The effect of whole wheat and wheat milling fractions on metabolic parameters of adiposity, glucose control, and lipid metabolism using a rat model of obesity with type 2 diabetes

THESIS

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Obesity prevalence is at an all-time high, with 1 in 3 Americans now classified as obese (1). Obesity is a debilitating condition that is associated with various co-morbidities, including type 2 diabetes (T2D), cardiovascular disease, pulmonary dysfunction, gastrointestinal disease, various cancers, and osteoarthritis. T2D is one of the most significant co-morbidities of obesity, as it often leads to other diseases such as non-alcoholic fatty liver disease (NAFLD) and hyperlipidemia.

There are now 347 million people worldwide with T2D (2).The incidence of newly diagnosed cases of T2D in America is at its highest of 1.9 million per year and, according to the American Diabetes Association, and if the trend continues, 1 in 3 Americans will have diabetes by the year 2050 (3). There are many factors that increase the risk of developing T2D, some which include being overweight and physically inactive, having a genetic predisposition, and high blood pressure. In addition, there are complications that develop gradually over a period of time during T2D if blood glucose sugar levels are not properly controlled. Long-term poor glucose control puts patients at a higher risk for complications such as cardiovascular disease (CVD), neuropathy, nephropathy, and retinopathy.

Obesity and diabetes are having a substantial impact on the economic state of the health care system. The total estimated cost of diagnosed diabetes in 2012 was $245 billion. This includes $176 billion in direct medical costs and $69 billion in reduced productivity, encompassing lower labor force participation from chronic disability and premature mortality (4). In addition to the economic burden, there are substantial intangible costs, such as a reduced quality of life for those people with diabetes, and often for their families and friends as well.

Several epidemiological studies suggest that whole grain consumption is associated with a lower risk of obesity and T2D. Whole grain consumption has been found to have an inverse association with fat mass and insulin resistance in both humans and rats, possibly through several different mechanisms, involving mechanical, hormonal, and anti-inflammatory processes. The mechanical mechanism focuses on alterations in caloric density and absorption of nutrients, the hormonal mechanism on changes in hormone synthesis and secretion due to nutrient availability, and lastly, anti-inflammatory and anti-oxidative components in the whole grain. However, it is unclear which milling fraction of the whole grain, the bran, germ, or endosperm, may be responsible for these health benefits.

Therefore, the aim of this study was to examine the effects of whole wheat and wheat milling fractions, specifically bran, germ, and endosperm, on glucose control, insulin resistance, fatty liver, and adiposity using an animal model of obesity with T2D, the Zucker Diabetic Fatty (ZDF) rat. Male ZDF rats were fed either a cornstarch-based diet (AIN-93G; obese control), or diets containing 64% whole wheat flour, 54% refined wheat, 9.4 % wheat bran, 1.6% normal wheat germ, or high 15% wheat germ for 5 weeks. Lean ZDF littermates fed a standard AIN-93G diet served as a negative control. All animals were fed ad libitum. The refined wheat, wheat bran, and wheat germ were present in the diets in the same concentration as would be found in the whole wheat diet. The high wheat germ diet had amounts of germ 10 times that of the normal wheat germ diet. It was found that after 5 weeks, the whole wheat, refined wheat, and high and normal wheat germ groups all showed a significant improvement ($p<0.05$) in area under the curve during glucose and insulin tolerance tests. There were no differences in body

weight or fat pad weight among the ZDF groups; however, there was a significant difference ($p=0.031$) in fat mass % between the whole wheat group and the obese control. The whole wheat group and all wheat fraction groups decreased the concentration of liver lipids compared to the obese control, and the bran and germ groups also had lower liver cholesterol concentration. Only the whole wheat group had a significantly lower cecum pH ($p<0.0001$) and greater cecal weight ($p<0.0001$) compared to the obese control, indicating greater fermentation of the diet. There were no significant differences in plasma adiponectin and resistin levels among the ZDF diet groups. In conclusion, the results of this study suggests that none of the wheat milling fractions stand out as responsible for the metabolic effects seen with consumption of whole wheat and that individual milling fractions of wheat are just as effective in improving insulin resistance and fatty liver as whole wheat.

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Chapter 1: Literature Review

Chronic Disease, Obesity, and Type 2 Diabetes

Obesity

Obesity is defined as having an excessive amount of adipose tissue, indicated by a body mass index (BMI) of 30 or higher, and is associated with an increased risk for numerous diseases and health complications, such as cardiovascular disease (CVD), type 2 diabetes (T2D), high blood pressure, stroke, cancer, sleep apnea, depression, gallbladder disease, infertility, skin problems, and delayed wound healing issues, all of which are significant health problems and reduce quality of life (1).

Obesity occurs when an individual consumes more energy than is expended through normal daily activities, exercise, and basal metabolic rate (BMR), causing the body to store the excess energy as fat. An individual's genetic background can also be a risk factor for obesity, as it may influence how the body responds to nutrients which may cause weight gain in some instances (2). Genetic and hormonal influences such as genetic mutations and hypothyroidism can affect body weight (3). There are also some more unusual causes that may be a result of underlying endocrine disorders. For example, Cushing's syndrome, polycystic ovary syndrome, and Prader-Willi syndrome all lead to decreased physical activity and therefore, weight gain (4, 5).

There are many approaches to treating obesity. Weight loss can generally be achieved with lifestyle changes such as increasing physical activity and altering eating behavior (6). Some of these diet modifications include following a low calorie diet, eating more fiber-rich foods such as whole grains, more plant based foods such as fruits and

vegetables, more lean sources of protein like beans, lentils, fish, lean beef, and meal replacements (7). Secondly, there are surgical methods that have been shown to be very effective at inducing weight loss such as gastric bypass and sleeve cut surgery. Lastly, pharmacological products such as Orlistat can assist in weight loss by inhibiting the absorption of fats from diet and therefore reducing the calories taken in by the body (8).

Type 2 Diabetes

T2D affects the body's ability to control blood glucose levels. The primary defect in T2D is the reduced effectiveness of the hormone insulin. Insulin is released from pancreatic β-cells into the bloodstream and targets the insulin receptor on muscle or adipose cells to cause the uptake of glucose (9). Several tissues show a reduced response to insulin and contribute to the development of T2D. The muscle and adipose tissue develop dysfunctional insulin signaling pathways, thereby preventing the uptake of glucose from the blood into the tissue cells (10). Additionally, the liver develops a selective insulin resistance that increases hepatic glucose output due to a failure in the glucose sensing mechanism (11). Without glucose uptake into the tissues, excessive glucose circulates in the blood stream and can glycosylate proteins, causing damage to the organs and nervous system (12).

When we consume a meal during normal, non-diabetic conditions, blood glucose levels rise and the pancreas is able to sense this through the glucose receptor GLUT2. β-cells in the pancreas then release insulin into the circulation, signaling the muscle and adipose tissue to absorb glucose from the blood via the GLUT4 receptor (13). When an individual is constantly eating foods high in glucose and/or highly processed foods, where starches

are rapidly digested into glucose, there is continually high blood glucose and blood lipids (pre-diabetic state) which with time, develops into T2D (14).

There are four stages to the progression of T2D. The first stage starts with normal insulin sensitivity but with high blood glucose levels, followed by the second stage, impaired glucose tolerance, which is due to a slight insulin resistance by insulin-sensitive tissues. As the cells become more insulin resistant, the third stage of hyperinsulinemia occurs, as the body tries to compensate for the insulin resistance by releasing more insulin into the blood. Eventually, in the last stage, there is an inadequate amount of insulin secreted (hypoinsulinemia) as the β-cells in the pancreas die due to lipid accumulation and oxidative stress (15).

Normal fasting blood glucose levels are typically below 100 mg/dL, while in a pre-diabetic state, blood glucose levels would range between 100 to 125 mg/dL. After an oral glucose tolerance test, if blood glucose remains lower than 140 mg/dL after 2 hours, then it is considered normal, but if it is between 140 to 199 mg/dL it is considered pre-diabetic, meaning impaired glucose tolerance. Higher than 200 mg/dL is considered diabetic (16).

There are various types of medications on the market to treat type 2 diabetes. These medications are divided into 4 groups as they work using different mechanisms. There are some that work by sensitizing the body to insulin (Biguanides and Thiazolidinediones), others that decrease hepatic glucose production (Dipeptidy peptidase-4 (DPP4) inhibitors) (17), some that stimulate the pancreas to make more insulin (Meglitinides and Sulfonylureas) (18) and lastly, others that slow absorption of

starches from the intestine (Alpha-glucosidase inhibitors), which then slows the rate of glucose entering the blood stream (19).

Dietary and lifestyle changes are known to reduce the risk of T2D as well as maintain a healthy blood glucose level if already diagnosed with T2D. Some of these changes include exercise, consumption of monounsaturated and polyunsaturated fatty acids such as omega-3's, which can decrease free fatty acids (FFA) in the blood stream, a known contributor to inflammation and insulin resistance (20), consumption of fiber and whole grains, and more extreme dietary methods such as caloric restriction and invasive surgeries like gastric bypass (21-23).

