Control 2 was added to the next two sets of wells. [EQC1 and EQC 2 contained various peptides including GLP-1 (7-36 amide) in QC buffer].

One hundred microliters of each of the subject's samples were added in duplicate to the remaining wells. The plate was gently shaken to ensure proper mixing. The plate was covered with plate sealer and incubated overnight for 20 to 24 hours at 4 °C.

Day Two Glucagon-Like Peptide-1 Procedures

Liquid was decanted from the plate and excess fluid tapped on absorbent paper towels. The plate was washed five times with 300 microliters wash buffer per well with a five-minute incubation at room temperature in wash buffer with the fourth wash. Excess buffer was tapped out onto absorbent paper towels after the fifth wash.

Two hundred microliters detection conjugate was immediately added. (Detection conjugate contained anti GLP-1-alkaline phosphatase conjugate). The plate was covered and incubated for two hours at room temperature. Afterwards, the liquid was decanted from the plate. The plate was washed three times with 300 microliters wash buffer. Excess buffer was tapped out onto absorbent paper towels.

Two hundred microliters diluted substrate (containing 4-methylumbelliferyl phosphate, hydrated in 1 ml deionized water and used in a 1:200 dilution) was added to each well. Wells were incubated for 20 to 45 minutes in the dark at room temperature. The ELISA plate was placed on a FLx800 Microplate Fluorescence Reader (Bio-Tek InstrumentTM Winooski, Vermont) to ensure there was significant signal-to-noise ratio with the lowest point on the standard curve (2 pM) and the highest standard point (100 pM). When sufficient fluorochrome had been generated, 50 microliters of stop solution was added to each well in the same order as the substrate was added. The plate was incubated at room temperature for an additional five minutes in the dark to arrest phosphatase activity. After incubation, the plate was read on FLx800 Microplate Fluorescence Reader (Bio-Tek InstrumentTM Winooski, Vermont) fluorescence plate reader with an excitation/emission wavelength of 355nm/460nm.

The lowest level of GLP-1 that could be detected was 2 pM. The intra assay and inter assay coefficients of GLP-1 were 7 and 12, respectively (Linco Research, Inc. St. Charles, MO 63304, USA).

Determination of Insulin Human Insulin ELISA Kit #EZH1 (Linco Research, Inc. St. Charles, MO, 63304)

Whole blood was collected by venipuncture and drawn into a Vacutainer serum tube that contained no anticoagulant from each participant after an overnight fast of ten to twelve hours. Blood was allowed to clot at room temperature for 30 minutes.

Specimens were promptly centrifuged immediately at 2,000 to 3,000 xg for 15 minutes at 4 ± 2 °C. Serum samples were transferred and stored at -20 °C until the insulin assay was performed.

The 10X concentrated horseradish peroxidase (HRP) in wash buffer (50mM tris buffered saline containing tween-20) was diluted ten-fold by mixing the entire buffer content with 270 mLs of deionized water. The assay plate was removed from the foil pouch and each well was filled with 300 microliters of diluted HRP wash buffer. The plate was incubated at room temperature for five minutes. The wash buffer was decanted and the residual amount was removed from all wells by inverting the plate and tapping it smartly onto absorbent paper towels several towels. Wells were not allowed to dry out before proceeding to the next step.

The human insulin detection antibody solution (pre-titered biotinylated monoclonal mouse anti-human insulin antibody) was transferred to a reagent reservoir and 20 microliters was added to each well with a multi-channel pipette. Twenty microliters matrix solution (heat-treated stripped off the clot human serum) was added to the non-specific binding (NSB), standard, and control wells. Twenty microliters human insulin standards (2, 5, 10, 20, 50, 100, and 200 μ U/mL) were added in duplicate in the order of ascending concentration to the appropriate wells. Twenty microliters of Quality Control

1 (QC1) and Quality Control 2 (QC2) were added to the appropriate wells. QC1 and QC2 were various levels of purified recombinant human insulin in assay buffer. Twenty microliters of each sample was added sequentially in duplicate to the remaining wells within one hour. The plate was covered with the plate sealer and incubated at room temperature for one hour on a plate shaker (Ocelet Model 2603000™, Fisher Scientific) set to rotate at moderate speed (approximately 400 to 500 rpm).

The plate sealer was removed and solutions decanted from the plate. The plate was tapped as before to remove residual solutions from the wells. The wells were washed with 300 microliters HRP wash buffer three times. HRP wash buffer was decanted and tapped after each wash to remove residual buffer. One hundred microliters of enzyme solution (pre-titered streptavidin-horseradish peroxidase conjugate in buffer) was added to each well. The plate was covered with plate sealer and incubated for 30 minutes on a plate shaker (Ocelet Model 2603000™, Fisher Scientific) set to rotate at moderate speed (approximately 400 to 500 rpm). The plate sealer was removed and solutions decanted from the plate. The plate was tapped as before to remove residual solutions from the wells. Wells were washed five times with three hundred microliters of HRP each time. The wells were decanted and tapped after each wash to remove residual buffer. One hundred microliters of substrate solution (3, 3', 5,5'-tetramethylbenzidine in buffer) was added to each well.

The plate was covered with plate sealer and incubated for eight to ten minutes on a plate shaker (Ocelet Model 2603000™, Fisher Scientific) set to rotate at moderate speed (approximately 400 to 500 rpm). A blue color formed in the participants and insulin standard wells with intensity proportional to increasing concentrations of insulin. The

plate sealer was removed and 100 microliters of stop solution (0.3 M HCL) was added to each well. The plate was shaken by hand to ensure complete mixing of solution in all of the wells. The blue color turned yellow after acidification. The wells were examined to ensure there were no air bubbles. The plate was gently shaken to remove air bubbles. The absorbance was measured at 450 nm on the uQuant Universal Microplate Spectrophotometer (Bio-Tek InstrumentsTM, Winooski, Vermont).

The sensitivity of the insulin assay was 2.0 $\mu\text{U/mL}$. The intra- and inter assay coefficients of the variation were 6.94 (insulin mean of 7.0 $\mu\text{U/mL}$) and 10.2 (insulin mean of 6.2 $\mu\text{U/mL}$), respectively (Linco Research, Inc. St. Charles, Missouri, 63304).

Determination of Glycated Hemoglobin as measured by the Hemoglobin A1c (HbA1c) Method. Glycohemoglobin Pre-Fil®, Procedure #P350, Standbio Laboratory, Boerne, Texas 78006).

Glycated Hemoglobin is formed progressively and irreversibly in the erythrocyte red blood cell during the 90 to 120 day life cycle of the red blood cell. The red cell glycohemoglobin concentration is based on the average blood glucose concentration during its life cycle and is stable for the life of the cell. Increased glucose levels result in more glycated hemoglobin. The measurement of glycohemoglobin as a percent of total hemoglobin is a valuable tool used to evaluate long-term glycemic control.

Whole blood was collected in blood tubes containing EDTA from each participant. Five hundred microliters of lysing reagent (potassium cyanide, 10 mmol/L and surfactants) was pipetted into tubes labeled standard, patient sample numbers, and control. One hundred microliters of well-mixed blood sample was pipetted into appropriately labeled tubes and vortexed on the S/P Vortex Mixer, Baxter Scientific Products (speed was set at six). The tubes stood at room temperature (15-30 °C) for five minutes in order to complete hemolysis.

Pre-filled resin tubes were labeled standard, patient sample numbers, and control. One hundred microliters of the previously prepared hemolysate was pipetted into appropriately labeled resin tubes. A resin separator was positioned in the pre-filled tubes so the rubber sleeve of the resin separator was approximately one to two centimeters above the liquid level. The tubes were mixed on a rocker for five minutes. After the five minute mixing, resin separators were pushed into the tubes until all of the resin was firmly packed into the bottom of each thirteen millimeter tube. The supernate was poured directly into separate cuvettes for absorbance readings. The percent of

glycohemoglobin for the standard, patient samples, and controls was obtained from absorbance readings taken with a Thermo Spectronic Genesys 10uV™ spectrophotometer that was set at 415 nm. The spectrophotometer was blanked with water and all readings were recorded within 60 minutes.

Five mLs of deionized water was pipetted into tubes labeled standard, patient sample numbers and control in order to perform the total hemoglobin assay. Twenty microliters of previously prepared hemolysate was pipetted into appropriately labeled tubes. The tubes were vortexed to ensure proper mixing and transferred to cuvettes for absorbance readings. The total hemoglobin for the standard, patient samples, and controls was obtained using a spectrophotometer that was set at 415 nanometers. The spectrophotometer was blanked with water and all readings were taken within 60 minutes. Calculations were performed according to Stanbio Glycohemoglobin Pre-Fil® Procedure No. P350 (Boerne, Texas 78006) and located in the appendix for blood analysis and calculations (Appendix B). Results were reported as Hemoglobin A1c.

The intra assay coefficients of variation for Glycohemoglobin A were 2.7 percent for a normal HbA1c blood sample of 7.8 percent and 1.7 percent for an abnormal glycohemoglobin value of 13.4 percent. Inter assay coefficients of variation for Glycohemoglobin A were 4.1 percent for normal (7.6%) Glycohemoglobin A blood samples and 4.6 percent for abnormal Glycohemoglobin A blood samples (13.0%) (Stanbio Laboratory data, Boerne, Texas 78006).

Determination of Fasting Plasma Glucose Stanbio Glucose Liquicolor® Procedure #1070 (Stanbio Laboratory, Boerne, Texas 78006).

The fasting plasma glucose (FPG) test is the preferred screening method for diabetes (ADA, 2002). This test requires fasting (water allowed) for at least eight to ten hours before the sample of blood is drawn. FPG measures the amount of glucose present in the blood. If the test results are greater than 100mg/dL but less than 126 mg/dL for readings taken on two separate days, pre-diabetes also known as impaired fasting glucose is indicated (ADA, 2003).

In order to obtain FPG levels from each participant, blood was allowed to clot; however, serum was removed from clot by centrifugation within 30 minutes of collection in order to prevent glycolysis. Serum samples were refrigerated a maximum of 40 hours before they were assayed. This time period was followed according to the manufactures protocol (Stanbio Glucose Liquicolor Procedures No. 1070, Boerne Texas). All reagents and serum were allowed to come to room temperature before the assay was performed.

One mL of reagent containing 0.2 mmol/L 4-aminoantipyrine, 15.0 U/mL glucose oxidase, 1.2 U/mL peroxidase and 4.0 mmol/L was pipetted into culture tubes labeled reagent blank, standard, and participant's samples were identified with randomly assigned numbers. Ten microliters of standard was pipetted into the appropriately labeled culture tube. Ten microliters of participant sample was pipetted into the appropriately labeled culture tubes. All culture tubes were vortexed on the S/P Vortex Mixer, Baxter Scientific Products (speed was set at six) to ensure proper mixing and incubated (Fisher Scientific Isotemp Incubator™) at 37 °C for five minutes. Reagent blank, standards, participant samples were all transferred to cuvettes and absorbance was read at 500

nanometers using a Thermo Spectronic Genesys 10 uVTM spectrophotometer.

Calculations were performed according to Stanbio Laboratory Glucose LiquiColor® Procedure #1070 (Boerne, TX) which is located in the appendix for blood analysis and calculations (Appendix B).

The intra assay coefficients of variation for fasting serum glucose were 1.6 and 1.2%. The inter assay coefficients of variation for fasting serum glucose were 3.0 and 2.0% (Stanbio Laboratory data, Boerne, Texas 78006).

Determination of Fructosamine Reagent Set Pointe Scientific, Inc. Lincoln Park, Michigan, 48416.

Serum was separated from cells as quickly as possible by centrifugation. Serum specimens were stored at 2 to 8 °C and analyzed within one week.

Culture tubes were labeled standard, control, and sample. One millimeter of reagent (nitroblue tetrazolium (NBT) 0.57mM) was pipetted into all culture tubes and pre-warmed for five minutes at 37 °C in a Fisher Scientific Isotemp™ Incubator. Fifty microliters of standard, control, and participant sample was added to each pre-warmed culture tube in duplicate. All culture tubes were placed in the incubator at 37 °C for ten minutes. After the ten-minute incubation period elapsed, the liquid from each tube was placed in a cuvette and the absorbance was read on a Thermo Spectronic Genesys 10uV Spectrophotometer™ at 550nm. The observed absorbance readings were recorded.

Cuvettes were return to the Fisher Scientific Isotemp Incubator™, which was set at 37 °C for another five minutes. After exactly five minutes of incubation, cuvettes were read again at 550 nanometers. Calculations were made according to Pointe Scientific's Fructosamine Reagent Set Protocol (Lincoln Park, Michigan) and are located in the appendix for blood analysis and calculations Appendix B.

The assay range of fructosamine was 1.0 to 10.0 mmol/L. The within day coefficient of variation for fructosamine mean 1.97 was 2.0% and the coefficient of variation for within day fructosamine mean of 5.57 was 1.8% (n=20). The day to day coefficient of variation for fructosamine mean 1.91 was 3.1%. The day to day coefficient of variation for fructosamine mean of 5.72 was 2.4 % (n=20) Pointe Scientific, Lincoln Park, Michigan 48146, USA.

DETERMINATION OF SHORT CHAIN FATTY ACIDS IN SERUM BY GAS CHROMATOGRAPHY (Laboratory analysis performed in Dr. Thomas D.M. Wolever's Laboratory, University of Toronto, Canada (Kervan Rivera Laboratory Specialist)).

Serum Short Chain Fatty Acids (SCFA's) were measured by Gas Chromatography (GC) after previous micro filtration and vacuum distillation. 2-ethylbutyric acid was used as internal standard (ISTD).

EXPERIMENTAL PROCEDURE

Cleaning filters

The filters (concentrator bodies) were filled with water and spun at 2200 g for ten minutes. The filtrate was discarded. This procedure was repeated three times. This step removed trace amounts of glycerol and sodium azide used as membrane preservatives. Each filtrate tube (conical) was rinsed three times with water and dried by flushing air. The concentrator bodies were dried by spinning the whole filtration unit upside down for five minutes at 100-200 g. Filters can be filled with water and kept under refrigeration overnight.

Filtering

The sufficient amount of filters required for a run (one filter per sample) was labeled and one to one and one-half mLs of serum was added to each using disposable small pasteur pipettes. The technician was careful to avoid the formation of bubbles inside the filter. The filtration units were capped and inserted into the centrifuge. When fixed angle rotors were used, the filters were angled so that the printed windows face upwards/outwards. The filters were centrifuged at 5000 g for 90 minutes at 4 °C. The concentrator bodies were discarded and the tubes containing the filtrate were capped. Filtrates can be kept at least three weeks at -70 °C before distilling.