Obesity related type 2 diabetes

During obesity, a risk factor for T2D as mentioned previously, there is a state of low-grade chronic inflammation which is thought to be a contributor to the progression of T2D. Excess white adipose tissue (WAT) can cause hypertrophy in the adipocytes leading to insulin resistance and increased adipogenesis (24, 25). This can result in an efflux of fatty acids from the adipocyte into the blood, which then circulate through the body and become deposited in the liver, muscle, and beta cells, causing disruptions in the insulin signaling pathway and therefore, cause insulin resistance (26-28). Increased amounts of WAT can also stimulate an immune response leading to release of pro-inflammatory cytokines such as tumor necrosis factor α (TNF-α) and interleukin-6 (IL6) from the adipose tissue and from macrophages that have infiltrated into the adipose tissue from the blood, causing a low-grade inflammation (29). This inflammation also contributes to the insulin resistance already present in the liver and muscle, worsening

T2D. In addition to plasma FFA and macrophage-derived pro-inflammatory cytokines, the WAT also secretes the hormone leptin, which is involved with regulation of appetite and energy balance (30). During obesity leptin becomes ineffective as its receptors develop resistance due to the presence of saturated fats and ceramides that prevent its signaling from controlling appetite and body weight (31). Adipose tissue also secretes other adipokines, such as adiponectin and resistin, however when there is expanding WAT, the normal plasma concentration of these adipokines becomes altered (24). Adiponectin levels decrease with increased fat mass and insulin resistance (32). Adiponectin is known to improve insulin resistance by binding to the adiponectin receptors AdipoR1 and AdipoR2 and then activating adenosine monophosphate dependent kinase (AMPK) and PPAR-α (33). This activation then causes the cells in the liver and muscle to increase energy production pathways such as β-oxidation. Resistin on the other hand is correlated with increased body fat and is thought to increase insulin resistance (34, 35), but other studies have not found this (36). Lastly, during obesity, oxidative stress may increase as a result of an oversupply of FFA and glucose in the mitochondria which are then oxidized. As a result, there is greater reactive oxygen species (ROS) production which can activate protein kinase C (PKC) and c-Jun N-terminal kinase (JNK), leading to interruption of the insulin signaling pathway (37).

Insulin Resistance

Insulin resistance during T2D is defined as the impairment of glucose uptake into insulin sensitive tissues such as muscle, liver, and adipose tissue. It can lead to hyperinsulinemia as the β-cells in the pancreas secrete greater quantities of insulin to control the excess glucose in the blood (15). The main defect in insulin resistance in the muscle is an

impaired signaling pathway, caused by the inhibition of serine phosphorylation on IRS-1, prohibiting downstream signaling and in the end, preventing glucose uptake through GLUT4 (38). The inhibition of serine IRS-1 phosphorylation can be caused by inflammatory pathways, activated by the cytokine TNF-α, and also by the accumulation of intracellular lipids such as free fatty acids, diacylglycerol (DAG), and ceramides (39). These lipids, which act as signaling agents, are increased during obesity and T2D and have been shown to activate the JNK and IκB kinase (IKK) stress response pathways to interrupt the insulin signal (40). Also, large amounts of ROS are produced through oxidation in the mitochondria due to the excess of fatty acids and glucose, which can cause mitochondrial dysfunction as well as mitochondrial death (41). Additionally, the liver becomes insulin resistant, and is no longer able to properly control glucose output while, at the same time, over-produces lipids from the excess glucose. Insulin resistance can also occur in the adipose tissue where it causes uncontrolled lipolysis, releasing more fatty acids into the circulation, further contributing to the accumulation of lipids in the liver and muscle (26-28). There are many mechanisms that cause insulin resistance but the focus here will be on three: inflammation, oxidative stress, and lipid accumulation in the liver.

Inflammation

A significant factor in the relationship between obesity and the development of insulin resistance is a chronic low-grade inflammation, particularly in the adipose tissue (42). During nutrient overload in adipose tissue, many stress pathways are triggered that stimulate the release of pro-inflammatory cytokines and adipokines into the circulation which contribute to the development of insulin resistance. Cytokines like TNF-α and IL-6

can act locally in the adipose tissue, initiating the recruitment of macrophages, which in turn secrete more cytokines and promote adipocyte cell death (43, 44). This creates a feed-forward loop that causes further inflammation. These pro-inflammatory cytokines circulate to other tissues, causing a constant, low-grade systemic inflammatory state (44, 45).

The low-grade chronic inflammatory state begins as a result of various effectors, some of which include endoplasmic reticulum (ER) stress, low adiponectin levels, higher leptin levels, uncontrolled lipolysis, macrophage infiltration, and adipocyte death (46). TNF-α is a pro-inflammatory cytokine produced mainly by macrophages and lymphocytes, however it can also be produced by adipose tissue in small amounts (47). TNF-α is thought to play an important role in insulin resistance through activation of stress kinases such as MAPK and JNK, which are able to phosphorylate serine residues on IRS-1, thereby stopping the insulin signaling pathway in the muscle (41). During obesity, the adipose tissue can over-express TNF-α and cause inflammation and a decrease in insulin sensitivity (46). TNF-α can also inhibit the nuclear receptor PPAR-γ, a transcriptional regulator of adipogenesis. As PPAR-γ controls the transcription of genes involved in the synthesis of triglycerides, reduced activity results in greater free fatty acid release from the adipocytes into the circulation, which then build up in the muscle and liver to cause insulin resistance (48). Inflammation mediators such as C-reactive protein (CRP) are also released through the action of TNF-α by the liver (64).

IL-6 is a multifunctional inflammatory cytokine produced mainly by adipose tissue and is involved in the progression of insulin resistance in the liver and muscle tissue (49). It belongs to the cytokine class I receptor family that are responsible for activating the

JAK/STATs inflammatory pathways. IL-6 levels in the blood are increased during obesity and can directly affect lipid metabolism in the liver by inducing VLDL secretion and causing hypertriglyceridemia (46) resulting in further inflammation and insulin resistance. It is unclear as to how this happens but it has been suggested to involve either tyrosine phosphatase activation, which is necessary for proper insulin signaling, or interactions between suppressors of cytokine signaling proteins and the insulin receptor. However, not all findings support a role for IL-6 in promoting insulin resistance, as it has been reported by Nieto-Vazquez et al. (50) that mice treated with IL-6 for 3 h stimulated glucose uptake in skeletal muscle, suggesting that acute exposure to IL-6 can actually improve insulin resistance.

Altered secretion of adipokines

The secretion patterns of adipokines are also altered during obesity and inflammation, such that adiponectin decreases and resistin increases, further contributing to insulin resistance. Resistin is produced in small amounts by the adipose tissue while the majority is secreted by macrophages and monocytes (46). The function of resistin is currently not completely understood but it is thought that it can induce insulin resistance in the muscle and liver (34). In-vitro studies using adipocytes have shown that resistin reduces insulin-stimulated glucose transport as well as inhibits adipocyte differentiation, which suggests a link between obesity and insulin resistance within the adipose tissue (51). Obese mice, who are insulin resistant, have increased resistin levels (34). Further, it has been shown that infusion of resistin into lean animals induces insulin resistance (34). However, the relationship between resistin and insulin resistance remains uncertain, as it has been

reported that there is a decrease in the gene expression of resistin during insulin resistance (52).

Adiponectin is another adipokine that is highly expressed in adipose tissue and released in large amounts into the circulation (53). Plasma adiponectin levels are inversely associated with obesity and insulin resistance, with lower concentrations present in obese and diabetic individuals and greater concentrations in insulin-sensitive individuals, and with increases during weight loss and (54, 55). Administration of adiponectin has been shown to reverse insulin resistance caused by adiponectin deficiency (32, 56). Adiponectin is believed to improve insulin resistance in the liver and muscle by activating AMP-activated protein kinase (AMPK), a kinase that acts as the master regulator for intracellular energy balance (57). Activated AMPK increases fatty acid β-oxidation in order to remove harmful intracellular lipids and promote insulin sensitivity in the muscle as well as decreasing intracellular malonyl CoA in the liver, which leads to lower rates of lipogenesis (58, 59). Adiponectin is also known to regulate liver glucose production by decreasing expression of the key gluconeogenic enzymes phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) (60). In addition, it is also thought that adiponectin can reduce inflammatory responses caused by TNF-α and reduce the secretion of TNF-α from macrophages (61).

Oxidative Stress

Oxidative stress is a result of an imbalance between the production and disposal of ROS produced during energy metabolism. In the insulin resistant state, this imbalance is due to greater oxidation of fatty acids and glucose in the mitochondria (62-64) . Some ROS produced include free radicals, such as superoxide ($\cdot O_{2-}$), hydroxyl ($\cdot OH$), peroxyl

(·RO), and hydroperoxyl (·HRO_{2-}), as well as non-radical species such as hydrogen peroxide (H_2O_2) and hydrochlorous acid (HOCl) (65). These species can cause oxidation of proteins, lipids, and DNA and also inhibit enzymes in the electron transport chain, blocking mitochondria respiration and therefore diminishing normal cellular functions (41). All of these effects can cause cell injury and may lead to cell death. Increased ROS production and peroxidation of cellular components can lead to mitochondrial dysfunction and a reduction in mitochondria number (66). This dysfunction can then contribute to FFA and lipid accumulation, which may lead to the interruption of the insulin signaling pathway (67). Additionally, ROS themselves are known to inhibit the insulin signal by activating the PKC, JNK, and NF-κB inflammatory pathways, as well as activating TNF-α and glucocorticoid signaling (64, 68).

Lipid Accumulation in the Liver (Nonalcoholic Fatty liver)

Non-alcoholic fatty liver disease (NAFLD) is a co-morbidity that accompanies T2D and obesity. This disease is classified as the accumulation of excess fat in the liver, accompanied by increased inflammation, which can eventually lead to more serious conditions such as liver scarring (69). Diagnosis of this disease requires evidence of hepatic steatosis, either by imaging or by histology, as well as another cause of secondary hepatic fat accumulation, hepatitis C infection, or medications (69). Diagnosis can be done by ultrasound imaging and measuring plasma levels of aminotransferase or, less commonly, by liver biopsy. World prevalence of NAFLD ranges from 6.3% to 33% in the general population whereas in obese and in T2D patients, the prevalence jumps to 90% and 69% respectively (70). NAFLD is associated with many metabolic risk factors

such as a high BMI, visceral obesity, diabetes mellitus, dyslipidemia, and hepatic carcinoma (69). The cause of NAFLD is believed to be due to an initial accumulation, due to increased influx from adipose tissue and increased conversion of glucose to fatty acids in the liver, of triglycerides in the liver, followed by increased oxidative stress in the hepatocytes, likely due to greater fatty acid β-oxidation (71). Sanyal et al. (71) found

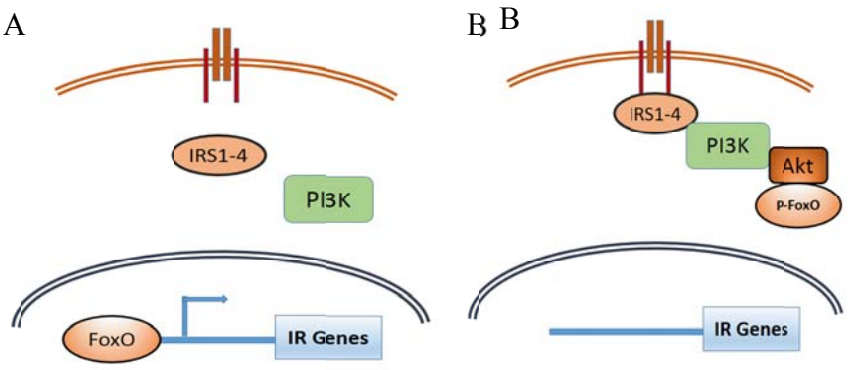

Figure 1-1. The regulation of gluconeogenesis phosphorylation of the FoxO transcription factor. Panel A shows transcriptional activation by FoxO of insulin-responsive (IR) genes, including those of gluconeogenesis. Panel B shows that insulin results in phosphorylation of FoxO, leading to a decreased transcription of IR genes. Redrawn from(73).

that patients with NAFLD had greater peripheral insulin resistance and increased plasma FFA derived from adipocytes. Increased plasma FFA, originating from uncontrolled lipolysis in insulin-resistant adipose tissue, are deposited in the liver and accumulate over time. NAFLD often occurs alongside hepatic insulin resistance, in which the liver goes through increased lipogenesis, generating an excess of triglycerides that remain in the

liver (11). The liver has selective insulin resistance during T2D in which there is increased glucose output through gluconeogenesis while, at the same time, greater fatty acid production from lipogenesis. In the normal situation, as shown in Figure 1-1, when insulin is low, the transcription factor FoxO binds to the promoter region for gluconeogenic genes, increasing their transcription and ultimately, increasing glucose production and secretion into the plasma. When insulin is in high concentration, such as after a meal, insulin can suppress gluconeogenesis by causing the phosphorylation of FoxO, which can no longer bind the promoter region (72). However, in the insulin resistant state, insulin is unable to cause the phosphorylation of FoxO, and inappropriately high rates of gluconeogenesis occur (73).

Additionally, the increase in insulin due to insulin resistance also activates the transcription factor SREBP-1c, which increases lipogenesis (11). These synthesized lipids either remain in the liver or are released into the blood within VLDL, as the liver cannot sense the high plasma glucose and attempts to send fat to other parts of the body to be used as energy (74).

This buildup of hepatic triglyceride can lead to a continuous β-oxidation of fatty acids in the liver, which can interfere with the normal function of mitochondria. This creates oxidative stress, which, when accompanied by adipose-derived pro-inflammatory cytokines such as TNF-α, is believed to play an important role in the progression of liver damage in NAFLD (75).

There are four stages to NAFLD (figure 1-2 (76)). The first stage is simple steatosis, second is non-alcoholic steatohepatitis (NASH), third is liver fibrosis, and the last stage is

cirrhosis (77). During the first stage of this disease, excess triglyceride accumulates in the hepatocytes, which is generally not considered harmful, but triggers the initial stages of inflammation. In the second stage, the development of NASH is characterized by greater inflammation and hepatocyte injury. In stage three, NASH develops into fibrosis, a persistent type of inflammation that results in the production of fibrous scar tissue around the hepatocytes and blood vessels. The last and most severe stage is liver cirrhosis where scar tissue develops from clumps of dead hepatocytes and debris from immune cells. This stage is irreversible and over time will cause liver failure (78). Treatment for this disease includes weight loss through exercise, an improved diet to reduce energy intake, and possibly an increase in consumption of bioactive compounds that may be beneficial in reducing oxidative stress (79).

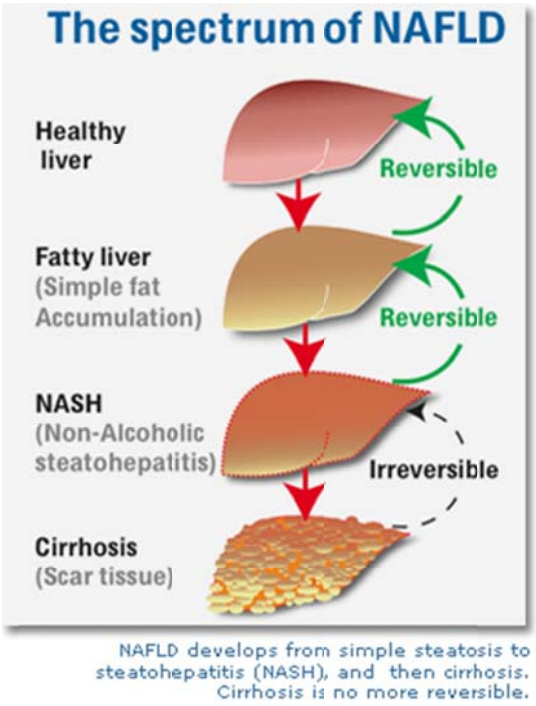

Figure 1-2. The stages of non-alcoholic fatty liver disease. I would like to acknowledge figure 1-2, as designed by the Kaori Minehira group

Whole Grains

There is growing evidence showing that whole grains play an important role in reducing the risk of chronic diseases like T2D and obesity, as well as reducing overall mortality rates, through improvements in glucose homeostasis (80, 81). Whole grains are composed of various vitamins, minerals, fiber, and bioactive phytochemical compounds which may have significant beneficial effects to health. Due to the large number of potentially beneficial components within whole grains, it is difficult to pin-point the actual component responsible, and therefore the mechanisms by which the whole grains may act to promote better health (80).

It has been reported that whole grain consumption is associated with a lower mortality rate relative to refined flours (82). As a result, there have been numerous studies conducted to examine the differences between diets high in whole grains and diets high in refined grains. With wheat being one of the most consumed grains in the United States, whole grain wheat has been widely used in research and is seen as a viable food production path for making changes that increase the overall health of the population. Observational studies (83-86) (87) and animal studies (88, 89) have demonstrated that whole wheat consumption is beneficial for reducing risk of obesity and T2D, but there is minimal research on identifying which component of whole wheat is responsible for the positive biological effects. Many researchers suggest that the health benefits could be due to the synergistic action of many compounds in the wheat (90, 91).

Wheat and Wheat Fractions

Wheat has become the predominate grain used in the United States food supply, as it has ideal properties for making consumer products such as breads, pastas, and baked goods. In addition, wheat is also consumed in side dishes and breakfast cereals as either wheat flakes or other forms like wheat berries (wheat kernels). There are two main varieties of wheat used in US food manufacturing, Triticum turgidum durum (durum wheat) and Triticum aestivum vulgare (bread wheat) (92). Bread wheat is described as soft or hard depending on what season the crop was harvested. Based on the color of the kernel, it is further classified as red or white (92). White wheat is most commonly used for making cookies, cakes, and pastries as it contains the least amount of gluten. It is also the most aesthetic in terms of color, has milder flavor characteristics, and less sweetener is needed to get the same level of sweetness than if red wheat was to be used. Red wheat has strong-flavored tannins compared to white wheat and has a higher percentage of gluten, and therefore, isn't widely used for products other than bread, making it somewhat less commonly consumed by the population. Hard wheat has more protein and is a winter crop grown in southern climates while soft wheat is a spring crop that is harvested in the fall (91). The wheat kernel is divided into 3 fractions, the bran, the endosperm and the germ, which can be separated by milling. These fractions are then sold and used for various food products such as bread making.

Whole wheat

A kernel of wheat is composed of roughly 80-85% endosperm (starch, i.e., refined wheat or white flour), 12-18% wheat bran and 2-3% wheat germ, each of which have different

macronutrient and micronutrient compositions that serve various functions for germination and growth of the plant (90). Due to the inclusion of germ and bran, whole wheat is considered to be more nutrient dense when consumed compared to the refined wheat fraction.