Standard curve

On the same day of the analysis 40, 80, 160 and 240 microliter of SCFA working standard were added into two mL chromatographic vials. Then 100 microliter of ISTD was added to each vial and filled to one mL with water. The gas chromatograph was started and the standard curve was injected while the distillation progressed.

Distillation (Based on the procedure described by Tollinger *et al.*, (1979) with modifications.)

The distilling system was set by turning on the vacuum pump at least 20 minutes before the distillation. The principal vacuum valve (lever-operated) was kept closed during this time. Two clean test tubes per sample were prepared to be used as distilling tubes. Twenty-five microliters (μL) of (internal) ISTD working standard and 225 μL of sample filtrate were transferred to one of the clean test tubes. Tubes were mixed well by swirling. Duplicate blank and control serum tubes were included. Two hundred twenty-five μL of water was used instead of serum for the blank.

The same number of clean test tubes was prepared to be used as receiver tubes. The duplicate receiver tubes were connected to the right side of each “U” tube. In order to facilitate the distillation process, the sample was evenly dispersed and frozen inside the distilling tube. The samples were then spread by hand swirling and immersed immediately into Styrofoam cups filled with liquid nitrogen.

Once frozen, the sample tubes were connected to the left sides of the “U” tubes and immersed in approximately 75 percent of the tube height into liquid nitrogen. The vacuum release valve was closed (not overly tight). The lever-operated principal vacuum valve was opened. It was important to wait until the entire system reached vacuum.

Once the vacuum gauge indicated at least 8×10^{-2} torr, the Teflon stopcocks were closed. After this was done, the principal lever-operated vacuum valve was closed. The vacuum was then released from the upper manifold. The vacuum was now confined to the “U” tubes.

The collection tubes were immersed in liquid nitrogen at this time. The distillation was started by immersing the sample tubes in water at room temperature, for approximately four to six seconds. The sample tubes were removed from water and allowed to distill for four to ten seconds. This procedure was repeated until a dry residue was obtained. Completeness was confirmed when the tubes were not cold to the touch. The sample tubes containing the dry residue were disconnected and discarded. The collection tubes containing the distillates were stored in dry ice.

Gas Chromatograph (GC) Analysis

The receiver tubes were allowed to thaw at room temperature and the distillates were transferred to properly labeled GC vials, using pasteur pipettes. They were sealed using clear PTFE/red natural rubber septa caps. The vials were transferred to the injector tray following the programmed sequence.

A 30m x 0.53mm x 1.0 μ m column was used. The helium flow rate was set at 107cm/sec for the carrier gas. The oven was set at 80 °C for 0.1 minutes, 80 °C to 125 °C at 10 °C per minute, 125 °C to 137 °C at 5 °C per minute, 137 °C for 0.1 minutes, 127 °C to 220 °C at 45 °C per minute and 220 °C for two minutes. The injector temperature was 130 °C and the detector was 250 °C.

**Determination of Resistant Starch AOAC Method 2002.02/AACC Method 32-40
Megazyme International Ireland Ltd., Bray Business Park, Bray, Co. Wicklow
Ireland.**

The resistant starch (RS) content of the control and resistant starch bread loaves consumed by study participants was analyzed according to the AOAC Method 2002.02, using the Megazyme™ Resistant Starch Assay Procedure (Wicklow, Ireland). The AOAC Method 2002.02 (McCleary & Monaghan, 2002; McCleary *et al.*, 2002) is the only internationally accepted method for determining the amount of resistant starch in foods (Brown, 2004). All resistant starch and non-resistant (soluble starch) were reported on a dry weight basis. Holm and Bjorck (1992) reported the average resistant starch in Western diets ranged from 2.5 to 5 grams per day.

Preparation of Test Samples

Approximately 50 grams of bread sample from each week of the feeding study were ground separately in a Black and Decker Smart Grind Coffee Grinder small enough to pass a 1.0 mm sieve. The ground bread samples were transferred to a wide-mouthed plastic jar (urine specimen jar). Ground bread samples were mixed well by shaking and inversion (all of the transferred material). Samples were freeze-dried for 48 hours and the moisture content was determined.

Determination of non-resistant starch

One hundred plus or minus five micrograms of bread sample was accurately weighed and placed directly into a 16 x 125 mL corning culture tube screw cap. The tube was gently tapped to ensure the sample fell to the bottom. Four mLs of pancreatic L-amylase (10 mg/ml) containing amyloglucosidase (3 U/ml) was added to each tube.

Tubes were tightly capped and mixed on a vortex mixer. After mixing tubes were attached horizontally on a shaking water bath.

The tubes were incubated at 37 °C with continuous shaking (200 strokes/min for **exactly sixteen hours**). After exactly sixteen hours, the tubes were removed from the water bath and excess surface water was removed with a paper towel. The tube caps were removed. Tube contents were treated with four mL of 99% ethanol OR 99% (Industrial Methylated Spirits; denatured ethanol OR ethanol; 95% ethanol plus 5% methanol) IMS with vigorous stirring on a vortex mixer for ten seconds. Non-capped tubes were centrifuged at 1,500 g (~ 3,000 rpm) for ten minutes.

Supernatants were carefully decanted and pellets were resuspended in two milliliters of 50% ethanol with vigorous stirring on a vortex mixer for approximately ten seconds. Another six milliliters of 50% IMS was added. The tubes were mixed and centrifuged again at 1,500g for ten minutes.

The supernatants were decanted and the previous suspension and centrifugation steps were repeated one more time. The supernatants were carefully decanted and tubes inverted on absorbent paper to drain excess liquid.

Measurement of Resistant Starch

A 5 x 15 millimeter magnetic stirrer bar and two mLs of 2M KOH was added to each tube. The pellets were resuspended. The resistant starch was dissolved by stirring for approximately 20 minutes in an ice/water bath over a magnetic stirrer. This suspension was not allowed to be mixed on a vortex mixer as this may have caused the starch to emulsify.

Tube contents were vigorously stirred as the KOH solution was added to avoid formation of a lump of starch material that would have been difficult to dissolve. Eight mLs of 1.2 M sodium acetate buffer (pH 3.8) was added to each tube with stirring on the magnetic stirrer. Immediately added 0.1 mL of Amyloglucosidase (AMG) (3300 U/mL) and mixed well. Tubes were placed in a water bath at 50 °C and incubated for 30 minutes with intermittent mixing on a vortex mixer.

Determination of resistant starch content for samples containing > 10% RS

The contents of the tube were quantitatively transferred to a 100 mL volumetric flask (using a water wash bottle). An external magnet was used to retain the stirrer bar in the tube while washing the solution from the tube with the water wash bottle. The volume was adjusted to 100 mL with water and mixed well. An aliquot of the solution was centrifuged at 1,500 g for ten minutes.

Determination of resistant starch content for samples containing < 10 % RS

Tubes were directly centrifuged at 1,500 g for ten minutes, without being diluted. The final volume in the tube was approximately 10.3 mLs (the appropriate allowance for volume was made in the calculations). Duplicate 0.1 mL aliquots of samples containing more than 10% RS were transferred into 16 x 100 mm glass test tubes. These samples were then treated with three mLs of glucose oxidase/peroxidase (GOPOD) reagent color reagent) and incubated at 50 °C for 20 minutes. The absorbance of each solution was measured at 510 nm against the reagent blank.

Reagent blank solutions were prepared by mixing 0.1 mL of 0.1 M sodium acetate buffer (pH 4.5) and 3.0 mL of GOPOD reagent (Megazyme International Ireland Ltd).

Glucose standards were prepared in quadruplicate by mixing 0.1 mL of glucose (1mg/mL and 3.0 mL of GOPOD reagent). Calculations were performed according to Megazyme AOAC Method 2002.02/AACC Method 32-40 Megazyme International Ireland Ltd., Bray Business Park, Bray, Co. Wicklow Ireland. Calculations are located in the appendix for blood analysis and calculations (Appendix B).

Measurement of Non-Resistant (Solubilised) Starch

The supernatant solutions obtained on centrifugation of the initial incubation were combined. The 0.1 mL aliquots of this solution (in duplicate) were incubated with 3.0 mL of GOPOD reagent for 20 minutes at 50 °C. The absorbance was measured at 510 nm against a reagent blank. The content of non-resistant (solubilised) starch was calculated. The total starch content is the sum of resistant starch plus non-resistant (solubilised) starch. Calculations are located in the appendix for blood analysis and calculations (Appendix B).

Homeostatic model assessment (HOMA) for assessing Beta-cell function (HOMA-Beta) and insulin resistance index (HOMA-IR)

The homeostatic model assessment (HOMA) for assessing Beta-cell function (HOMA-Beta) and insulin resistance index (HOMA-IR) were calculated according to the formulas originally described by Matthews *et al.*, (1985) and modified by Osei *et al.*, 2003 are located in the appendix for blood analysis and calculations (Appendix B).

Block Dietary Questionnaire

Participants were given the Block Dietary Questionnaire (Block Dietary Data Systems, Berkeley California) two times during the study. This Block Dietary Questionnaire was filled out by each participant after written and verbal instructions were provided in order to assess usual dietary practices and establish baseline nutrient intake values. Nutrient intake values were also obtained at the culmination of the 14-week study in order to evaluate dietary practices during the study period. The Block Dietary Questionnaire is a quantitative food frequency questionnaire. It contains more than 100 food items and corresponds to 90 percent of the nutrient intakes listed in the National Health and Nutrition Examination Survey III database (DHHS Pub. No. (PHS) 94-1308, 1994).

Statistical Model Description

Statistical analysis was performed using the Statistical Analysis System for Windows, (SASTM, version 9.1). The SASTM Proc Mixed procedure was used for calculations (SAS Institute, Cary NC). All results are presented as means \pm standard error of the mean (SEM). A nested design with gender and sequence at the main level and a crossover design at the nested level were performed. Following the baseline data collection, the within subject crossover design was separated by a washout period (**Figure 3.2**). The full model contained the gender, sequence (either resistant starch or control bread first), their interaction, the treatment, the interaction with gender and a carryover effect nested in treatment. The models were reduced to determine the significant effects by excluding interactions or the carry-over effect if not significant at the 25% level for participant's fasting serum glucose, fructosamine, C-reactive protein, Glucagon-like peptide-1, insulin, HOMA-Insulin Resistance and HOMA-Beta cell function, serum acetate, propionate, and butyrate levels.

Statistical analyses were performed using Student's t-test (paired) for the dietary data and ANOVA with repeated measures, where appropriate. The Bonferroni method was used for *post hoc* testing. Hemoglobin A1c statistical analysis was also obtained from a Student's paired t-test and a two sample t-test for gender of the paired differences. A *P* value less than 0.05 was considered statistically significant.

Statistical Data Analysis for Resistant Starch & Non-Resistant Solubilized Starch

Duplicate samples were analyzed. Absorbance data were converted by reference formulas (Appendix D) into g/100g dry weight for resistant starch and soluble starch. Results were expressed as means \pm standard deviation.

CHAPTER THREE

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CHAPTER FOUR

RESULTS

Resistant Starch

The resistant starch content of the control loaves of bread for the study ranged from 1.91 g/100 g to 3.21 g/100g, with a mean of 2.64 g/100g and standard deviation of 0.42. Whereas, the resistant starch content of the resistant starch loaves of bread for the study ranged from 8.68 g/100g to 11.01 g/100g with a mean RS level of 10.17 g/ 100g and a standard deviation of 0.69, as shown in Table 4.1.

The (non-resistant) solubilised starch content of the control loaves of bread for the study ranged from 65.38 g/100g to 70.60 g/100g, with a mean of 67.22 g/100g and a standard deviation of 1.99. The soluble starch content of the resistant starch loaves of bread for the study ranged from 58.48 g/100g to 69.14 g/100g, with a mean of 64.09 g/100 and a standard deviation of 3.72, as observed in Table 4.1. Complete resistant starch laboratory results are outlined in Appendix D.

Table 4.1 Resistant Starch and Soluble Starch Content of Bread Loaves

Control Bread	Resistant Starch Content	Soluble Starch Content
	(g/100g)	(g/100g)
Mean	2.64	67.22
Standard Deviation	0.42	1.99
Standard Error of Mean	0.13	0.63
Resistant Starch Bread	Resistant Starch Content	Soluble Starch Content
	(g/100g)	(g/100g)
Mean	10.17	64.09
Standard Deviation	0.69	3.72
Standard Error of Mean	0.23	1.18

Anthropometric Data

Anthropometric characteristics for male and female subjects combined are presented in Table 4.2. No significant differences were found for age, weight, height, body mass index, or systolic and diastolic blood pressure. Waist circumferences were statistically less at weeks six, eight, and fourteen ($P \leq 0.05$). See Tables 4.5 A & B for detailed waist circumference data. Complete data tables are also available for weight (Tables 4.3 A & B), and body mass index see Tables 4.4 A & B. Participant's complete systolic and diastolic blood pressure measurement data are located in Tables 4.6 A & B and 4.7 A & B, respectively.

Table 4.2: Anthropometric Data for Male and Female Participants

Variable	Baseline	wk 6	wk 8	wk 14
Age (y)	36.6 ± 1.55 (n=15)	36.6 ± 1.55 (n=15)	36.6 ± 1.55 (n=15)	36.6 ± 1.55 (n=15)
Weight (lb)	256.0 ± 18.1 (n=13)	254.2 ± 15.7 (n=15)	255.7 ± 15.9 (n=15)	253.6 ± 15.4 (n=15)
Height (in)	69.17 ± 1.2 (n=15)	69.17 ± 1.2 (n=15)	69.17 ± 1.2 (n=15)	69.17 ± 1.2 (n=15)
BMI (kg/m ²)	37.1 ± 2.0 (n=13)	37.2 ± 1.9 (n=15)	37.7 ± 1.9 (n=15)	37.4 ± 1.8 (n=15)
WC (in)	43.9 ± 2.1 (n=12)	43.7 ± 1.7 (n=15)	43.3 ± 1.8 (n=15)	43.2 ± 1.8 (n=15)
SBP (mmHg)	119.7 ± 6.98 (n=9)	117.9 ± 3.6 (n=15)	117.5 ± 3.2 (n=15)	119.7 ± 3.6 (n=15)
DBP (mmHg)	79.6 ± 2.3 (n=9)	78.0 ± 2.2 (n=15)	76.1 ± 2.6 (n=15)	81.6 ± 1.7 (n=15)

Data are shown as mean ± SE. BMI: body mass index, WC: waist circumference, SBP: systolic blood pressure, DBP: diastolic blood pressure.