Bran

Wheat bran is a multilayer substance composed of various fibrous components: outer pericarp, inner pericarp, testa, nucellar epidermis, and the aleurone layer. Both pericarps are made up of branched heteroxylans, inulin, cellulose, and lignins that contain many cross-links between polymer chains due to the high content of ferulic acid dimers (80). The nucellar epidermis contains arabinoxylans, which contain the phenolic acids and other antioxidants. The aleurone layer makes up 50% of the bran fraction and is mainly composed of cell walls, which contain β-glucans (soluble fiber), some protein, arabinoxylans, cellulose (insoluble fiber), and ferulic acid. It is also rich in nutrients such as phenols, phytates, minerals, and B vitamins like niacin and folate.

Endosperm

Wheat endosperm is primarily composed of starchy carbohydrate and comprises 80-85% of the wheat kernel (80). Refined flour is predominantly wheat endosperm and during the milling process, the endosperm is separated from the bran and the germ, most of the nutrients are removed except for the carbohydrates. The endosperm contains starch, small amounts of proteins, iron, and various B vitamins and vitamin E. In the United States, almost all of the refined wheat flour is enriched to replace certain nutrients that are lost when the bran and germ are removed in the milling process (93).

Germ

The germ is 2.5-3.8% of the wheat kernel and is a potentially nutritious food supplement. It is a concentrated source of various healthy lipids, proteins, sugars, minerals, vitamins (especially tocopherols and B vitamins), phytosterols, policosanols, carotenoids, and flavonoids. The germ fraction contains relatively high amounts of unsaturated fatty acids and, because of this, can oxidize and become rancid quickly (94).

Whole Grains and Chronic Disease

Consumption of whole grain cereal products are associated with reduced obesity and lower insulin resistance, however the exact mechanism of action is unclear, as whole grains contain an enormous number of bioactive compounds that could be involved, such as phytochemicals, fiber, omega-3 fatty acids, trace elements, choline containing compounds, phytosterols, oligosaccharides, and B and E vitamins (90). Each of these compounds may have a beneficial impact on factors related to obesity and T2D, therefore it is difficult to pinpoint which individual components are responsible. As well as the individual compounds found in whole wheat, wheat is also separated into 3 different milling fractions, which can bring more uncertainty as to where the benefits are coming from. There have been few studies that compare the individual fractions of wheat using well-controlled designs, making it difficult to understand which milling fraction of whole wheat is most significant in lowering risk of chronic disease.

Epidemiological Studies

There have been a number of epidemiological studies that have reported an inverse association between whole grain intake, including wheat, and chronic diseases such as obesity and T2D. In a meta-analysis, consumption of high amounts of whole grain products, such as whole-wheat bread, were associated with a 20-30 % reduction in the risk of T2D (87).

In a prospective cohort study conducted in 2013, Parker et al. (83) found that whole grain consumption in postmenopausal women was associated with a decreased incidence of T2D. Women who consumed more than two servings per day of whole grains had a 43% reduction in the incidence of diabetes compared to women who did not eat any whole grains. Even those consuming only one serving per day showed a significant decrease in the hazard ratio for T2D incidence.

Another recent cohort study conducted by Wirstrom et al. (84) also reported an inverse relationship between high whole grain intake and the risk of diabetes. In this study, they investigated whether a higher intake of whole grain (59.1 g/d compared with 30.6 g/d) protected against the development of T2D in men and women 35-56 years of age. They found that higher whole grain intake (> 59.1g/d as opposed to 30.6 g/d) was associated with a 34% lower risk of T2D.

A prospective cohort study by Liu et al. (95) examined the associations of dietary fiber and whole or refined grain products with weight gain over 12 years, using food frequency questionnaires to collect dietary information. The results showed that, overall, women who consumed more whole grains weighed less (on average 1.5 kg) than women who

consumed fewer whole grains. The results suggest that with a dietary intake of 12 g of fiber there was less weight gain (8 lb) compared to those who had the smallest increase in intake, over the 12 year follow-up period. Weight gain was inversely associated with whole grain consumption but was also positively related to refined grain intake, suggesting that whole grains may be more beneficial for maintaining body weight than refined grains. In another study conducted by Steffen et al. (86), they observed that whole grain intake was associated with lower BMI and decreased insulin resistance in adolescents, especially among the heaviest persons.

Thus, epidemiology studies suggest that individuals who consume high amounts of whole grains have better weight management and glucose control than those consuming lesser amounts of whole grains and/or greater amounts of refined grains.

Animal studies

There have been a number of animal feeding trials investigating the effects of whole wheat, refined wheat, and wheat bran on T2D and obesity (88, 89, 96), however, there are relatively few studies on the effect of wheat germ.

In a study conducted by Adam et al. (88), the potency of different wheat milling fractions, whole wheat flour, white flour, and wheat bran, on lowering liver lipids and cholesterol as well as their effect on colonic fermentation, was evaluated in rats. The diet groups in this study consisted of each milling fraction. The whole wheat diet was composed of 70% whole wheat flour, the refined white flour diet was 48.6% refined flour and the wheat bran diet consisted of 21.4% wheat bran. The whole wheat-fed and bran-fed rats showed decreased hepatic cholesterol compared to the control group (fiber free)

and all wheat diets showed reduced liver triglycerides compared to the purified fiber-free control group. There were no differences in body weight after consuming the wheat diets for 3 weeks. Rats fed whole wheat flour and white flour showed a significant increase in cecum weight (+70% and +59%, respectively, $P < 0.001$) compared to control group, whereas rats fed the bran diet only showed a slight increase in cecum weight, suggesting greater fermentation in the 3 treatment diets. This study suggests that whole wheat flour, bran, and endosperm are all capable of reducing liver cholesterol and lipids, but have no effect on weight gain.

Another study conducted by Choct et al. (89) examined the effects of whole wheat and white (refined) flour from two wheat varieties that differed in fiber content on plasma cholesterol in rats. They also determined the concentration of soluble and insoluble fiber of the flours, which was different between the two wheat varieties, as this difference may result in altered digesta viscosity which may have an impact on cholesterol absorption. The flours were processed to a particle size of 2 mm and the diets were composed of 75% milled whole wheat and white (refined) flour and fed for 18 days. The results show that plasma cholesterol concentrations were lower in rats fed white wheat compared to whole wheat, regardless of wheat type, however there was no change in body weight. The amount of bile acids and cholesterol in the cecum was greater in rats fed the white wheat flours compared to the whole wheat flours but were unaffected by wheat variety. This study indicates that white wheat flour, in comparison to whole flour, may increase the excretion of cholesterol and bile acids in the feces, and that this may be the reason for the lower plasma cholesterol in these groups. The data from previously described animal studies are inconsistent, Adam et al. suggests that whole wheat, wheat bran and refined

wheat can lower liver cholesterol. However Choct et al. suggests that refined wheat lowers plasma cholesterol more than whole wheat and therefore reduces the risk of cardiovascular disease. These studies did not find an effect on body weight gain by refinement state of flour, and thus do not support the epidemiological associations of lower body weight with greater consumption of whole wheat.

Previously in our laboratory, we have shown that whole wheat consumption has a modest effect on the development of type 2 diabetes in the Goto–Kakisaki rat (97). Rats that were fed 65% whole wheat flour compared to a standard rat diet, the AIN-93G diet, had reduced fasting blood glucose after 2 months but not after 5 months. Whole wheat did not improve other measure of glucose control, including insulin secretion, glycated hemoglobin, oxidative stress, and β-cell mass. This study also examined the effects of other whole grains like oats, barley and maize, which also showed no effect on glucose control compared to the control diet. In this polygenetic model of diabetes without obesity, whole wheat consumption did not provide long term protection against the development of T2D.

Clinical studies

Clinical trials have shown that regular intake of whole grain cereals can contribute to lower risk factors related to non-communicable chronic diseases such as diabetes and obesity. In a short-term, randomized cross-over design study conducted by Kim et al. (98), obese women consumed a meal containing fiber from only wheat (primarily insoluble fiber), from only barley (soluble fiber) or a combination of the two grains. After a two day control diet, the women were given the test meal and their blood glucose and

insulin responses were measured for 3 h. There was no significant difference in the plasma glucose AUC, however the insulin response was significantly lower in the barley group compared to the meal containing only whole wheat. As a lower glucose and insulin response are beneficial for controlling blood glucose, this study suggests that whole wheat may not be helpful for reducing the risk of T2D compared to diets that have more soluble fiber. Clinical trials that fed whole grains for longer periods of time and measured variables more informative of insulin resistance, such as HOMA (an index of insulin resistance), or glycated hemoglobin, a measure of long-term blood glucose control, show no benefit compared to refined grains (99-101). In a crossover design study by Kristensen et al. (99), refined wheat was replaced by whole wheat in the diets of 79 overweight or obese women for 12 weeks. No differences were found in measures of blood glucose control, including fasting plasma glucose, plasma insulin, HOMA, or glycated hemoglobin. They also observed no changes in bodyweight or BMI, but did note a slight reduction in fat mass. In a randomized control trial, subjects were fed diets high in either refined wheat or whole wheat for 12 weeks (100). At the end of the study, there were no differences in fasting plasma glucose or insulin, HOMA, or another index of insulin resistance called QUICKI, between the refined wheat group and whole wheat group. In a smaller crossover design study, Giacco et al. (101) fed 15 subjects whole wheat foods or refined wheat foods for a period of 3 weeks. Here again, as in previous studies, fasting plasma glucose, insulin, and HOMA were not different between the groups. Plasma free fatty acids and bodyweight were also similar. Two other clinical trials using whole grain treatments also show no difference in bodyweight or BMI (102, 103). There have been few clinical trials that examine the effect of individual wheat milling fractions. Jenkins et

al. (104) examined the effect of wheat bran on glycemic control in a randomized crossover trial. After 3 months of consumption of 19 g/d wheat bran, there were no significant differences between the control and wheat bran groups in body weight, fasting blood glucose, or glycated hemoglobin. There is also little evidence that wheat germ is beneficial. In a 4 week study by Cara et al. (105), healthy adults were fed diets supplemented with 30 g of wheat germ per day. No changes in body weight or fasting plasma glucose were found, however they did observe a significant reduction in plasma triglycerides. Thus, the results of clinical trials do not appear to support the epidemiological observations suggesting that whole wheat consumption improves insulin resistance or reduces obesity.

Possible mechanisms of action of wheat on diabetes and obesity

While clinical studies show no effect of whole wheat on glucose control, epidemiological and some animal studies suggest otherwise, therefore possible mechanisms need to be investigated. Studies have been conducted to investigate mechanisms potentially responsible for the health benefits of whole wheat, particularly for the diabetic and obese populations. It is thought that the mechanism responsible is through decreasing blood glucose levels, liver lipids, and a decrease in body weight (106).

Several mechanisms by which blood glucose levels and cholesterol are reduced by whole wheat have been proposed, including properties of dietary fiber such as colonic fermentation and viscosity, a decreased rate of starch digestion due to slower gastric emptying, particle size effects, or the actions of phytochemicals. Colonic fermentation of dietary fiber by the gut microbiota leads to the production of short chain fatty acids

(SCFA) which may also have an impact on the body's metabolism by increasing transit time through the digestive tract and perhaps the rate of absorption of glucose. Increased viscosity of intestinal contents by dietary fibers slows the absorption of glucose, thus reducing the glycemic index of a meal (107). Reduced gastric emptying and an increased particle size of the diet slow the digestion rate of starch which can reduce the postprandial glucose curve. Lastly, phytochemicals such as ferulic acid, which is the predominant phenolic acid in wheat, may have anti-inflammatory and anti-oxidative properties which help decrease inflammation and oxidative stress and in turn, insulin resistance.

Dietary Fiber

Dietary fiber has consistently been shown to have health benefits such as decreased cholesterol. Wheat fiber is mainly composed of insoluble fiber that is minimally fermented, but it also has a small amount of soluble fiber in the form of arabinoxylans (108). The insoluble part primarily assists with bowel movements by increasing stool mass (109). Soluble fiber has several well accepted health benefits, such as lowering plasma cholesterol and improving blood glucose in type 2 diabetes patients, and may assist with weight maintenance and decreasing low-grade inflammation by decreasing plasma C-reactive protein levels (101). A high consumption of dietary fiber has also been associated with a lower risk of developing coronary artery disease, hypertension, obesity, T2D, and colon cancer (110).

To understand how dietary fiber may reduce plasma cholesterol it is necessary to understand cholesterol metabolism. Cholesterol is obtained either from endogenous production in the liver or from exogenous sources, that is dietary cholesterol. Cholesterol homeostasis is maintained by the liver and can be used for the synthesis of steroid hormones and integration into cell membranes (111). Excess cholesterol is stored as cholesteryl esters in the liver. The main route for cholesterol catabolism is through the synthesis of bile acids (111). Cholesterol in the body can be lowered through excretion of cholesterol itself or by bile acids in the feces. As bile acids are synthesized from cholesterol in the liver, greater excretion forces the liver to continuously synthesize new bile acids from stored cholesterol, thereby lowering circulating cholesterol (111).

Soluble fibers such as β-glucans have been shown to lower plasma LDL cholesterol. There are several possible mechanisms for this effect (112). One is through its effect on postprandial glycemia due to the viscosity of soluble fibers, which reduces the rate of intestinal absorption of glucose and in turn, decreases the level of insulin in the blood (113). Reduced insulin levels may lead to decreased cholesterol synthesis, as insulin may increase the gene expression of HMG-CoA reductase, the rate-limiting cholesterol synthesizing enzyme, through SREBP-1c (114). However, this mechanism seems unlikely, since viscous fibers have been shown to increase hepatic cholesterol synthesis in rats (115). Another possibility is that increased viscosity in the small intestine can hinder the emulsion and interfere with the formation of small micelles, limiting the ability of the digestive tract to absorb all the cholesterol present (116).

Particle size, starch structure, and the glycemic index

Starch structure in wheat can have a profound effect on the postprandial glucose and insulin responses by slowing the absorption of glucose (117). Diets that yield a reduced glucose and insulin response are believed to lower the risk of obesity and T2D (118). In a study conducted by Jarvi et al. (119), the effect of glycemic index in a diabetic population was examined by altering food structure but maintaining identical nutrient composition. The patients were given two meals that differed in the structure of the starch sources. One meal consisted of whole red kidney beans, parboiled rice and bread made from whole wheat grains while the other meal was sticky rice, ground kidney beans and bread made from ground wheat grains. The results showed that the AUC for blood glucose and plasma insulin where significantly lower ($P< 0.001$) in patients who consumed the meal that had a preserved structure and bigger food particle size. Similarly, Holt et al. (120) found that subjects fed coarse flour and cracked grain, which have a larger particle size and a less-disrupted starch structure, had decreased glucose and insulin responses compared to those fed fine flour. Contrarily, meals that contained white or finely ground whole wheat flours resulted in similar glucose responses. However, the standard whole wheat flour particle size may have been milled too finely for a difference to be discernable (121).

Further study of the influence of particle size was conducted by Vincent et al. (122), who examined the effect of fine and coarse bran (15 g/day) on gastric emptying and small bowel transit in a three way crossover study. This study included males and females of age 19-23 who were given a meal of either 15 g of fine or course bran, along with labeled rice, with gastrointestinal transit time measured using scintigraphic imaging. The results

showed that the time to reach 50% gastric emptying was significantly delayed by the course bran compared to the fine bran. However, there was no difference in small bowel transit time. The mechanism of action suggested was a reduced pyloric flow with the coarse bran meal due to an increased viscosity of the gastric contents. Another mechanism may be a selective retention of large particles (>2 mm) by the pylorus in the stomach, including particles containing starch, thus slowing movement of starch into the small intestine. Surprisingly, the addition of coarse bran or small plastic particles to the meal can actually speed up transit time in the small bowel, once the material has passed through the stomach (123). This may explain the laxation effect of bran.

The ratio of amylose to amylopectin in wheat starch can also have significant effects on the glucose and insulin responses to a meal (124). High amylose products produce a lower blood glucose and insulin response when compared with similar products high in amylopectin, due to reduced branching and the lower solubility of amylose (117). In-vitro digestion of chewed barley samples, differing in amylose content, showed that samples with a low amylose content were hydrolyzed more quickly than grains with a high amylose content (125). In a study conducted by Kabir et al. (124) the consumption of mung bean containing 35% amylose and cornstarch containing 0.5% amylose was evaluated in rats. They found that the high amylose mung bean diet group produced a lower glucose response compared to the low amylose cornstarch group. In another study in which rats were fed either a high amylose/low amylopectin starch or a low amylose/high amylopectin starch, the low amylose/high amylopectin group had a greater glucose and insulin response as well as a greater bodyweight and higher insulin resistance compared to the high amylose/low amylopectin (126). Therefore the presence of high

amylose rather than amylopectin appears to reduce the response of glucose and insulin to a meal.

Colonic fermentation

Certain types of indigestible carbohydrates consumed in a meal can be fermented in the colon by various bacteria (127). This fermentation results in the production of microbial metabolites including the SCFAs, acetate, propionate, and butyrate, as well as carbon dioxide and hydrogen gases, and vitamins K and B_{12} (127). These microbiota-produced metabolites can interact with receptors on epithelial cells and sub-epithelial cells causing the release of hormones that affect food intake and energy metabolism of the host (127). There have now been several studies demonstrating that whole grains are able to change the gut microbiota composition in the colon, increasing species that produce SCFAs (128). It has been demonstrated that the presence of SCFA in the colon can promote the secretion of PYY and GLP-1, which are able to slow the rate of gastric emptying and small bowel movement (129). However, recent evidence suggests that only fermentation of fructans such as inulin, is able to increase the concentration of PYY in the blood compared to other fermentable fibers like β-glucans (130). Soluble fibers such as guar gum, inulin, and pectin are highly fermentable while insoluble fibers like cellulose and wheat bran are minimally fermented (131).