Anthropometric Data

Body weight

The mean \pm standard errors of weight are shown in Tables 4.3 A and B. Mean baseline weights for males and females combined were 250.6 ± 18.11 pounds (n=13). Male subjects had a mean weight of 270.04 ± 29.79 at baseline (n=7). Whereas female subjects had a mean baseline weight of 227.92 ± 16.52 (n=6).

The mean \pm standard errors of the mean body weight for male and female participants combined during consumption of resistant starch bread was 253.89 ± 15.73 pounds (n=15) and 253.92 ± 15.32 pounds during the control bread feeding period for all participants (n=15). The mean \pm standard errors of the mean body weight when male participants received resistant starch bread were 269.76 ± 24.96 pounds and 266.31 ± 24.18 pounds during the control bread periods. The mean \pm standard errors of the mean body weight when all female participants consumed resistant starch bread were 235.76 ± 17.40 pounds and 239.76 ± 18.21 pounds following control bread consumption. Adults, who gain weight, increase their risk of type 2 diabetes (Colditz et al., 1990; Hu et al., 2001; Carey et al., 1997; Chan et al., 1994).

The adjusted P values from statistical analysis of weight levels are given in Table 4.3 C (Appendix F). No significant differences were found in weight due to treatment or gender. No statistically significant differences were found for treatment, gender, or gender-treatment interactions during the fourteen-week study ($P > 0.05$).

**Table 4.3 A Resistant Starch and Control Bread Feeding Periods
Weight (Pounds) MEAN ± SEM**

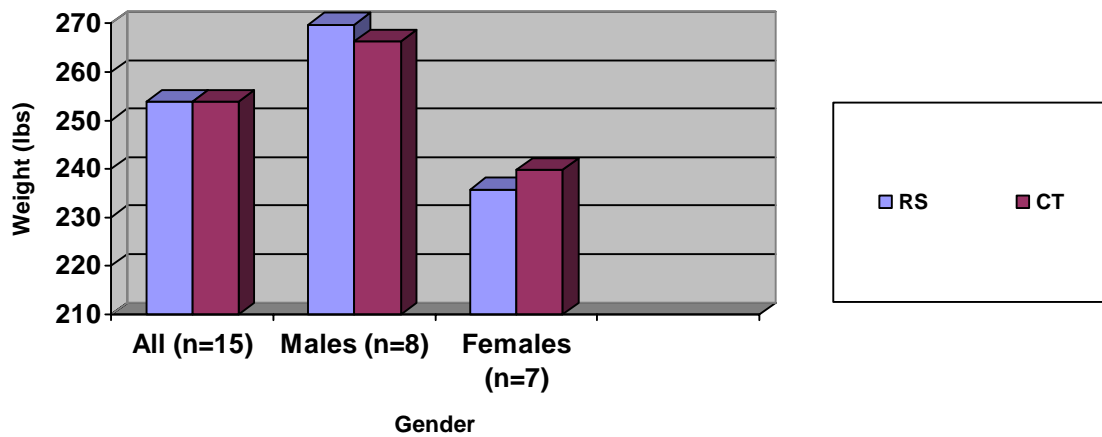
	All Subjects	Males	Females
RS Periods	253.89 ± 15.73	269.76 ± 24.96	235.76 ± 17.40
Sample size	(n=15)	(n=8)	(n=7)
CT Periods	253.92 ± 15.32	266.31 ± 24.18	239.76 ± 18.21
Sample size	(n=15)	(n=8)	(n=7)

RS Periods = Two six-week periods participants consumed bread containing added resistant starch

CT Periods = Two six-week periods participants consumed bread containing no added resistant starch

Graph 4.3 Resistant Starch & Control Bread Feeding Periods

Weight (lbs) Mean ± SEM



Body Mass Index

The mean \pm standard errors of body mass index (BMI; kg/m^2) data are shown in Tables 4.4 A and B. Mean baseline BMI measurements for males and females combined were 37.09 ± 2.02 ($n=13$). Male subjects had a mean BMI of 36.94 ± 3.41 at baseline ($n=7$). Whereas female subjects had a mean baseline BMI of 37.27 ± 2.23 ($n=6$).

The mean \pm standard errors of the mean BMI for male and female participants combined during consumption of resistant starch bread was 37.4 ± 1.82 (kg/m^2) ($n=15$) and 37.24 ± 1.83 (kg/m^2) during the control bread feeding period for all participants ($n=15$). The mean \pm standard errors of the mean BMI when male participants received resistant starch bread were 36.59 ± 2.82 (kg/m^2) and 35.91 ± 2.76 (kg/m^2) during the control bread periods. The mean \pm standard errors of the mean BMI when all female participants consumed resistant starch bread were 38.33 ± 2.40 (kg/m^2) and 38.76 ± 2.42 (kg/m^2) following control bread consumption.

According to the World Health Organization's Obesity Classifications, adults with a BMI ≥ 25 kg/m^2 are considered overweight (WHO, 2000). The World Health Organization further classifies obesity into categories identified as Grades I, II, and III.

Grade I adults have a BMI of 30 to 34.9 kg/m^2 . Grade II adult's BMI ranges from 35 to 39.9 kg/m^2 and Grade III is classified as ≥ 40 kg/m^2 . It has also been suggested this range may not be correct for all ethnicities (ADA, 2004). Obese adults (BMI ≥ 30 kg/m^2 and ≤ 39.99 kg/m^2) are three to seven times more likely to have type 2 diabetes (Mokdad et al., 2003; Field et al., 2001). In addition, adults with a BMI > 35 kg/m^2 increase their risk for developing type 2 diabetes by twenty percent when compared to their counterparts with a normal BMI between 18.5 and 24.9 kg/m^2 (Mokdad et al.,

2003). Adults with a BMI ≥ 40 kg/m² are considered morbidly obese (Mokdad et al., 2003).

The adjusted P values from statistical analysis of body mass index are given in Table 4.4 C (Appendix F). No significant differences were found for treatment and gender effects. No significant differences were found for gender-treatment interactions for body mass index levels during the fourteen-week study ($P > 0.05$).

**Table 4.4 A Resistant Starch and Control Bread Feeding Periods
BMI Values (kg/m²) MEAN ± SEM**

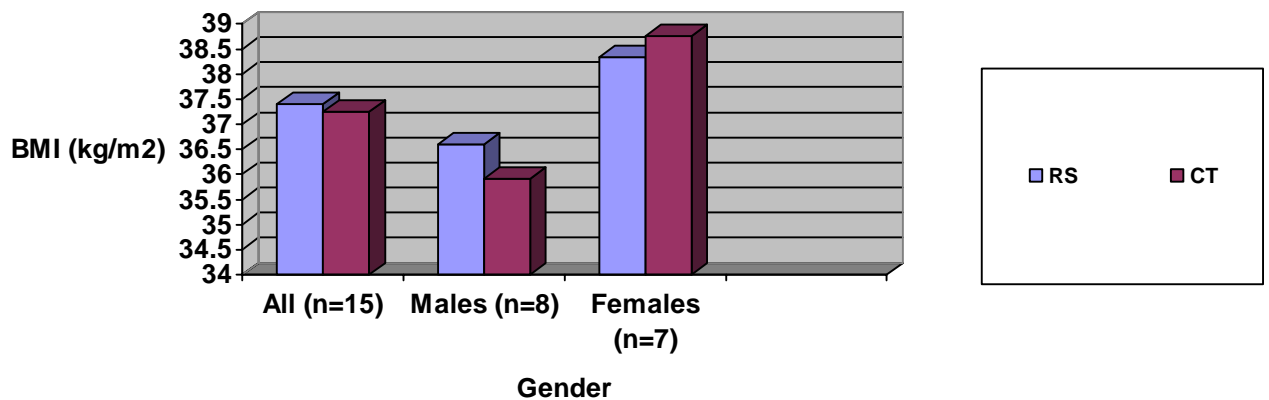
	All Subjects	Males	Females
RS Periods	37.4 ± 1.82	36.59 ± 2.82	38.33 ± 2.40
Sample size	(n=15)	(n=8)	(n=7)
CT Periods	37.24 ± 1.83	35.91 ± 2.76	38.76 ± 2.42
Sample size	(n=15)	(n=8)	(n=7)

RS Periods = Two six-week periods participants consumed bread containing added resistant starch

CT Periods = Two six-week periods participants consumed bread containing no added resistant starch

Graph 4.4 Resistant Starch & Control Bread Feeding Periods

BMI (kg/m²) Mean ± SEM



Waist Circumference

The mean \pm standard errors of waist circumference measurements are shown in Tables 4.5 A and B. Mean baseline waist circumference measurements \pm standard error for males and females participants in this study were 43.95 ± 2.13 inches (n=12). Mean baseline waist circumference measurement \pm standard error for males was 45.02 ± 3.57 inches (n=6). Female subjects reported a mean waist circumference measurement \pm standard error of 42.83 ± 2.59 inches (n=6) at baseline.

The mean \pm standard errors of the mean waist circumference for male and female participants combined during consumption of resistant starch bread was 43.36 ± 1.79 (inches) (n=14) and 43.57 ± 1.68 (inches) during the control bread feeding period for all participants (n=15). The mean \pm standard errors of the mean waist circumference when male participants received resistant starch bread were 44.41 ± 2.72 (inches) (n=7) and 44.61 ± 2.48 (inches) (n=8) during the control bread periods. The mean \pm standard errors of the mean waist circumferences when all female participants consumed resistant starch bread were 40.41 ± 2.46 (inches) and 42.37 ± 2.33 (inches) (n=7) following control bread consumption.

Waist circumference measurements are considered a predictive measurement of the independent effects of abdominal obesity (Wang et al., 2005; Zhou 2002; Seidell et al., 2001). The American Heart Association and the United States Department of Agriculture recommends a cutoff of 40 inches (102 centimeters) for a waist circumference for men and a cutoff of 35 inches (88 centimeters) for women (American Heart Association 1992; US Department of Agriculture (Publication 261-495/20124). Lower waist circumference cutoffs have been proposed for men (37 inches or 94

centimeters) and women (31.5 inches or 80 centimeters) (Han et al., 1995; Lean et al., 1995; Zhu et al., 2002).

The adjusted P values from statistical analysis of waist circumference measurements are given in Table 4.5 C (Appendix F). There were some significant differences in waist circumference levels due to treatment effects. Waist circumference levels of all fifteen subjects approached significance (0.059) after consumption of the control bread for six weeks when compared to baseline. Waist circumference levels of all subjects also approached significance ($P = 0.0578$) following consumption of resistant starch bread when compared to baseline values. In addition, waist circumference levels were statistically significant ($P = 0.0077$) following the two-week washout period compared to baseline.

**Table 4.5 A Resistant Starch and Control Bread Feeding Periods
Waist Circumference (inches) MEAN ± SEM**

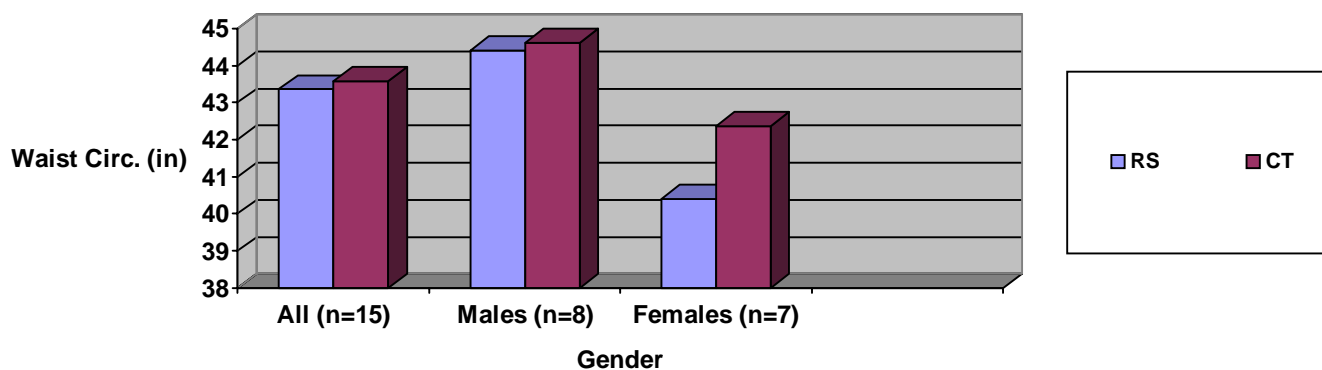
	All Subjects	Males	Females
RS Periods	43.36 ± 1.79	44.41 ± 2.72	40.41 ± 2.46
Sample size	(n=14)	(n=7)	(n=7)
CT Periods	43.57 ± 1.68	44.61 ± 2.48	42.37 ± 2.33
Sample size	(n=15)	(n=8)	(n=7)

RS Periods = Two six-week periods participants consumed bread containing added resistant starch

CT Periods = Two six-week periods participants consumed bread containing no added resistant starch

Graph 4.5 Resistant Starch & Control Bread Feeding Periods

Waist Circumference (in) Mean ± SEM



Systolic and Diastolic Blood Pressure

The mean \pm standard errors of systolic and diastolic blood pressure levels are shown in Tables 4.6 A and B and 4.7 A and B, respectively. Mean baseline systolic/diastolic blood pressure (SBP/DBP) measurements for males and females combined were 119/79.56 mmHg (n=9). Male subjects had a mean SBP/DBP measurement of 128/82.75 mmHg (n=4), whereas female subjects had a mean baseline SBP/DBP of 113/78 mmHg. The mean systolic/diastolic blood pressure for male and female participants combined during consumption of resistant starch bread were 118.4/78.93 mmHg and 119.27/80.67 mmHg during the time periods the participants consumed control bread (n=15).

**Table 4.6 A Resistant Starch and Control Bread Feeding Periods
Systolic Blood Pressure (mmHg) MEAN ± SEM**

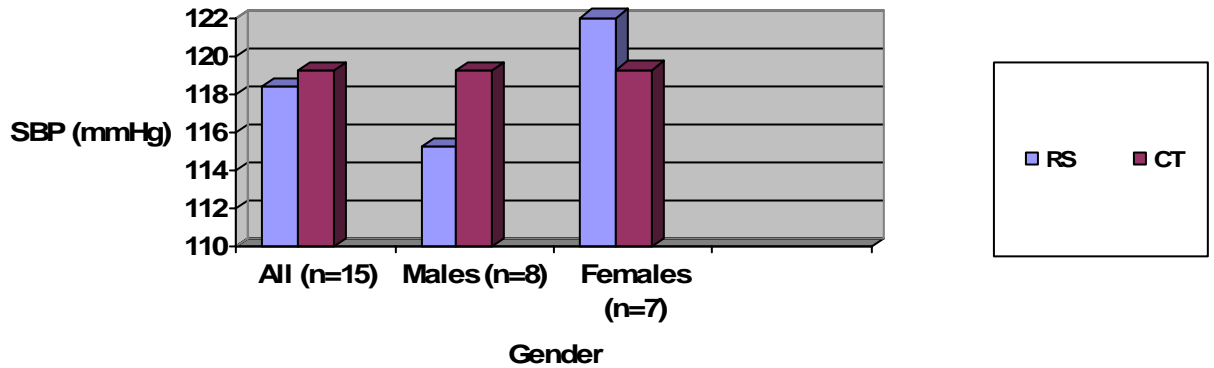
	All Subjects	Males	Females
RS Periods	118.4 ± 3.63	115.25 ± 4.36	122 ± 6.03
Sample size	(n=15)	(n=8)	(n=7)
CT Periods	119.27 ± 3.53	119.25 ± 3.78	119.29 ± 6.59
Sample size	(n=15)	(n=8)	(n=7)

RS Periods = Two six-week periods participants consumed bread containing added resistant starch

CT Periods = Two six-week periods participants consumed bread containing no added resistant starch

Graph 4.6 Resistant Starch & Control Bread Feeding Periods

SBP (mmHg) Mean ± SEM



**Table 4.7 A Resistant Starch and Control Bread Feeding Periods
Diastolic Blood Pressure (mmHg) MEAN \pm SEM**

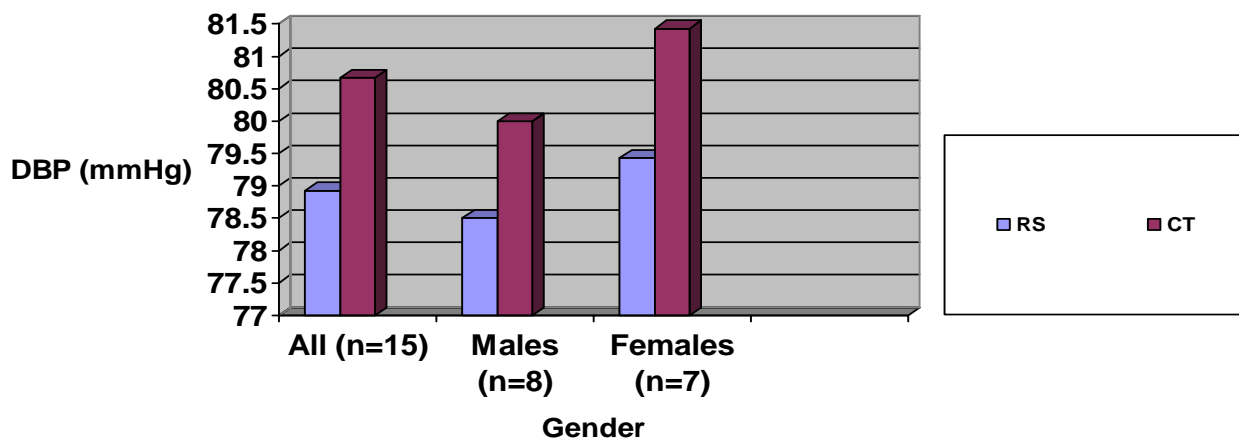
	All Subjects	Males	Females
RS Periods	78.93 \pm 1.47	78.5 \pm 1.24	79.43 \pm 29.5
Sample size	(n=15)	(n=8)	(n=7)
CT Periods	80.67 \pm 2.48	80 \pm 3.27	81.43 \pm 4.04
Sample size	(n=15)	(n=8)	(n=7)

RS Periods = Two six-week periods participants consumed bread containing added resistant starch

CT Periods = Two six-week periods participants consumed bread containing no added resistant starch

Graph 4.7 Resistant Starch & Control Bread Feeding Periods

DBP (mmHg) Mean \pm SEM



The adjusted P values from statistical analysis of systolic and diastolic blood pressure levels are given in Table 4.6 C and 4.7 C (Appendix F), respectively. No significant differences were found for treatment or gender effects for systolic or diastolic blood pressure levels during the fourteen-week study ($P > 0.05$).

According to the Seventh Report of the Joint National Committee (JNC 7) on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure, normal systolic and diastolic blood pressure levels are less than 120/80 mmHg. JNC 7 introduced a new classification including normal and borderline “prehypertension”. The most recent classifications for blood pressure values are listed in Table 4.8.

Table 4.8 Blood Pressure Classification

JNC 7 Category	SBP/DBP
NORMAL	<120/80
NORMAL PREHYPERTENSION	120-129/80-84
BORDERLINE PREHYPERTENSION	130-139/85-89
HYPERTENSION	≥ 140/90
STAGE 1	140-159/90-99
STAGE 2	160-179/100-109
STAGE 3	≥ 180/110

SBP = systolic blood pressure. DBP = diastolic blood pressure.

Table 4.8 adapted from The Seventh Report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure (2003).

Dietary Data

The mean \pm standard error of daily macronutrient and micronutrient status are shown in Table 4.9. Dietary analysis demonstrated no significant difference between baseline values (defined as one year pre-intervention) and follow-up (defined as week's one through fourteen), with one exception; fruit servings were significantly greater during the follow-up period ($P = 0.03978$) than at baseline.

Table 4.9 Baseline & Follow-Up Data

† Daily Macronutrient & Micronutrient Status of Male & Female Subjects

Variable	Baseline - Previous Year (n=14)	Follow-Up (Week 1 - 14) (n=14)	P value
Energy (kcal)	1932.9 ± 208.1	2080.6 ± 243.2	1
Protein (g)	79.55 ± 8.7	77.6 ± 9.6	1
Fat (g)	94.6 ± 10.6	96.9 ± 11.6	1
Carbohydrate (g)	195.3 ± 22.7	195.3 ± 22.7	0.12275
Calcium (mg)	585.2 ± 68.2	229.4 ± 26.9	1
Phosphorus (mg)	1177.9 ± 116.5	1227.1 ± 148.5	1
Iron (mg)	12.5 ± 1.2	13.5 ± 1.4	1
Sodium (mg)	2634.9 ± 246.4	27199.9 ± 309.3	1
Potassium	2423.2 ± 249.6	2723.5 ± 319.4	1
Vitamin A (RE)	867.9 ± 140.76	843.49 ± 108.9	1
Vitamin A (IU)	6361.1 ± 1119.5	5836.2 ± 752.5	1
Riboflavin (mg)	1.46 ± 0.16	1.6 ± 0.21	1
Vitamin C	83.4 ± 11.8	101.7 ± 16.1	1
Niacin (mg)	23.2 ± 2.3	23.13 ± 2.8	1
Folate (µg)	318.2 ± 30.9	338.4 ± 34.3	1
Fiber (g)	14.2 ± 1.3	15.9 ± 1.6	1
Isoflavone	4.7 ± 2.7	4.6 ± 2.6	1
Glycemic Index Glucose	56.7 ± 1.3	56.4 ± 1.12	1
Glycemic Load Glucose	103.9 ± 13.1	120.7 ± 14.6	0.18278
Glycemic Index Bread	79.4 ± 1.8	78.9 ± 1.56	1
Glycemic Load Bread	145.49 ± 18.3	169.0 ± 20.45	0.18278
Fruit Serving*	0.56 ± 0.08	0.79 ± 0.10	0.03978
Grain Serving	4.14 ± 0.53	4.31 ± 0.52	1
Global Vegetable	3.58 ± 0.48	3.46 ± 0.48	1
Global Cereal	1.57 ± 0.25	2.08 ± 0.29	1
Global Fruit	2.29 ± 0.16	2.34 ± 0.23	1
Whole Grain	0.64 ± 0.15	1.07 ± 0.21	0.07715

Data are shown as Mean ± SEM

***Significantly different by paired t-test (P < 0.05) Bonferroni test statistic**

† Dietary data were calculated using the Block Dietary Questionnaire (Block 1998)

Blood glucose

The means \pm standard errors of the mean fasting plasma glucose (FPG) levels are shown in Tables 4.10 A and B. The mean fasting plasma glucose levels, for male and female participants combined were 98.8 ± 3.11 mg/dL during the six-week resistant starch bread periods and 92.13 ± 2.39 mg/dL during the six-week control bread periods. The mean \pm standard errors of the mean baseline fasting plasma glucose level for all male subjects (n=8) was 103.38 ± 3.43 mg/dL. Whereas, the mean \pm standard errors of the mean baseline fasting plasma glucose level for all female subjects (n=7) was 95.86 ± 3.76 mg/dL. The mean \pm standard errors of the mean fasting plasma glucose levels when male participants received resistant starch bread were 95.5 ± 3.11 mg/dL and 92.13 ± 3.58 mg/dL during the control bread periods. The mean \pm standard errors of the mean fasting plasma glucose levels when all female participants consumed resistant starch bread were 102.57 ± 5.57 mg/dL and 92.14 ± 3.40 mg/dL following control bread consumption.

Four of the eight male participants had FPG levels higher than 100 mg/dL, which is clinically recognized by the ADA as pre-diabetes (ADA 2003). These data are shown in Table 4.10 B (Appendix F). In addition, at baseline, four of the seven female participants also had FPG levels above the recommended 100 mg/dL by the American Diabetes Association (ADA 2003) as indicated in Table 4.10 B.

Of particular interest was male subject number one, who was the only participant that had been previously clinically diagnosed with pre-diabetes. His fasting plasma glucose (FPG) level was 122 mg/dL at baseline, which was just below the 126 mg/dL recognized by the American Diabetes Association (ADA) as clinical type 2 diabetes (ADA 2003). Male subject number one's FPG level was lowered to 105 mg/dL after

consuming resistant starch bread for six weeks. Following the two-week washout period, male subject one's FPG was lowered to 98 mg/dL, which is within the clinically recognized normal range for blood glucose values (≤ 100 mg/dL) (ADA 2003). His blood glucose level increased to 106 mg/dL, in the pre-diabetic range, following the six-week consumption of control bread as observed in Table 4.10 B. The Expert Committee on the Diagnosis and Classification of Diabetes Mellitus distinguished persons with Fasting Plasma Glucose (FPG) or Fasting Serum Glucose (FSG) levels between 100 and 125 mg/dL as having pre-diabetes and or impaired glucose tolerance (ADA 2003).

The adjusted P values from statistical analysis of FPG levels are given in Table 4.10 C (Appendix F). There were some significant differences in FPG levels due to treatment effects. FPG levels of all fifteen subjects were significantly lower ($P = 0.0179$) after consuming the control bread for six weeks than at baseline. FPG levels of all subjects were also significantly lower ($P < 0.0001$) after the two-week washout period than at baseline. FSG levels were significantly higher ($P < 0.0001$) after consuming the resistant starch bread for six weeks than after the washout period. FPG levels of all fifteen subjects due to consumption of the resistant starch versus the control bread approached significance ($P = 0.0574$).

As indicated in Table 4.10 C, there were no significant differences in the FPG levels of male and female subjects at any time during the study. Significant differences in FPG levels were observed due to gender-treatment interactions. FPG levels of the eight male subjects were significantly lower ($P = 0.0005$) after the two week washout period than at baseline. FPG levels of the eight male subjects approached significance

($P = 0.0674$) when the baseline period was compared to the resistant starch period. The seven female subjects had significantly higher ($P = 0.0068$) FPG levels following six weeks consumption of the resistant starch bread when compared to control bread consumed for the same time period. FPG levels of female subjects were also significantly higher ($P = 0.0002$) after six weeks on the resistant starch bread than after the washout period.

**Table 4.10 A Resistant Starch and Control Bread Feeding Periods
Fasting Plasma Glucose mg/dL MEAN ± SEM**

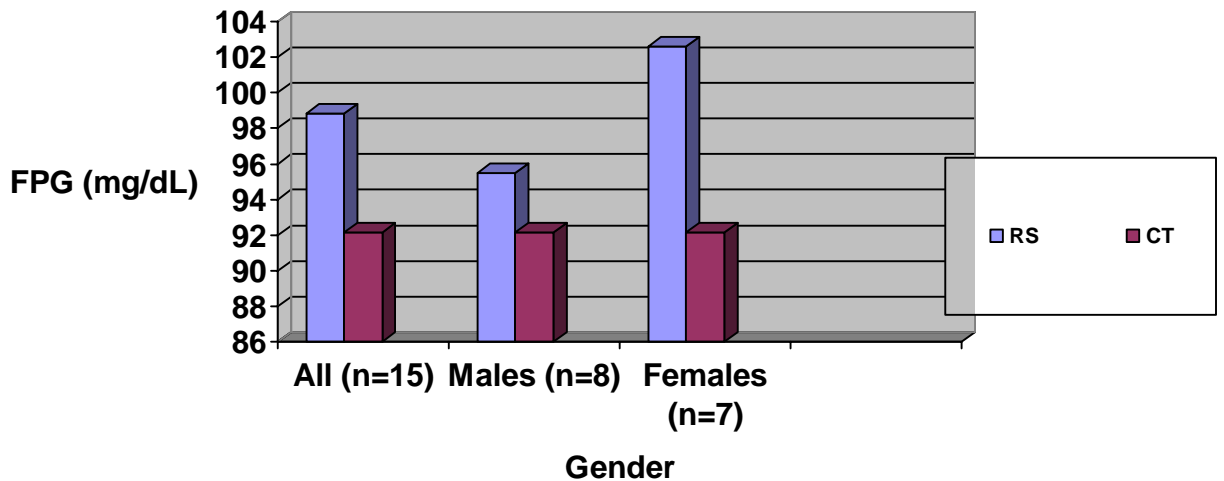
	All Subjects	Males	Females
RS Periods	98.8 ± 3.11	95.5 ± 3.11	102.57 ± 5.57
Sample size	(n=15)	(n=8)	(n=7)
CT Periods	92.13 ± 2.39	92.13 ± 3.58	92.14 ± 3.40
Sample size	(n=15)	(n=8)	(n=7)

RS Periods = Two six-week periods participants consumed bread containing added resistant starch

CT Periods = Two six-week periods participants consumed bread containing no added resistant starch

Graph 4.10 Resistant Starch & Control Bread Feeding Periods FPG (mg/dL)

Mean ± SEM



Fructosamine

The means \pm standard errors of the mean of fructosamine levels for all participants are shown in Tables 4.11 A and B. The mean \pm standard errors of the mean fructosamine levels when male and female results were combined was $2.48 \text{ mmol/L} \pm 0.12$ during the two time periods resistant starch bread was consumed and $2.58 \pm 0.16 \text{ mmol/L}$ during control bread feeding periods. Mean \pm standard errors of the mean fructosamine levels for males was $2.59 \pm 0.19 \text{ mmol/L}$ during the resistant starch feeding periods and $2.83 \pm 0.27 \text{ mmol/L}$ during the time periods males consumed control bread. The mean fructosamine level observed for female participants was $2.36 \pm 0.14 \text{ mmol/L}$ when they consumed resistant starch bread and $2.31 \pm 0.12 \text{ mmol/L}$ during the time periods female participants consumed control bread.