Cani et al. (132) has shown that supplementation of the fructans oligofructose, a short chain fermentable fiber, in the diet of high fat-fed diabetic mice for 4 weeks, improved their glucose tolerance, fasting blood glucose, and glucose-stimulated insulin secretion, in addition to reduced body weight gain. However, in a long-term study conducted by Isken

et al. (133), mice fed the soluble fiber guar gum for 45 weeks showed a significant increase in body weight as well as increased plasma glucose AUC during an insulin tolerance test compared to mice fed insoluble fiber. It was suggested that soluble fiber increased energy extraction from the diet, causing weight gain. Lastly, in a human study by Luo et al. (134), type 2 diabetic subjects who consumed 20 g/d of short-chain fructooligosaccharides for 4 weeks had no improvement in their fasting plasma glucose, plasma glucose AUC during an insulin tolerance test, or glycated hemoglobin compared to a control group who were fed sucrose. At this time the data suggest that fermentation of soluble dietary fibers may not be able to reduce the risk of T2D.

Ferulic acid in the prevention of diabetes

Ferulic acid (FA) is a phenolic acid found in the seeds and leaves of most plants, and is the predominant phenolic acid found in wheat bran (135). FA is an antioxidant that neutralizes free radicals such as superoxide, nitric oxide, and hydroxyl radicals that may cause oxidative damage to cell membranes and DNA (136, 137). Whole grains such as wheat and oats have a high concentration of ferulic acid. However, the ferulic acid is mostly bound to the cell wall in the grains and therefore is only slightly bioavailable due to limited release by gut microbiota (138). However, ferulic acid can be freed from cell wall components through processing methods such as fermentation and alkaline treatment. Ferulic acid supplementation at relatively low doses has been shown to increase the activities of antioxidant enzymes, thus neutralizing free radicals which, in diabetes, are a primary cause of accelerated tissue damage (139). While ferulic acid is a strong antioxidant, it is unlikely that the amount absorbed from wheat, where it is bound and unavailable, has a meaningful effect on overall antioxidant status through classical

mechanisms as a free radical scavenger (139). However, supplementation of ferulic acid has beneficial effects on various chronic diseases, especially type 2 diabetes (135, 140). In streptozotocin-induced diabetic rats, supplementation of purified ferulic acid (10 mg/kg/d) for 6 weeks resulted in lower fasting blood glucose, TBARS, and free fatty acids and greater pancreatic islet mass (141). In another study using OLETF rats, a less severe model of insulin resistance than streptozotocin-induced diabetes, ferulic acid (10 mg/kg/d) supplemented for 20 weeks resulted in a decrease in the plasma glucose AUC during glucose and insulin tolerance tests and greater plasma adiponectin, compared to rats not supplemented with ferulic acid (135). In high fat-fed mice, ferulic acid supplementation for 7 weeks decreased body weight and plasma and liver triglycerides (140). These studies suggest that supplementation of ferulic acid may be a viable therapeutic method for reducing the progression of obesity and T2D.

Phytosterols as a mechanism in decreasing cholesterol

Studies have shown that plant sterols are able to decrease intestinal cholesterol absorption and therefore reduce LDL cholesterol in the plasma (142, 143). Plant sterols are similar in structure to cholesterol and therefore use the same mechanism for solubilization in the lumen and for absorption from the intestine lumen into the enterocytes. Plant sterols are more hydrophobic compared to the cholesterol and therefore have a higher affinity for incorporation into the micelles. This causes the cholesterol to remain in the lumen instead of the micelles, therefore reducing intestinal absorption and increasing cholesterol excretion in the feces (144). The presence of plant sterols in the wheat germ could therefore have an impact in lowering LDL cholesterol.

Study Aim

Evidence from epidemiological and animal studies suggests that whole grain intake is beneficial in decreasing obesity and insulin resistance, whereas clinical studies do not show the same result. Further work is necessary in this area to more fully understand the underlying health benefits of whole grains, particularly whole wheat. Currently there are no studies that investigate the effect of individual wheat milling fractions, compared to whole wheat, on T2D and obesity. Therefore, the aim of the following study was to examine the effects of whole wheat and wheat milling fractions on glucose control, insulin resistance, and fatty liver using an animal model of obesity with type 2 diabetes.

Chapter 2: Whole Wheat and Wheat Milling Fractions Improve Glucose Control and Reduce Non-Alcoholic Fatty Liver in the Zucker Diabetic Fatty Rat

Purpose: Whole wheat has been shown to improve glucose control and insulin resistance, and decrease fatty liver. However, which milling fraction is responsible for these positive effects is unknown. Therefore, this study examined the effects of whole wheat flour and its milling fractions, bran, germ and endosperm (refined wheat), on glucose control, insulin resistance, fatty liver, and adiposity, in an animal model of obesity with type 2 diabetes, the Zucker Diabetic Fatty (ZDF) rat.

Methods: Male ZDF rats were fed ad libitum either 64% whole wheat flour, 54% refined wheat, 9.4 % wheat bran, 1.6% wheat germ or 15% wheat germ (hi-germ) for five weeks. ZDF obese control and lean ZDF littermates (negative control) groups were fed a purified diet containing 50% cornstarch. The refined wheat, wheat bran and wheat germ diets were present in the same concentration as would be found in the whole wheat diet.

Results: After 5 weeks, the whole wheat, refined wheat, wheat germ, and high-germ groups showed significant reductions in plasma glucose AUC during glucose and insulin tolerance tests compared to the obese control ($p<0.05$). There were no differences in body weight or fat pad weight among the ZDF groups but there was a significant reduction in fat mass % in whole wheat group compared to the obese control group ($p=0.031$). The whole wheat group and all wheat fraction groups decreased the concentration of liver lipids compared to the obese control and the bran and both germ groups had lower liver cholesterol ($p<0.05$). Only the whole wheat group had both a greater cecum pH ($p<0.0001$) and cecal weight ($p<0.0001$) compared to the obese control, indicating greater fermentation of whole wheat diet. There were no significant differences in plasma resistin concentrations among the ZDF diet groups. However, plasma adiponectin

concentrations were significantly lower in the whole wheat group compared to the obese control group ($p= 0.032$).

Conclusion: The results show that each individual milling fraction is as beneficial as whole wheat itself in slowing development of insulin resistance and reducing fatty liver, and that the effects of the milling fractions are not additive.

Introduction

Chronic non-transmittable diseases (NCD) such as obesity and type 2 diabetes (T2D) previously occurred predominantly in developed nations but are now becoming more common in developing countries, outnumbering transmissible diseases (3). There are now 347 million people worldwide with T2D. In the United States alone there are 18.8 million people with T2D and another 79 million people in a pre-diabetic state (3). The incidence of newly diagnosed cases of diabetes is at its highest with 1.9 million per year. It is predicted that 1 in 3 Americans will have diabetes by the year 2050 if these trends continue (145). Often accompanying T2D, obesity is also at an all-time high with 1 in 3 Americans being obese (1). Due to the magnitude of the prevalence of obesity and T2D, they are now in the top ten causes of death in North America, and therefore are among the greatest public health concerns. (1).

Changes in lifestyle, food habits, and decreased physical activity are implicated in the rapid increase in obesity and T2D. The combination of diabetes and obesity is particularly harmful as it increases the risk of comorbidities, such as heart disease, kidney disease, nerve damage, dyslipidemia, blindness, and nonalcoholic fatty liver disease (NAFLD), drastically increasing mortality rates. Occurring in 70% of type 2 diabetes patients, NAFLD results from increased fat accumulation in liver cells (146). This can cause inflammation and increase the rate of gluconeogenesis which will aggravate hyperglycemia and increase the risk of T2D (147).

Several epidemiological studies suggesting that whole grain consumption can decrease the risk of obesity and T2D. A recent observational cohort study found that people who ingested whole grain cereals had 30% less risk of developing type 2 diabetes (84). Whole grain consumption has been found to be inversely associated with body fat mass and insulin resistance in both humans and rats (83). However, it is unclear which milling fraction of the whole grain, the bran, germ, or endosperm, is responsible for these health benefits. The bran contains high concentrations of dietary fiber, which is thought to reduce obesity and diabetes by slowing down the digestion and absorption of digestible carbohydrates as well as increasing feelings of fullness (148). It also contains large quantities of ferulic acid which has been suggested to have anti-inflammatory and hepatoprotective properties and to improve glucose control (135, 140). The germ is high in vitamins, minerals and essential fatty acids which may reduce inflammation and improve immune system function (149). Currently there are no studies that investigate the effect of individual wheat milling fractions, compared to whole wheat, on T2D and obesity. Therefore, the aim of this study was to examine the effects of whole wheat and wheat milling fractions on glucose control, insulin resistance, and fatty liver using an animal model of obesity with type 2 diabetes.

Methods and Materials

Animal Model

Choosing the correct animal model for the research objective is very important, as it will dictate the reliability of the results. The Zucker Diabetic Fatty (ZDF) rat was chosen in this experiment because it is a good model for studying the initial stages of type 2

diabetes with obesity. This rat model has a nonsense mutation in the leptin receptor gene, which is normally expressed most abundantly in the brain (150). This results in a defective leptin receptor, resulting in an absence of leptin signaling in the brain and causing hyperphagia. Characteristics of this model include obesity, insulin resistance, hyperinsulinemia, type 2 diabetes, hyperlipidemia, fasting hyperglycemia and hypercholesterolemia (151). At 5 weeks of age their initial weight ranges from 80 to 140 g and at week 10, from 280-380 g (152). The ZDF rats have lean ZDF counterparts which are much smaller and do not develop obesity or T2D. Since our research objective was to assess metabolic changes associated with the onset of obesity and T2D during a whole wheat and wheat milling fraction dietary intervention, the ZDF rat appeared to be an appropriate model.