The mean fructosamine level for all male subjects ($n=8$) was $3.7 \pm 0.19 \text{ mmol/L}$ at baseline. Whereas, the mean fructosamine level for all female subjects at baseline ($n=7$) was $3.2 \pm 0.14 \text{ mmol/L}$. Normal adult fructosamine levels are expected to range from 1.61 to 2.68 mmol/L (Tietz, 1996). Female subject one was the only participant with a normal fructosamine level at baseline. However, her fructosamine level approached the high-end of this normal range. Laboratory guidelines advise that fructosamine values should be compared to previous values in the same person rather than to reference interval values (Tietz, 1996; Wallach 2000).

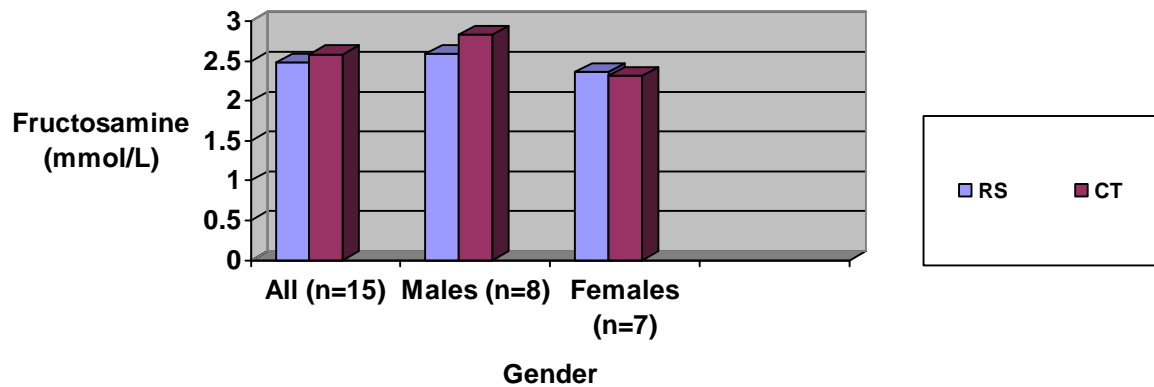
The adjusted P values from statistical analysis of fructosamine levels are given in Table 4.11 C (Appendix F). A few significant differences in fructosamine levels were observed due to treatment effects. Fructosamine levels of all fifteen subjects approached significance ($P = 0.0054$) after subjects consumed the control bread for six weeks

compared to baseline values. Fructosamine levels of all subjects were significantly lower ($P < 0.0012$) after the consumption of resistant starch bread for six weeks than at baseline. Fructosamine levels were also significantly higher ($P < 0.0001$) after the washout period than at baseline. There were no significant differences in fructosamine levels of all fifteen subjects due consumption of the resistant starch versus the control bread ($P = 0.9692$). Significant differences were observed in male and female subjects during the fourteen week study ($P = 0.0236$).

Table 4.11 A Resistant Starch and Control Bread Feeding Periods Fructosamine Levels mmol/L MEAN ± SEM			
	All Subjects	Males	Females
RS Periods	2.48 ± 0.12	2.59 ± 0.19	2.36 ± 0.14
Sample size	(n=15)	(n=8)	(n=7)
CT Periods	2.58 ± 0.16	2.83 ± 0.27	2.31 ± 0.12
Sample size	(n=14)	(n=7)	(n=7)
<p>RS Periods = Two six-week periods participants consumed bread containing added resistant starch</p>		<p>CT Periods = Two six-week periods participants consumed bread containing no added resistant starch</p>	

Graph 4.11 Resistant Starch & Control Bread Feeding Periods

Fructosamine (mmol/L) Mean ± SEM



Hemoglobin A1c

The means \pm standard errors of hemoglobin A1c (HbA1c) levels are shown in Table 4.12. Mean baseline HbA1c levels when male and female participants were combined was 6.9% (n=15). This value was slightly lowered to 6.79% (n=14) at the end of the fourteen-week study, although statistical significance was not found. The mean \pm standard errors for HbA1c values were 6.9% \pm 0.18% and 6.9% \pm 0.14% at baseline for the sequence groups, resistant starch first (n=7) and control treatment first (n=8) groups, respectively. The mean \pm standard error HbA1c values were 6.7% \pm 0.27% and 6.9% \pm 0.27% at the conclusion of the fourteen-week study for the sequence groups, resistant starch first group (n=7) and control treatment first group, respectively. The normal reference range according to the American Diabetes Association, as referenced by the Diabetes Control and Complications Trial (DCTT) (Rohlfing et al., 2002) ranges from 4.0 to 6.0 %.

The two sample t-test for paired differences between males and females was not statistically significantly different (P = 0.4622) between males and females. The paired t-test for differences in baseline hemoglobin A1c levels compared to the end of the study values revealed no significant difference (P = 0.7057).

Table 4.12 Resistant Starch and Control Bread Feeding Periods

Hemoglobin A1c Levels (%)

Hemoglobin A1c (%)	Treatment Sequence	Baseline Week 0	End of Study Week 14
Mean ± SE All Subjects		6.9 ± 0.11 (n=15)	6.79 ± 0.19 (n=14)
Mean ± SE All RS 1st Subjects		6.9 ± 0.18 (n=7)	6.7 ± 0.27 (n=7)
Mean ± SE All CT 1st Subjects		6.9 ± 0.14 (n=8)	6.9 ± 0.27 (n=7)
Male Subject 1	RS:CT	7.4	6.2
Male Subject 2	RS:CT	6.6	6.2
Male Subject 3	RS:CT	6.9	6.1
Male Subject 4	CT:RS	7.3	NS
Male Subject 5	CT:RS	7	7.6
Male Subject 6	RS:CT	6.5	7.5
Male Subject 7	CT:RS	6.3	6.7
Male Subject 8	CT:RS	6.6	7.5
Mean ± SE Male Subjects RS 1st		6.85 ± 0.20 (n=4)	6.5 ± 0.33 (n=4)
Mean ± SE Male Subjects CT 1st		6.8 ± 0.22 (n=4)	7.27 ± 0.28 (n=3)
Mean ± SE All Male Subjects		6.83 ± 0.14 (n=8)	6.23 ± 0.26 (n=8)
Female Subject 1	RS:CT	6.8	6.3
Female Subject 2	CT:RS	6.9	6.4
Female Subject 3	CT:RS	6.7	5.8
Female Subject 4	CT:RS	6.9	6.5
Female Subject 5	RS:CT	6.3	6.7
Female Subject 6	RS:CT	7.6	7.9
Female Subject 7	CT:RS	7.6	7.7
Mean ± SE Female Subjects RS 1st		6.9 ± 0.38 (n=3)	6.97 ± 0.48 (n=3)
Mean ± SE Female Subjects CT 1st		7.03 ± 0.20 (n=4)	6.6 ± 0.80 (n=4)
Mean ± SE All Female Subjects		6.97 ± 0.18 (n=7)	6.78 ± 0.29 (n=7)

RS = Resistant Starch CT = Control NS = No Sample

Insulin

The means \pm standard errors of insulin levels are shown in Tables 4.13 A and B. Normal insulin levels are expected to range from two to twenty-five $\mu\text{U}/\text{mL}$ for adults less than sixty years of age following a twelve-hour fast (Burtis et al., 1996).

The mean \pm standard error of the mean insulin levels when male and female results were combined were $10.99 \pm 1.43 \mu\text{U}/\text{mL}$ during the two time periods resistant starch bread was consumed and $9.84 \pm 1.42 \mu\text{U}/\text{mL}$ during control bread feeding periods. Mean \pm standard error of the mean insulin levels for males were $11.6 \pm 2.45 \mu\text{U}/\text{mL}$ during the resistant starch periods and $9.94 \pm 2.55 \mu\text{U}/\text{mL}$ during the time periods males consumed control bread. The mean insulin levels observed for female participants were $10.29 \pm 1.44 \mu\text{U}/\text{mL}$ when they consumed resistant starch bread and $9.73 \pm 1.17 \mu\text{U}/\text{mL}$ during the time periods female participants consumed control bread. The mean insulin level for all male subjects ($n=6$) was $17.3 \pm 5.04 \mu\text{U}/\text{mL}$ at baseline. Whereas, the mean insulin level for all female subjects at baseline ($n=7$) was $10.51 \pm 1.96 \mu\text{U}/\text{mL}$.

While male subject number one had the second highest insulin level at baseline ($29.5 \mu\text{U}/\text{mL}$) (because male subject number four had a baseline insulin level of $31.9 \mu\text{U}/\text{mL}$), male subject one's insulin level was lowered to $6.8 \mu\text{U}/\text{mL}$ after ingesting resistant starch for six-weeks. These values increased to $16.1 \mu\text{U}/\text{mL}$ after the two-week washout period. Male subject number four's insulin levels were lowered by more than 50% after ingesting control bread for six-weeks ($12.5 \mu\text{U}/\text{mL}$). All other participant's whose serum insulin measurements were available had normal insulin levels at baseline. No washout data was available for this participant and while still normal, his insulin

levels increased to 19.6 $\mu\text{U}/\text{mL}$ following a six-week consumption of resistant starch bread.

The adjusted P values from statistical analysis of insulin levels are given in Table 4.13 C (Appendix F). No significant differences were found for treatment, gender, or sequence effects for insulin levels during the fourteen-week study ($P > 0.05$).

**Table 4.13 A Resistant Starch and Control Bread Feeding Periods
Insulin $\mu\text{U}/\text{mL}$ MEAN \pm SEM**

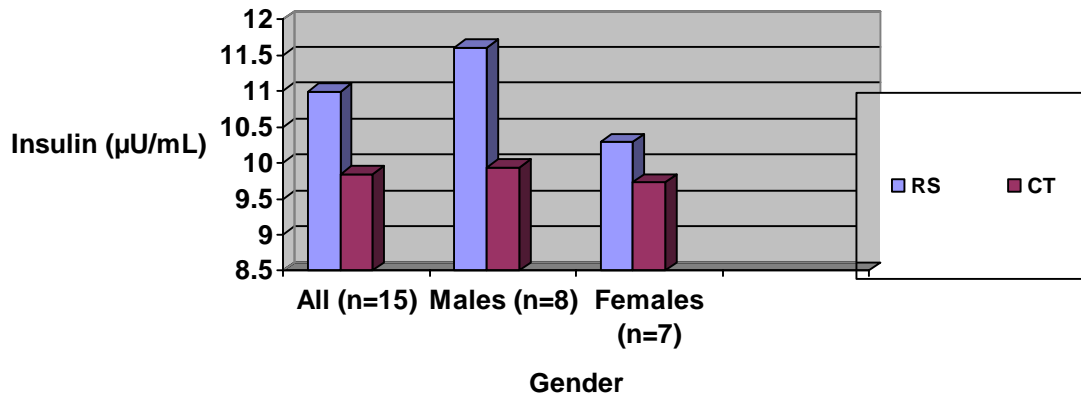
	All Subjects	Males	Females
RS Periods	10.99 \pm 1.43	11.6 \pm 2.45	10.29 \pm 1.44
Sample size	(n=15)	(n=8)	(n=7)
CT Periods	9.84 \pm 1.42	9.94 \pm 2.55	9.73 \pm 1.17
Sample size	(n=15)	(n=8)	(n=7)

RS Periods = Two six-week periods participants consumed bread containing added resistant starch

CT Periods = Two six-week periods participants consumed bread containing no added resistant starch

Graph 4.13 Resistant Starch & Control Bread Feeding Periods

Insulin ($\mu\text{U}/\text{mL}$) Mean \pm SEM



C-reactive protein

The mean \pm standard errors of C-reactive protein (CRP) levels are shown in Table 4.14 A and B. Baseline mean and standard errors CRP levels for male and female combined results were 0.62 ± 0.16 mg/dL (n=15). Mean CRP levels, which represent the overall average of the two time periods male and female participants received either resistant starch bread or control bread were 0.53 ± 0.12 mg/dL for resistant starch bread and 0.64 ± 0.21 mg/dL for control bread. Mean CRP levels during the two time periods females received resistant starch bread was 0.54 ± 0.18 mg/dL and 0.59 ± 0.22 mg/dL for control bread. Whereas, male participants had mean CRP levels of 0.52 ± 0.18 mg/dL during resistant starch bread consumption periods and 0.69 ± 0.37 mg/dL during control bread periods.

Median normal concentrations of C-reactive protein levels are 0.08 mg/dL. Ninety percent of presumable healthy persons have CRP values 0.3 mg/dL or below. In addition, ninety-nine percent of seemingly healthy persons have CRP values 1.2 mg/dL or below (Pepys MB, 1996).

The adjusted P values from statistical analysis of C-reactive protein levels are given in Table 4.14 C (Appendix F). No significant differences were found for treatment, gender, or sequence effects for C-reactive protein levels during the fourteen-week study ($P > 0.05$).

**Table 4.14 A Resistant Starch and Control Bread Feeding Periods
C-Reactive Protein mg/dL MEAN ± SEM**

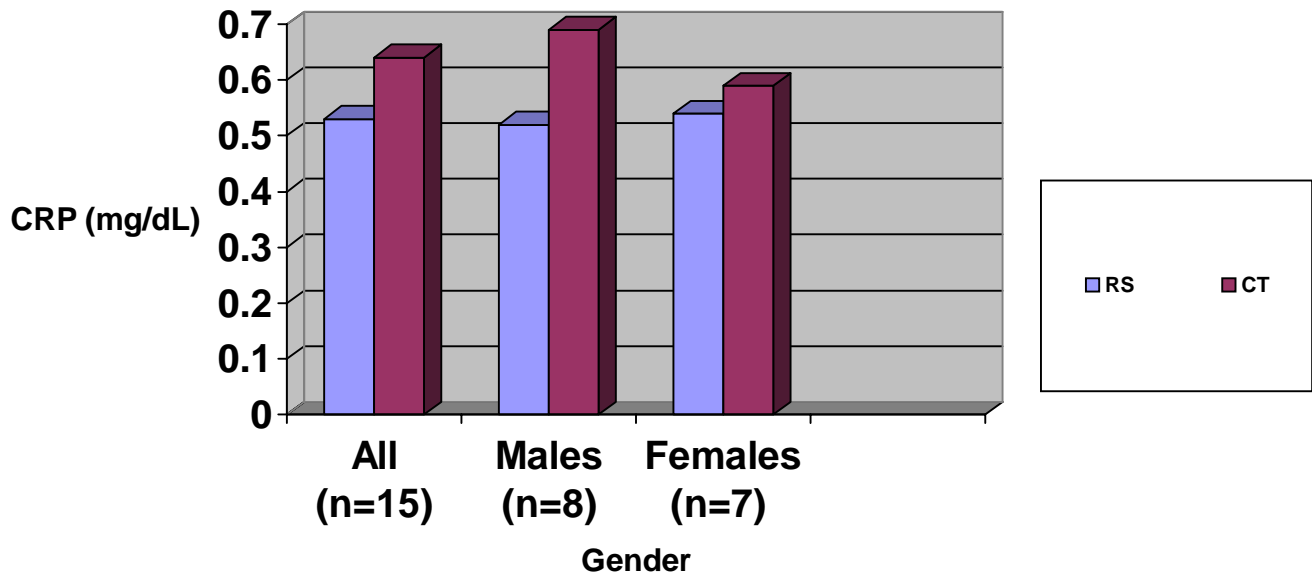
	All Subjects	Males	Females
RS Periods	0.53 ± 0.12	0.52 ± 0.18	0.54 ± 0.18
Sample size	(n=15)	(n=8)	(n=7)
CT Periods	0.64 ± 0.21	0.69 ± 0.37	0.59 ± 0.22
Sample size	(n=15)	(n=8)	(n=7)

RS Periods = Two six-week periods participants consumed bread containing added resistant starch

CT Periods = Two six-week periods participants consumed bread containing no added resistant starch

Graph 4.14 Resistant Starch & Control Bread Feeding Periods

CRP (mg/dL) Mean ± SEM



Glucagon-Like Peptide-1

The mean \pm standard errors of the mean of Glucagon-like peptide-1 (GLP-1) levels are shown in Tables 4.15 A and B. Mean \pm standard error of the mean GLP-1 levels, which represent the overall average of the two time periods male and female participants received either resistant starch bread or control bread were 8.48 ± 0.55 pM for resistant starch bread and 8.31 ± 0.66 pM for control bread. Mean \pm standard error of the mean GLP-1 levels during the two time periods females received resistant starch bread were 8.2 ± 0.81 pM and 8.74 ± 1.27 pM for control bread. Whereas, male participants had mean GLP-1 levels of 8.73 ± 0.80 pM during resistant starch bread consumption periods and 7.94 ± 0.63 pM during control bread periods.

The adjusted P values from statistical analysis of Glucagon-like peptide-1 levels are given in Table 4.15 C (Appendix F). No significant differences were found for treatment, gender, or sequence effects for Glucagon-like peptide-1 levels during the fourteen-week study. Baseline mean data were unavailable for Glucagon-like peptide-1 ($P > 0.05$).

**Table 4.15 A Resistant Starch and Control Feeding Periods
Glucagon Like Peptide-1 (pM) MEAN ± SEM**

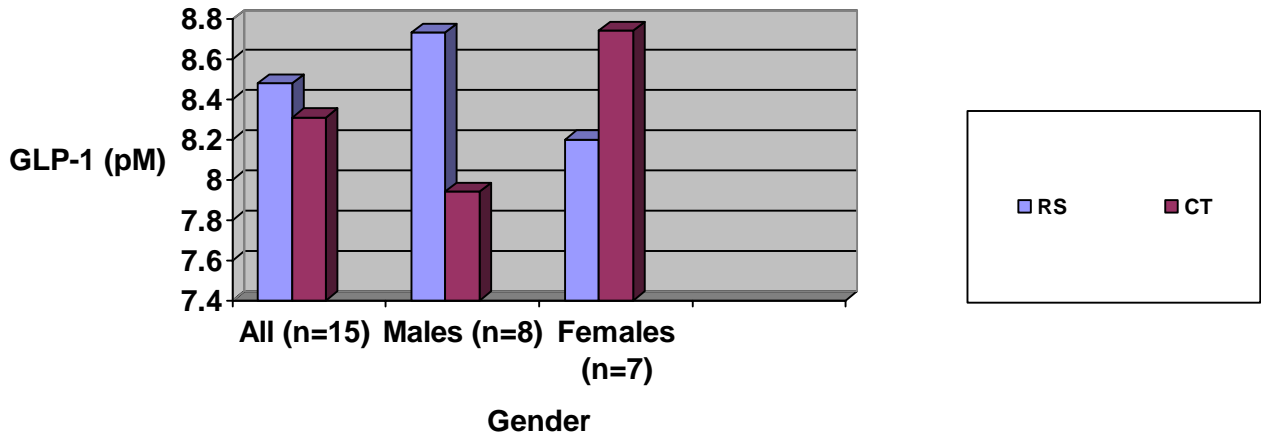
	All Subjects	Males	Females
RS Periods	8.48 ± 0.55	8.73 ± 0.80	8.2 ± 0.81
Sample size	(n=15)	(n=8)	(n=7)
CT Periods	8.31 ± 0.66	7.94 ± 0.63	8.74 ± 1.27
Sample size	(n=15)	(n=8)	(n=7)

RS Periods = Two six-week periods participants consumed bread containing added resistant starch

CT Periods = Two six-week periods participants consumed bread containing no added resistant starch

Graph 4.15 Resistant Starch & Control Bread Feeding Periods

GLP-1 (pM) Mean ± SEM



Short Chain Fatty Acids

Serum Acetate

The mean \pm standard errors of the mean of serum acetate levels are shown in Tables 4.16 A and B. The mean \pm the standard errors of the mean serum acetate levels when male and female results were combined was 95.81 ± 6.27 $\mu\text{mol/L}$ during the two time periods resistant starch bread was consumed and 102.68 ± 11.13 $\mu\text{mol/L}$ during control bread feeding periods. Mean \pm standard errors of the mean serum acetate levels for males was 91.37 ± 5.43 $\mu\text{mol/L}$ during the resistant starch feeding periods and 102.20 ± 17.74 $\mu\text{mol/L}$ during the time periods males consumed control bread. The mean serum acetate levels observed for female participants was 101.74 ± 12.71 $\mu\text{mol/L}$ during the time periods female participants consumed resistant starch bread. Female participants had a mean of 103.17 ± 14.66 $\mu\text{mol/L}$ after both control bread periods.

The adjusted P values from statistical analysis of serum acetate levels are given in Table 4.16 C (Appendix F). No significant differences were found for treatment or gender effects for serum acetate levels during the fourteen-week study. Sequence effects were statistically significant ($P = 0.0129$).

**Table 4.16 A Resistant Starch and Control Bread Feeding Periods
Serum Acetate ($\mu\text{mol/L}$) MEAN \pm SEM**

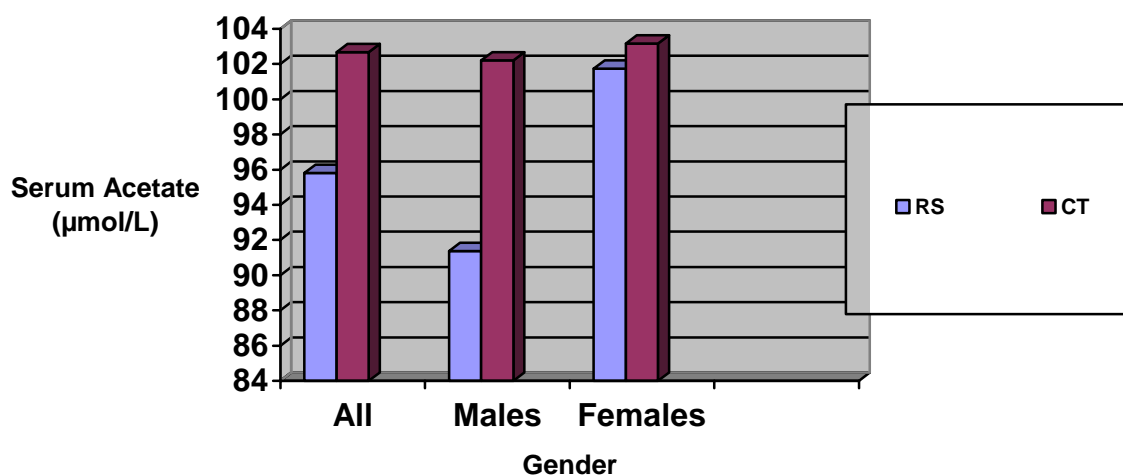
	All Subjects	Males	Females
RS Periods	95.81 \pm 6.27	91.37 \pm 5.43	101.74 \pm 12.71
Sample size	(n=14)	(n=8)	(n=6)
CT Periods	102.68 \pm 11.13	102.20 \pm 17.74	103.17 \pm 14.66
Sample size	(n=14)	(n=7)	(n=7)

RS Periods = Two six-week periods participants consumed bread containing added resistant starch

CT Periods = Two six-week periods participants consumed bread containing no added resistant starch

Graph 4.16 Resistant Starch & Control Bread Feeding Periods

Serum Acetate ($\mu\text{mol/L}$) Mean \pm SEM



Propionate

The mean \pm standard errors of the mean of serum propionate levels are shown in Tables 4.17 A and B. The mean \pm the standard errors of the mean serum propionate levels when male and female results were combined was 6.99 ± 0.43 $\mu\text{mol/L}$ during the two time periods resistant starch bread was consumed and 6.17 ± 0.36 $\mu\text{mol/L}$ during control bread feeding periods. Mean \pm standard errors of the mean serum propionate levels for males was 7.54 ± 0.66 $\mu\text{mol/L}$ during the resistant starch feeding periods and 6.25 ± 0.42 $\mu\text{mol/L}$ during the time periods males consumed control bread. The mean serum propionate levels observed for female participants was 6.26 ± 0.43 $\mu\text{mol/L}$ during the time periods female participants consumed resistant starch bread. Female participants had a mean of 6.08 ± 0.62 $\mu\text{mol/L}$ after both control bread periods.

The adjusted P values from statistical analysis of serum propionate levels are given in Table 4.17 C (Appendix F). Gender effects were statistically significant ($P = 0.0818$) and sequence effects approached significance ($P = 0.0595$). No significant differences were found for treatment effects.

**Table 4.17 A Resistant Starch and Control Bread Feeding Periods
Serum Propionate ($\mu\text{mol/L}$) MEAN \pm SEM**

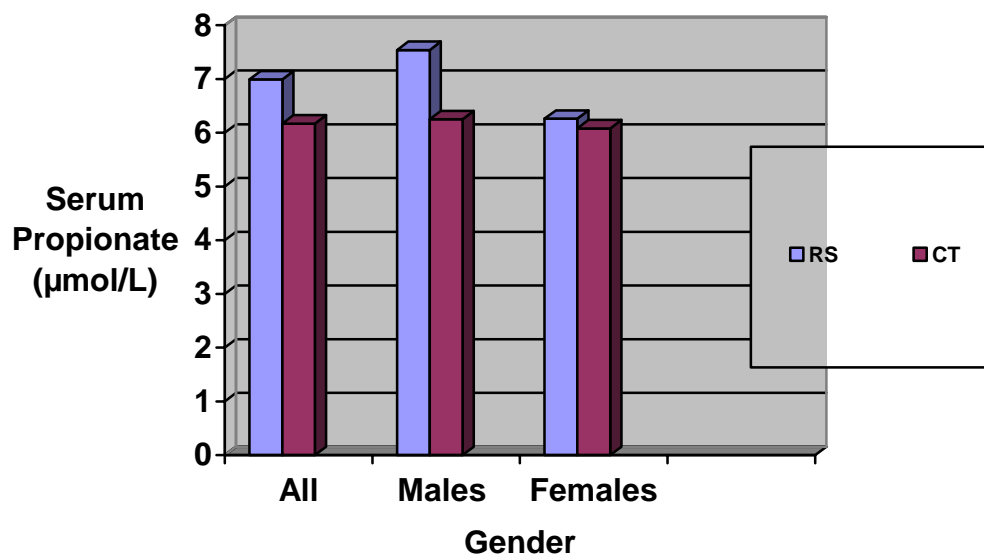
	All Subjects	Males	Females
RS Periods	6.99 \pm 0.43	7.54 \pm 0.66	6.26 \pm 0.43
Sample size	(n=14)	(n=8)	(n=6)
CT Periods	6.17 \pm 0.36	6.25 \pm 0.42	6.08 \pm 0.62
Sample size	(n=14)	(n=7)	(n=7)

RS Periods = Two six-week periods participants consumed bread containing added resistant starch

CT Periods = Two six-week periods participants consumed bread containing no added resistant starch

Graph 4.17 Resistant Starch & Control Bread Feeding Periods

Serum Propionate ($\mu\text{mol/L}$) MEAN \pm SEM



Serum Butyrate

The mean \pm standard errors of the mean of serum butyrate levels are shown in Tables 4.18 A and B. The mean \pm the standard errors of the mean serum butyrate levels when male and female results were combined was $0.71 \pm 0.34 \mu\text{mol/L}$ during the two time periods resistant starch bread was consumed and $0.74 \pm 0.06 \mu\text{mol/L}$ during control bread feeding periods. Mean \pm standard errors of the mean serum butyrate levels for males was $0.75 \pm 0.06 \mu\text{mol/L}$ during the resistant starch feeding periods and $0.82 \pm 0.11 \mu\text{mol/L}$ during the time periods males consumed control bread. The mean serum acetate levels observed for female participants was $0.66 \pm 0.04 \mu\text{mol/L}$ during the time periods female participants consumed resistant starch bread. Female participants had the same mean of $0.66 \pm 0.04 \mu\text{mol/L}$ after both control bread periods, even though the sample size was increased by one.

The adjusted P values from statistical analysis of serum butyrate levels are given in Table 4.18 C (Appendix F). No significant differences were found for treatment or gender effects for serum butyrate levels during the fourteen-week study. Sequence effects were statistically significant ($P = 0.0495$).