Housing and care

The ZDF rats were purchased from Charles River Laboratories (Wilmington, MA) at 5 weeks of age. They were housed individually with a 12h light and dark cycle with food and water provided ad libitum. The animals were handled according to the National Institutes of Health guidelines and experimental procedures were approved by the University of Minnesota Animal Care and Use Committee.

Diet

There were 7 diet groups of 10 animals each. The positive control (ZDF) and the negative control (ZDF lean) were fed the AIN-93G purified diet (153). The remaining 5 ZDF

groups were fed diets containing either 64% whole wheat flour, 54% refined wheat, 9.4 % wheat bran, 1.6% wheat germ, or 15% wheat germ for 5 weeks (Table 1).

Soft white wheat and its milling fractions were sourced from a milling company (Star Of the West Milling Co., Frankenmuth, MI) and stored at 4º C. A proximate analysis was conducted to determine the protein, carbohydrate, lipid, total dietary fiber, and ash content of the whole wheat and milling fractions (Medallion Laboratories, MN, USA). The results of the analysis were used to formulate the diets, ensuring that they were matched for macronutrient content and therefore were isoenergetic. A final analysis of the diets was conducted to check for the concentration of resistant starch and fibers. The diets were formulated so that the amount of the milling fraction present in the bran, refined, normal wheat germ diets were the same as they would be present in the whole wheat diet. The high wheat germ diet had 10 times the amount of germ present in the normal germ diet.

Experimental design

The animals were fed the diets ad libitum for 5 weeks. A meal tolerance test was conducted at the end of week 1, an insulin tolerance test and a glucose tolerance test at the end of week 3, a urine collection at the end of week 4, and a 48 h fecal collection was done at the beginning of week 5. In addition, body composition was determined by dual x-ray absorptiometry (DXA) scanning (Lunar Prodigy Advance, GE Healthcare, Piscataway, NJ) at the end of week 4. At the end of week 5, 12 h fasted rats were anesthetized by isoflurane, blood collected by cardiac puncture into tubes containing EDTA (1 mg/mL), and the kidneys, liver, epididymal, inguinal, and retroperitoneal fat

pads were harvested and stored at -80° C. The blood was centrifuged, plasma collected, and then stored at -80° C until analysis. The pH of the cecal contents was measured using a combination spear-tip pH electrode (model 81-63, Orion Research, Boston, MA) and then the cecum was emptied of contents, flushed with distilled water and weighed. Feces were freeze-dried, weighed and stored at -50 °C. At 4 weeks, rats were placed in metabolic cages with free access to food and urine collected over 24 h which was then frozen at -50 °C until analysis. Body weight was measured weekly and food intake was measured at weeks 1, 2, and 3.

Plasma analysis

Plasma glucose was measured in whole blood using a glucometer (AlphaTrak, Abbott Laboratories, Abbott Park, IL) calibrated for rodents. Fasting plasma adiponectin was measured using an ELISA kit (Millipore, Billerica, MA) and fasting plasma resistin was measured using an EIA kit (Cayman Chemicals, Ann Arbor, MI). Fed-state plasma insulin was measured using a rat-specific radioimmunoassay kit (Millipore).

Meal, glucose, and insulin tolerance tests

During the meal tolerance test, 12 h fasted rats were fed 2.5 g (1.33 g digestible carbohydrate) of their respective diets and allowed 20 minutes to consume the meal. Blood glucose was measured at time intervals of 0, 15, 30, 60, 90, and 120 minutes after presentation of the meal and ~250 μL blood collected at time points 0, 30, 60, 90, 120, and 180 minutes to measure plasma insulin.

During the glucose tolerance test, 12 h fasted rats were given glucose via gavage (0.5 g\kg) and blood glucose was measured using a glucometer at time points 0, 15, 30, 60, 90, 120, and 180 minutes. Area under the curve (AUC) was calculated by the trapezoidal rule, using a blood glucose concentration of zero as a baseline.

During the insulin tolerance test, insulin (1 IU/kg) was injected into the abdominal cavity and blood glucose was measured at time points 0, 15, 30, 60, 90, 120, and 180 minutes and AUC calculated as above.

TBARS and urinary ferulic acid analysis

Urinary thiobarbituric acid-reactive substances (TBARS) were measured using a previously described method (154).

Urinary ferulic acid was measured by HPLC after deconjugation using a modification of the method of Bunzel et al. (155) Briefly, ferulic acid was deconjugated by mixing 5% of 24 h collected rat urine with the same volume of 1 M sodium acetate buffer (pH 4.9), 300 µL of sulfatase solution (Sigma, S9626-10KU, ST. Louis, MO), and 100 µL of 0.1 mg/mL alpha coumaric acid solution (in 50% aqueous methanol). The mixture was incubated 37 °C for 3 h. The reaction was stopped with concentrated hydrochloric acid 0.3 mL and ferulic acid extracted with a total of 4 mL ethyl acetate (2 mL, 1 mL, 1 mL). The pooled ethyl acetate phases were extracted with 5 mL of 5% sodium hydrogen carbonate (2 mL, 2 x 21.5 mL) and phases separated by centrifugation. The combined sodium hydrogen carbonate phases were acidified with 0.5 mL of concentrated hydrochloric acid until the pH was <2, and then extracted with 3 x 2.5 mL ethyl acetate.

The ethyl acetate phases were collected and dried under nitrogen. The dried extract was reconstituted in 200 μL 50% methanol and 1:5 diluted for HPLC analysis.

Quantification of ferulic acid was achieved using a Shimadzu HPLC system with auto sampler and SPD-M20A PDA detector. HPLC conditions are as follows: A Luna Phenyl-Hexyl column (250 x10 mm; 4.6 μm particle size) was used for separation at a flow rate of 1 mL/min and temperature of 45° C. Mobile phases were 0.1 mM trifluoroacetic acid (TFA) (mobile phase A), 90% methanol/10% TFA (mobile phase B) and 90% acetonitrile/10% TFA (mobile phase C). The following gradient program was used (156): 87% A and 13% C for 10 min; change over 10 min to 77% A, 3% B, and 20% C; change over 5 min to 70% A, 5% B, and 25% C; and finally change over 5 min to 25% A, 25% B, and 50% C. Detection wavelengths were 280 and 325 nm.

Liver lipids and cholesterol

Liver lipids were extracted with organic solvents using the method of Fölch et al. (157). The solvent was evaporated under nitrogen, and lipids determined gravimetrically. Liver cholesterol concentrations were determined enzymatically as previously described by Gallaher et al. (158) and fecal bile acids extracted and purified by the method of Locket and Gallaher (159) and quantified using the method of Sheltawy (160).

Statistical analysis

The results were analyzed by ANOVA using the SAS statistical program (SAS 9.3, SAS Institute, Cary, NC). The group means were compared by Duncan's multiple range test

and comparison of fat mass % between the obese control and whole wheat groups was done using Student's t-test. Final body weight of the obese control and whole wheat groups was compared using Student's t-test. Statistical significance was taken at $p<0.05$.

Results

Whole wheat and milling fractions do not alter body weight and food intake

At the end of 4 weeks, the ZDF groups had significantly greater body weight compared to the lean group, as expected due to the animal model. However, there were no significant differences in weight gain among the ZDF groups (Table 2). Additionally, while the ZDF groups did have a greater mean food intake than the lean group, there were no significant differences in food intake among the ZDF groups (Table 2).

Whole wheat produces greater fermentation

Rats fed the whole wheat diet showed a significant decrease in cecum pH compared to the obese control, suggesting greater fermentation of this diet (Table 2). The cecum weight was significantly greater in the wheat based diets compared to the obese control except for the wheat bran group (Table 2). Among the wheat diet groups, the whole wheat showed a significantly greater cecum weight compared to the other wheat diets, further suggesting greater fermentation in this group.

Whole wheat, but not milling fractions, reduce fat mass %

The obese control had greater epididymal, inguinal, and retroperitoneal plus perirenal fat pad weights compared to the lean group, as expected, but there were no significant differences among the ZDF groups in fat pad weights (Table 2).

Likewise, a DEXA scan did not show significant differences in fat mass or lean mass percentage among the ZDF groups (Table 2). However, when compared by Student's t-test, the whole wheat group showed a significant decrease in fat mass percentage compared to the obese control ($p=0.025$).

Whole wheat and wheat milling fractions reduce insulin resistance.

A meal tolerance test was conducted at week 1 to examine the effect of the diets on the uptake of glucose and the insulin response to the diet. There were no differences in the plasma glucose AUC during the meal tolerance test between the obese control and the wheat milling fraction diet groups, although wheat bran did have a significantly greater AUC compared to the high wheat germ (Table 2). The obese control group had a significantly greater insulin AUC than the lean control but there were no differences among the ZDF groups in the insulin AUC.

After 3 weeks on the experimental diets, the fasting plasma glucose concentration of the whole wheat diet and refined wheat groups was significantly less compared to the obese control group while wheat bran, wheat germ, and high wheat germ were not different (Table 2).

During the GTT at week 3, all wheat diets, except for wheat bran, had a reduced plasma glucose AUC compared to the obese control group (Table 2). Similarly, during an ITT,

also performed at week 3, the plasma glucose AUC was significantly lower in all ZDF wheat diet groups compared to the obese control (Table 2).