**Table 4.18 A Resistant Starch and Control Bread Feeding Periods
Serum Butyrate ($\mu\text{mol/L}$) MEAN \pm SEM**

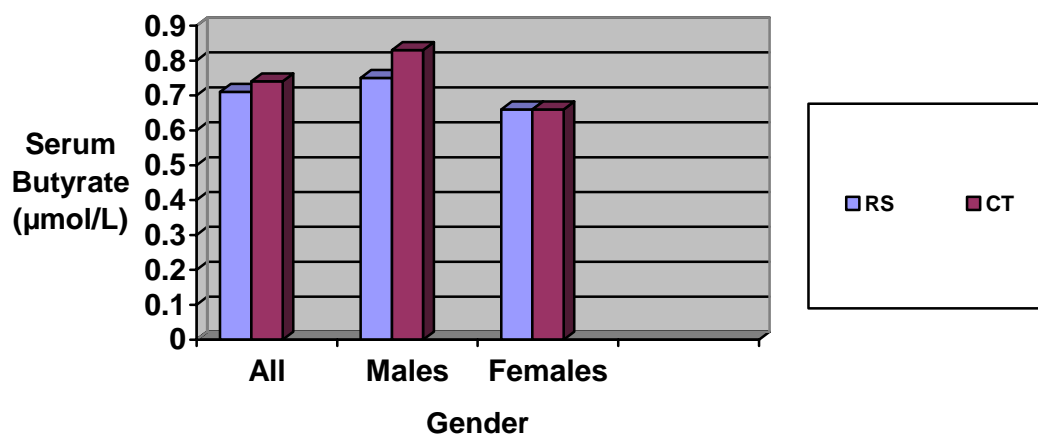
	All Subjects	Males	Females
RS Periods	0.71 \pm 0.36	0.75 \pm 0.06	0.66 \pm 0.04
Sample size	(n=14)	(n=8)	(n=6)
CT Periods	0.74 \pm 0.06	0.83 \pm 0.11	0.66 \pm 0.04
Sample size	(n=14)	(n=7)	(n=7)

RS Periods = Two six-week periods participants consumed bread containing added resistant starch

CT Periods = Two six-week periods participants consumed bread containing no added resistant starch

Graph 4.18 Resistant Starch & Control Breeding Feeding Periods Serum Butyrate ($\mu\text{mol/L}$)

Mean \pm SEM



Homeostasis Model Assessment (HOMA) Insulin Resistance (IR)

The mean \pm standard errors of HOMA Insulin Resistance (HOMA-IR) are located in tables 4.19 A and B. Values greater than 2.5 for HOMA-IR indicate insulin resistance in adults (Wallace 2004).

Mean HOMA-IR for all participants after consuming resistant starch bread for each of the six-week periods was 2.69 ± 0.35 and 2.23 ± 0.29 after consuming control bread during the six-week periods. Male HOMA-IR data indicated a mean level of 2.69 ± 0.55 after resistant starch bread consumption and 2.19 ± 0.48 following control bread consumption (n=8). On the other hand, female participants reported a mean HOMA-IR of 2.68 ± 0.46 after consuming resistant starch bread and 2.27 ± 0.35 after consumption of control bread (n=7).

The mean baseline HOMA-IR \pm the standard error of the means for male and female participants combined were 3.51 ± 0.76 (n=13). Mean baseline values \pm the standard error for male participants only were 4.59 ± 1.45 (n=6), whereas, mean baseline values \pm the standard error for female participants were 2.58 ± 0.55 (n=7) as indicated in Table 4.19 B (Appendix F).

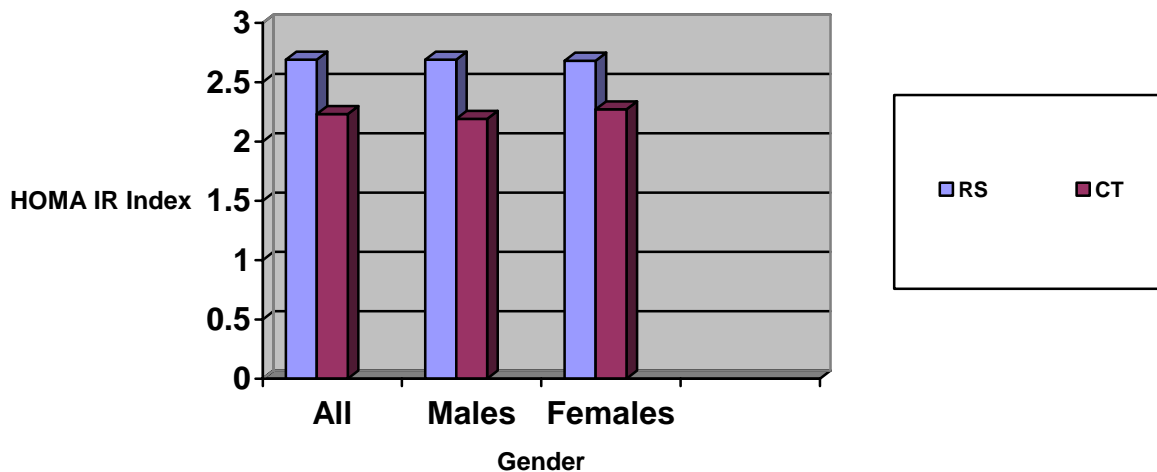
No significant differences were found for sequence (P = 0.6739), treatment (P = 0.1396) or gender (P = 0.7785) effects for HOMA-IR levels during the fourteen-week study (data not shown).

**Table 4.19 A Resistant Starch and Control Bread Feeding Periods
HOMA Insulin Resistance (IR) Index MEAN ± SEM**

	All Subjects	Males	Females
RS Periods	2.69 ± 0.35	2.69 ± 0.55	2.68 ± 0.46
Sample size	(n=15)	(n=8)	(n=7)
CT Periods	2.23 ± 0.29	2.19 ± 0.48	2.27 ± 0.35
Sample size	(n=15)	(n=8)	(n=7)
	RS Periods = Two six-week periods participants consumed bread containing added resistant starch	CT Periods = Two six-week periods participants consumed bread containing no added resistant starch	

Graph 4.19 Resistant Starch & Control Bread Feeding Periods

HOMA Insulin Resistance (IR) Index



Homeostasis Model Assessment (HOMA) Beta-cell Function

The mean \pm standard errors of HOMA Beta-cell function (HOMA-Beta %) are located in tables 4.20 A and B. Mean HOMA- derived % Beta cell function for all participants after consuming resistant starch bread for each of the six-week periods was 36.99 ± 5.70 and 35.73 ± 6.69 after consuming control bread during the six-week periods (n=15) as shown in Table 4.20 A. Male HOMA-derived % Beta cell data indicated a mean level of 41.28 ± 10.16 after resistant starch bread consumption and 37.31 ± 12.59 following control bread consumption (n=8). Analysis of female data revealed a mean HOMA-derived % Beta-cell function of 32.08 ± 4.16 after consuming resistant starch bread and 33.92 ± 3.42 after consumption of control bread (n=7).

The mean baseline HOMA-derived % Beta cell function \pm the standard error of the means for male and female participants combined were 43.26 ± 8.07 (n=13). Mean baseline values \pm the standard error for male participants only were 53.12 ± 15.88 (n=6), whereas, mean baseline values \pm the standard error for female participants were 34.81 ± 6.02 (n=7) as indicated in Table 4.20 B (appendix F).

No significant differences were found for sequence (P = 0.5264), treatment (P = 0.5923) or gender (P = 0.8577) effects for HOMA-derived % Beta-cell function levels during the fourteen-week study (data not shown).

**Table 4.20 A Resistant Starch and Control Bread Feeding Periods
HOMA derived Beta-cell Function MEAN \pm SEM**

	All Subjects	Males	Females
RS Periods	36.99 \pm 5.70	41.28 \pm 10.16	32.08 \pm 4.16
Sample size	(n=15)	(n=8)	(n=7)
CT Periods	35.73 \pm 6.69	37.31 \pm 12.59	33.92 \pm 3.42
Sample size	(n=15)	(n=8)	(n=7)

RS Periods = Two six-week periods participants consumed bread containing added resistant starch

CT Periods = Two six-week periods participants consumed bread containing no added resistant starch

Male Participant Number One

Male participant number one was the only subject who had been previously diagnosed with clinical pre-diabetes prior to enrolling in this study. His fasting serum glucose, fructosamine, hemoglobin A1c, insulin, and C-reactive protein levels were lowered after six weeks of resistant starch bread consumption. Short chain fatty acids levels should increase after ingestion of resistant starch and did not show a consistent trend. These data are shown in Table 4.21.

Table 4.21 Male Participant One (Previously Diagnosed with Pre-Diabetes)
 14 week Feeding Study Blood Parameter & Anthropometric Data

BLOOD PARAMETERS	Baseline	End 1st 6 Wks	2 wk washout	End of Study
	Week 0	Week 6 (RS)	Week 8	Week 14 (CT)
Fasting Serum Glucose (mg/dL)	122	105	98	105
Fructosamine (mmol/L)	3.4	2.1	3.1	4
Hemoglobin A1c (%)	7.4			6.2
Insulin (μ U/mL)	29.5	6.8	16.1	9.9
C-reactive protein (mg/dL)	1.49	0.28	0.18	0.2
Glucagon-Like Peptide-1 (pM)	No Baseline Data	5.9	6.8	9.4
Serum Acetate (μ mol/L)	91.1	92.62	108.82	73.78
Serum Propionate (μ mol/L)	5.42	8.5	6.35	6.21
Serum Butyrate (μ mol/L)	0.71	0.54	0.65	0.55

ANTHROPOMETRICS	Baseline	End 1st 6 Wks	2 wk washout	End of Study
	Week 0	Week 6 (RS)	Week 8	Week 14 (CT)
Age (years)	43	43	43	43
Weight (pounds)	206.6	202.8	202.8	202.1
Body Mass Index (kg/m ²)	31.4	30.8	30.8	30.7
Waist Circumference (inches)	40	39.8	39.5	39.5
Blood Pressure Systolic (mmHg)	110	120	120	110
Diastolic Blood Pressure (mmHg)	80	70	74	80

CHAPTER FOUR

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CHAPTER FIVE

DISCUSSION

The purpose of this study was to examine the effects of daily consumption of approximately twelve grams of Hi-maizeTM 260 Resistant Starch, a high amylose starch, added to loaves of bread and consumed by a free-living population of fifteen African-American men and women, mean age 36.6 ± 1.55 years, who were at increased risk for type 2 diabetes as determined by factors such as family history, and anthropometric status. This discussion includes the references to the glycemic index and Homeostasis Model Assessment Insulin Index (HOMA-IR) and Homeostasis Model Assessment for Beta-cell function (HOMA Beta-cell function) levels along with examining clinical indicators and biochemical markers for risk of type 2 diabetes. Clinical indicators of type 2 diabetes include fasting plasma glucose, fructosamine, hemoglobin A1c, insulin, C-reactive protein, and glucagon-like peptide-one. An assessment of short chain fatty acids, including serum acetate, propionate, and butyrate was done to determine levels of resistant starch fermentation and also to determine if twelve grams of RS improved colonic metabolism enough to modify disease risk as measured by clinical diabetes markers in this African-Americans population at increased risk for type 2 diabetes. Dietary data was obtained from a food frequency questionnaire that assessed the macronutrient and micronutrient content of each participant's diet for the year prior to joining the study and during the fourteen-weeks each participant was involved in the study.

Health benefits such as reduced glycemic response and increased short chain fatty

acid concentration have been attributed to resistant starch and starchy foods that are either relatively or absolutely resistant to digestion in the small intestine (Jenkins *et al.*, 1998; Salmeron *et al.*, 1997 (a,b). Our research has shown no published data examining the long term effects on using resistant starch added to a dietary item such as bread in the African-American population.

African-Americans are a vulnerable population group with disproportionately elevated rates of type 2 diabetes (Day 1996; Harris *et al.*, 1998; National Diabetes Statistics 2003). The participants in this study fit the profile of ethnicity, family history, mean body mass index (BMI 37.1 ± 2.0 kg/m²), waist circumferences 43.95 ± 2.13 inches, hemoglobin A1c (6.9 ± 0.11 %), and fasting plasma glucose (99.86 ± 2.63 mg/dL) which indicated they were at increased risk for type 2 diabetes at baseline. Thus, the primary goal of this study was to assess the effects of resistant starch added to bread on blood parameters for pre-diabetes in this high-risk population.

Resistant Starch/Control Bread Solubilised and Non-Solubilised Starch

Analysis of digested material obtained from ileostomy patients demonstrated that physiological differences occur between individuals that may have ingested the same quantity of resistant starch (Langkilde *et al.*, 2002; Silvester *et al.*, 1995). Gallant *et al.*, 1992 reported the amount of time it takes carbohydrates to travel through each person's intestines and reach their colon varies (Gallant *et al.*, 1992). In addition, the diverse fermentation patterns of the four forms of resistant starch affect the potential health impact that can be expected from resistant starch consumption (Noah L, *et al.*, 1998). The Hi-maize™ 260 Resistant Starch utilized in the current study has been reported to

contain crystalline structures which minimize starch hydrolysis (Edwards *et al.*, 1996; Nordgaard *et al.*, 1995) rendering more starch available for fermentation because less starch is susceptible to rapid digestion. However, the physiological variations in human transit time as reported in some ileostomy patients, have resulted in increases in resistant starch fractions digested in persons whose digestive systems are slower and therefore takes longer for food to travel through the intestines and reach the colon. Since ileostomates are regarded as a reliable model to study carbohydrate digestion (Langkilde *et al.*, 1998; Silvester *et al.*, 1995; Muir *et al.*, 1993; Anderson 1992), it is reasonable to believe physiological differences in gut transit time may have affected the results in the current study.

Additionally, the solubilised (non-resistant starch) content of the control and resistant starch bread loaves were similar in this study. Mean levels of soluble starch content of the control and resistant starch bread loaves were, 67.22 g/100g and 64.09 g/100g, respectively. Solubilised fractions are hydrolyzed to glucose (AOAC Method 2002.02) and, as a consequence, are digestible and absorbable as glucose. Therefore, the soluble starch contents may have reduced the effects of the resistant starch content in this feeding study. This factor coupled with the differences in human gut physiology and the varying degrees of each subject's ability to maintain glucose homeostasis may have diluted the effects of the resistant starch in proportion to the carbohydrate intake obtained from other food sources in this study.

Fasting Plasma Glucose (FPG)

Careful examination of each participant's Fasting Plasma Glucose (FPG) data did

not reveal a consistent pattern following the six-week consumption of bread containing increased levels of resistant starch nor after the six-week consumption of the control bread. However, most participants FPG values were lowered when baseline values were compared to the end of the first six-weeks for both resistant starch bread consumption and control bread consumption.

Notably, fasting plasma glucose levels are more likely to be altered when postprandial levels are measured following acute dietary changes (such as reducing available carbohydrates) which occur with increased resistant starch, or fiber intake (Wolever 2003). A chronic and slow glucose delivery rate should have long-term, lowering effects on glucose and insulin parameters (Tapsell 2004), although this contradicts the findings in the current study.

Robertson *et al.*, (2003), assessed the effects of acute dosages of resistant starch in ten healthy subjects who consumed a diet supplemented with 60 grams of (Novelose 260) high-amylose resistant starch. Robertson and colleagues' findings revealed postprandial plasma glucose levels were significantly lower ($p=0.037$) for up to five hours. Robertson *et al.*, controlled for all nutrient consumption as well as prohibited smoking and exercising during each of the two, twenty-four hour, high or low resistant starch feedings.

While fasting plasma glucose levels reflect short-term changes in blood glucose levels, fructosamine and hemoglobin A1c (HbA1c) are indicators of longer-term blood glucose management, based on glycosylated hemoglobin (Bunn 1981; Jovanovic *et al.*, 1981; Goldstein *et al.*, 1982; Nathan *et al.*, 1984; Ko *et al.*, 1998). Fructosamine levels can be evaluated under free living conditions, whereas postprandial blood glucose assessments are typically determined during simulated, controlled experimental

conditions (Wolever *et al.*, 2003).

Fructosamine

Fructosamine values should, ideally, be compared with previous values within the same subject. There is currently no acceptable standardized value for fructosamine levels other than the reference intervals as indicated by each laboratory that provides commercially available kits (Wallach 2000) because the calibrators used to evaluate fructosamine levels are not the same for every clinical laboratory.

In this study fructosamine levels of all fifteen subjects were significantly lower ($P = 0.0054$) after subjects consumed the control bread for six weeks compared to baseline values. Fructosamine levels of all subjects were significantly lower ($P < 0.0012$) after the consumption of resistant starch bread for six weeks than they were at baseline. Fructosamine levels were also significantly lower ($P < 0.0001$) after the washout period than at baseline. There were no significant differences in fructosamine levels of all fifteen subjects due to consumption of the resistant starch versus the control bread ($P = 0.9692$).

Hemoglobin A1c (HbA1c)

Hemoglobin A1c (HbA1c) measures the concentration of hemoglobin molecules, which are found in red blood cells that have glucose attached to them. Glycation of the hemoglobin molecule results in its linkage with excess glucose in the blood. The more glucose that is circulating in blood, as opposed to reaching target organs and tissues, the more glycated hemoglobin becomes. The average life span of a red blood cell is approximately 120 days. Red blood cell glycohemoglobin concentration is dependent on

the average blood glucose concentration over a period of eight to twelve weeks and is stable for the life of the red blood cell.

Hemoglobin A1c (HbA1c), like fructosamine, is an indicator of longer-term blood glucose management, although it was not significantly different at baseline (day 0) and at the end of the fourteen-week study (day 98) for either males or females combined or males and females separately in this study. The participants in the current study had higher than normal mean HbA1c levels although the mean values are approaching less than 7%, which is the value that is recommended by the American Diabetes Association (ADA) for tight glycemic control in persons with type 2 diabetes (ADA 2006). These HbA1c values suggest that these participants may be experiencing problems with glucose control, although their fasting plasma glucose levels are normal.

Hemoglobin A1c levels were not measured during the cross-over and washout periods, although this contradicts findings by Tahara and Shima (1993) and Goldstein *et al.*, (1982) who reported that plasma glucose levels that occurred during the previous three to four weeks contribute to more than fifty percent of the final HbA1c levels whereas, plasma glucose levels from 90 to 120 days make up less than 10 percent of the HbA1c totals. Therefore, HbA1c levels can be clinically different as a result of changes in mean plasma levels as recent as thirty days before a blood glucose measurement is obtained. A possible explanation for the non-significant differences in hemoglobin A1c levels observed in our study, was that measurable changes could have occurred during weeks six and eight when HbA1c was not measured.

Glycemic Index (GI) and Homeostasis Model Assessment-Insulin Resistance (HOMA-IR)

Glycemic index (GI) is “the incremental area under the curve of blood glucose produced by a standard amount of carbohydrate in food, usually 50 grams, relative to the incremental area produced by the same amount of carbohydrate from a standard source, usually white bread or glucose” (Willett *et al.*, 2002). The GI pre and post study were not statistically different for this population. Glycemic Index levels may have been too high to appreciably compensate for the small amount of resistant starch that was able to be incorporated in the bread in this study. Foster-Powell *et al.*, 2002 reported GI levels of bread made from finely ground “wholemeal” flour were the same as white bread. These findings are consistent with the current study. Willett further reported that high GI diets can result in increased levels of insulin resistance.

Baseline HOMA-IR levels reported in the current study indicated participants were hyperinsulinemic (values > 2.5) (Wallace 2004). Mean HOMA-IR levels for all subjects who consumed resistant starch in the first six-week treatment group decreased to normal values after six-weeks of resistant starch. However, the same was true for the subjects who consumed the control bread during the first six-weeks of the study. In addition, the washout value increased to above normal levels in the group who consumed resistant starch bread first although no significant differences were found for treatment effect ($P = 0.5923$). Insulin resistance is affected by central adiposity, genetics, physical activity levels, as well as dietary practices (Willett *et al.*, 2002). Variations in individual responses to increased GI diets have been attributed to varying degrees of underlying insulin resistance. Insulin resistance and beta cell dysfunction are chief causative factors

associated with the development of type 2 DM (Beck-Nielsen *et al.*, 1994).

Insulin

No significant differences were found for treatment, gender, or sequence effects for insulin levels in the current study. Mean insulin levels were reported within the normal range of two to twenty-five microliters (2-25 μ L) (Painter *et al.*, 1996). Noakes *et al.* (1996) examined the effects of replacing twenty-five percent of dietary carbohydrate with high-amylose starch, oat bran, or low-amylose starch in twenty-three overweight hypertriglyceridemic subjects who were randomly assigned to each of these diets for four-weeks. Their findings were similar to the current study, as no significant effects were observed in insulin levels following each four-week starch replacement period. It is interesting to note however, that Noakes also included an acute dosage of resistant starch delivered via a test meal, where thirty-three percent of the carbohydrate content in a meal was replaced with high-amylose starch. A small biological reduction of seventeen percent in plasma insulin concentration was observed when the high-amylose starch test meal was compared to the low-amylose test meal ($p < 0.001$) (Noakes *et al.*, 1996). Wolever (1996) supported Noakes findings when he reported that the amount or source of dietary carbohydrate can alter insulin levels.

C-Reactive Protein (CRP), Glucagon-Like Peptide-One (GLP-1), and Homeostasis Model Assessment for Beta-cell Function (HOMA) Beta-Cell Function

Whereas, FPG, HbA1c, and fructosamine levels are clinical markers that can be used to assess glycemic control; neither CRP nor GLP-1 can be used for this type of assessment. CRP has, however, been implicated in the pathogenesis of type 2 diabetes

(Ford 1999; Pradhan *et al.*, 2001; Freeman *et al.*, 2002). No significant differences were found for treatment, gender, or sequence effects for CRP levels in the current study. While, these statistical results suggest there was no significant effect as a result of consumption of resistant starch or control bread, higher than normal mean CRP concentrations during this fourteen-week study are suggestive of possible trauma, tissue injury, microbial infection, or inflammation in the study participants at the four times CRP was analyzed.

It is currently known that CRP is produced in increased amounts in as few as six hours following tissue injury, surgery, infection, and or myocardial infarction (Pepys *et al.*, 2001). Levels can double every eight hours, peaking in a little more than two days if stimulus is sustained. Pepys also reported that CRP levels can reach normal levels almost as quickly as they reach abnormal levels. Although CRP is activated in acute situations, constant stimulus can result in higher CRP levels (Pepys *et al.*, 2001). These interactions may also explain the findings in the current study.

de Matt *et al.*, 1996; Macy *et al.*, 1997; as well as Kluft *et al.*, 2001 have observed variations in CRP levels in individuals over time, similar to the current study. These researchers were able to approximate stable basal rates by plotting the CRP concentrations of each subject at each point CRP was measured during the length of the studies. These three researchers also reported outliers in some of their subjects' CRP concentrations.

Yudkin *et al.*, 1999 and de Maat *et al.*, 2001 reported that obesity was positively associated with increased CRP levels, primarily because interleukin-6 (IL-6) is synthesized in adipose tissue and IL-6 levels can increase CRP gene expression (de Matt

2001). All of the participants in the current study were classified as overweight (BMI ≥ 25 kg/m²) and eleven of the fifteen (missing data for two participants) were classified as obese (BMI ≥ 30 kg/m²) at baseline. However, the interaction between increased CRP concentrations and obesity was not assessed in the current study. Yudkin *et al.*, 1999 and Hak *et al.*, 1999 reported an association between CRP, insulin resistance and obesity in healthy subjects. Marques-Vidal *et al.*, 2002 found elevated CRP levels in persons with insulin resistance syndrome.

Females have been reported as having higher CRP levels than males (Gram *et al.*, 2000; Cook *et al.*, 2000) due to fluctuations in CRP and cytokine concentrations that occur during menstruation (Jilma *et al.*, 1997). These findings were not evident in the current study.

According to Hui and colleagues GLP-1 is capable of stimulating insulin secretion in response to meals (Hui *et al.*, 2005). In support of Hui's study, Valasquez-Mieyer *et al.* (2003) in their study of GLP-1 in African-Americans compared to Caucasians reported that in addition to stimulating insulin secretion GLP-1 has been shown to have other profound biological effects on beta cells: a) it makes resistant islets glucose sensitive and b) it promotes regeneration of islet cells. Further, while HOMA Beta-cell function in the current study did not reveal any statistical significance for either treatment, gender, or sequence interactions, the model implies, that the participants' beta cells were producing insulin at either normal or compensatory levels. It is important to note, however that baseline data were not available for GLP-1 in the current study.

Short Chain Fatty Acids (SCFA's): Serum Acetate, Serum Propionate, Serum Butyrate

Resistant starches “resist” being digested in the small intestine and but travel, undigested, into the colon where fermentation takes place (Higgins 2004). In adult humans the principal products of fermentation are SCFA together with gases and some heat. A measurement of short chain fatty acids (SCFA's) can be taken from the blood serum which can be used to monitor large bowel events (Topping and Clifton 2001). Acetate is the main SCFA in mixed venous blood. Propionate and butyrate concentrations are sometimes so low that measurement is difficult (Wolever *et al.*, 1997), although measurement was possible in this study.

In regard to the current study no significant differences were found for treatment or gender effects for serum acetate levels. However, sequence effects were significant ($P = 0.0129$). While treatment effects were not significant for propionate, gender effects were significant ($P = 0.0818$) and sequence effects approached significance ($P = 0.0595$). Serum butyrate was the smallest measurable SCFA in this study. No significant differences were found for treatment or gender effects. Sequence effects were statistically significant ($P = 0.0495$). These data suggest there were undigested carbohydrates present in the study population.

SCFA concentrations may not have increased in the current study following consumption of Hi-maize™ 260 Resistant Starch for several reasons: 1) Readily digestible carbohydrates, such as soluble starch have been reported as reducers of SCFA production (Cummings 1992). 2) The amount of soluble starch in the control bread and resistant starch bread were similar in this study. 3) Gallant *et al.*, 1992 found that gut

transient time varies in humans. 4) As indicated by, Macfarlane 1998 SCFA production is enhanced by increased transit time in the large intestine. 5) Diet, ageing, stress as well as disease affect SCFA formation (Macfarlane and Macfarlane 2003). 6) Twelve grams of Hi-maizeTM 260 Resistant Starch daily for six-weeks may not have been enough RS to elicit an increased response in SCFA production in the current study. The combination of all of these factors may have contributed to the findings that SCFA levels were not increased with consumption of resistant starch. The culmination of these factors may be overshadowed by the possibility of whether or not the participants in the current study were compliant with the given protocol.

Observations

The only participant that had been previously diagnosed with clinical pre-diabetes prior to enrolling in this study demonstrated decreased clinical indicators of type 2 diabetes in response to resistant starch and control bread intake. His FPG levels were lowered to the same values after six weeks of resistant starch and control bread consumption. Fasting Plasma Glucose was normal during the washout period. Fructosamine levels also returned to normal after six-weeks of resistant starch consumption. This level increased to above normal during washout and after six-weeks of control bread consumption. The long term indicator of glycemic control, HbA1c, was lowered to nearly normal levels (6.2%) at the end of the study. This level is also recognized as tight glycemic control in patients with type 2 diabetes. His baseline insulin levels suggest hyperinsulinemia prior to his enrollment in the study. By the end of the six-week consumption of resistant starch bread his levels were reduced by 43%. His weight decreased by four pounds during the fourteen-week study. However, weight loss alone cannot explain these differences because his BMI levels were still clinically classified as obese (although slightly). The changes in this participant's clinical parameters were deemed by the author as worth mentioning in this evaluation with full knowledge that a sample size of one provides minimal if any, conclusive evidence.

Conclusions

A daily consumption of approximately twelve grams of Hi- maizeTM 260 Resistant Starch consumed by a free-living African American population at increased risk for type 2 diabetes did not consistently show significance in all clinical indicators and biochemical markers assessed. Based on a compliance sheet participants were asked to complete, a few participants indicated they consumed two slices of bread daily on some days instead of the three slices of bread they were asked to consume. Consumption of one slice of Hi-maizeTM 260 Resistant Starch would provide approximately four point one grams of resistant starch. This small dosage of resistant starch has not been indicated as an appropriate amount to elicit responses in the parameters that were evaluated in the current research.

On the basis of the evidence in this study we do not have evidence that this amount of resistant starch in this population's diet will prevent the onset of diabetes. However, results are suggestive that higher levels of resistant starch in a more controlled experiment could reduce blood indicators. The current study clearly could be useful for future research on diabetes prevention.

Implications for Further Study

This study was conducted using a population of African American men and women who were considered to be vulnerable to pre-diabetes based on identified risk factors. The following recommendations were suggested by the results of this study:

1. More research should be conducted to determine if a greater amount of resistant starch in a food matrix in which processing conditions do not

- reduce the initial amount of added resistant starch would be more effective.
2. More study is needed with persons clinically diagnosed with pre-diabetes.
 3. A similar study should be conducted in an area with a higher population of African Americans so that a larger sample can be studied for a longer period of time.
 4. A study should be conducted using a different ethnic population with similar risk factors for Type 2 DM.
 5. A similar study should be done with a comparison of results between genders.
 6. A similar controlled feeding study should be conducted, assessing, monitoring, and recording physical activity levels for each participant.
 7. A similar study should be conducted utilizing dual-energy X-ray absorptiometry (DEXA) to analyze total tissue, fat mass, and lean mass and or bioelectric impedance.
 8. A similar study should be conducted utilizing RS from intact grains (RS1) as well as Novelose 260™ (RS2).

Limitations

The area of Southwest Virginia, where the current study was conducted has a small population of African-Americans including, 3.7% in Montgomery County and 8.1% in Radford (<http://www.census.gov> accessed April 8, 2006). The census statistics for Pulaski County are not included because no persons from that county actually participated. Study criteria requiring persons at risk for type 2 diabetes and or pre-

diabetes candidates, further reduced the sample from this population due to the unfortunate finding that many were receiving medical care for clinical type 2 diabetes.

CHAPTER FIVE

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