A greater kidney weight as a percentage of body weight is considered a marker of poor blood glucose control (161). The kidney weight percentage was lower in the lean group compared to the ZDF groups, but there were no differences among the ZDF rats (Table 2).

Urinary 24 h TBARS was measured as a marker of whole body lipid peroxidation (Table 2). Urinary TBARS was significantly greater in the obese control group compared to the lean control group. Urinary TBARS was significantly greater in the whole wheat, refined wheat, wheat bran, and high wheat germ groups than the obese control group, and whole wheat was greater than all other groups except high wheat germ.

Plasma adiponectin, an adipokine that is generally inversely proportional to body fat and insulin resistance, was in fact greater in the obese control compared to the lean group (Table 2). The whole wheat group had a significantly lower plasma adiponectin concentration compared to the obese control whereas the individual wheat milling fraction groups were not different from the obese control group. Resistin, another adipokine, is known to increase in models of obesity and T2D and has been suggested to be a link between obesity and insulin resistance (162). Plasma resistin was greater in the obese control group compared to the lean group. Although there were no differences among the ZDF groups, plasma resistin in the whole wheat and wheat milling fraction groups did not differ from the lean group (Table 2).

Fatty liver is reduced with whole wheat and wheat milling fractions.

The liver weights of the lean group were significantly less than the ZDF groups, yet there were no differences among the ZDF groups (Table 3). The whole wheat and wheat fraction diets, however, had lower total liver lipids compared to the obese control.

Total liver cholesterol concentration was significantly lowered in the wheat bran, germ, and high germ groups compared to the obese control, whereas the whole wheat and the refined wheat only showed a trend in decrease (Table 3). Liver free cholesterol concentration was not different among the ZDF groups. There was no difference in bile acid excretion between the obese control and lean groups, yet among the wheat groups the whole wheat and wheat bran groups had significantly greater fecal bile acid excretion compared to both the lean and obese control groups (Table 3). Fecal bile acid excretion did not correlate with liver cholesterol concentration (r^2 =0.05, p = 0.67).

Whole wheat and wheat germ groups had the greatest absorption of ferulic acid

Ferulic acid, a phenolic acid found in wheat, has been suggested to slow the progression of diabetic complications (135, 163). The whole wheat group had the greatest intake of total ferulic acid followed by wheat bran, high wheat germ, refined wheat and, lastly, wheat germ (Figure 1). As there were differences in the relative concentration of total and free ferulic acid in the wheat diets, the daily intake of free ferulic acid was slightly different. The refined wheat group had the greatest free ferulic acid intake, followed by whole wheat, high wheat germ, wheat bran, and wheat germ (Figure 1). Urinary excretion of ferulic acid, a measure of ferulic acid absorption, mirrored the concentration present in the individual diets, with whole wheat having the greatest urinary ferulic acid excretion, followed by bran, high wheat germ, refined wheat and, lastly, wheat germ.

Discussion

There is considerable epidemiological evidence indicating that consumption of whole grains, of two or three servings per day, in comparison to more refined grains, are associated with a lower risk of obesity (164, 165) (decrease in body weight and a change in body composition) and type 2 diabetes (85, 149), although there are also studies that show no effect (166). When wheat is milled, the bran and germ are removed, and most of the dietary fiber, vitamins, minerals, essential fatty acids, and phytochemicals are lost. It is unknown which milling fraction of the whole wheat, the bran or the germ, may be responsible for the putative health benefits. Therefore, we investigated the effect of whole wheat and its individual milling fractions, endosperm, bran, and germ, on parameters of glucose control, insulin resistance, adiposity, and fatty liver using an animal model of the initial stages of diabetes with obesity, the ZDF rat.

After 5 weeks consuming the diets, there were no differences in body weight or fat pad weights among the ZDF groups, suggesting that neither whole wheat nor its bran or germ milling fractions were more effective than endosperm (refined wheat) at minimizing weight gain and body fat. Although whole grain intake is highly associated with a lower body weight in observational studies (95, 167), a direct relationship is not supported in clinical trials (102, 103). In the present study, no decrease in body weight was observed with consumption of whole wheat, yet a decrease in body fat percentage in the whole wheat group compared to the obese control was found by DEXA scanning. These findings are similar to a study conducted by Kristensen et al. (99), in which overweight or obese women fed isoenergetic diets containing either refined or whole wheat for 12

weeks showed no difference in body weight but did have a decrease in body fat percentage by DEXA, implying a change in body composition. A study in women conducted over 20 months reported that for every 1g increase in total fiber consumed a day body weight decreased by 0.25 kg and body fat by 0.25% (168). However, in the present study all diets were isofibrous, so changes in dietary fiber consumption does not explain the reduction in body fat experienced by the whole wheat group, as measured by DEXA scanning. A possible reason as to why no differences in the fat pad weights were seen could be the greater variability in fat pad weights compared to DEXA scanning, as a result of the challenge in precise removal of fat pads in these extremely obese animals. There were no differences in body weight or body fat percentage with the milling fractions compared to the obese control. This is consistent with other studies reporting that wheat bran fed to obese Zucker rats did not alter either final body weight or fat pad weight (169). Although glucose intolerant subjects given wheat bran as a supplement to their diet experienced a slight but statistically significant decrease in body weight (170), body weight in patients with diverticular disease experienced no change in weight with wheat bran consumption (171).

A possible explanation for the reduced fat mass percentage in the whole wheat group could be the effect of fermentation. After 5 weeks on the diets, the whole wheat diet group showed a significant decrease in pH level and greater cecum weight, suggesting greater fermentation of the diet. In a similar study pigs were fed different whole grains, including whole wheat, and saw an increase in fermentation which was believed to be due to fermentation of the soluble fiber arabinoxylans present in cereals (172). Adam et al. (88) also saw decreased pH and increased cecum weight in rats fed whole and refined

wheat, but not with wheat bran, which can be attributable to the small amounts of soluble fiber present in bran. Fermentation increases production of short chain fatty acids (SCFA), which have been shown to increase the release of the satiety peptides PYY and GLP-1 in some cases (173) but not others (130), depending on the type of fermentable fiber.

The reduction in fasting blood glucose and glucose AUC during an ITT and GTT suggests that all wheat diets led to a significant improvement in glucose control and reduction in insulin resistance compared to the obese control group. However, there was only a trend for a lower GTT AUC and fasting blood glucose in the wheat bran group compared to the obese control group. A decrease in kidney weight as a percentage of body weight is considered a secondary maker of blood glucose control (161). In this study, there were no differences in relative kidney weight among the ZDF groups, even though there was a decrease in insulin resistance in the whole wheat or wheat fraction-fed groups. It is interesting that both whole wheat and refined wheat were able to improve insulin resistance to the same degree compared the control diet. This result brings into question the recommendation to consume whole grains over refined grains to reduce the risk of diabetes. Several clinical trials that examined the effect of whole grains versus refined grains also did not show improved glucose control (100-102). The inconsistent findings of improved insulin resistance and decreased BMI with whole grain consumption in observational studies and the lack of such an effect in clinical trials may be due to factors that were not controlled for such as healthier lifestyles in general. Participants who ate more whole grains in observational studies may have a better diet and may be more physically active (174). We suggest that the greater insulin sensitivity

of the whole wheat and the refined wheat groups, compared to the obese control, is due to the type of starch present. The obese control diet contained only purified corn starch whereas the whole wheat and refined wheat contained mostly wheat starch. Unpurified wheat starch contains proteins and more complex starch granules compared to purified corn starch (175). This, along with small amounts of soluble dietary fiber in the form of arabinoxylans, could slow glucose absorption, which is known to reduce the progression of insulin resistance (176, 177). The differences between the wheat diets and the control diet, which used corn starch as the main carbohydrate source, maybe be due to differences in the ratio of amylose to amylopectin, as amylose has a lower glycemic index than amylopectin (117). The percent of amylose and amylopectin in wheat starch is 25% and 75% respectively (178) whereas in corn starch it is 5% and 95% (124). Although wheat bran was able to decrease insulin resistance, as shown by the decreased AUC during the ITT, there was not a corresponding change in the GTT or fasting blood glucose. This is consistent with a well-controlled study by Jenkins et al. (104), who found that consumption of wheat bran for 3 months did not alter measures of blood glucose control.

One characteristic of whole grains that has been suggested to improve insulin sensitivity is their low glycemic index. This is believed to improve glucose control by limiting large spikes in blood glucose and insulin (179). Interestingly, during the MTT in week 1, there was no difference in the AUC of the postprandial plasma glucose or insulin curves of the wheat diets compared to the obese control. This might suggest that the reduced insulin resistance observed in the wheat diets is not to be due to a lower glycemic index compared to the control diet. However, it may be that in this ZDF model the differences

in postprandial insulin and glucose caused by the wheat diets are too small to be observed from a meal tolerance test, but still result in improvements in glucose control by the end of 3 weeks of feeding.

It is unclear why wheat germ and high wheat germ improve insulin sensitivity and glucose control. The effect of wheat germ on insulin resistance is not widely studied, but in a clinical trial by Cara et al. (105), subjects who consumed 30 g of wheat germ for 4 weeks had no change in fasting blood glucose. There have been studies that suggest that particle size of the diet could be responsible for the differences in glucose control. It has been shown that larger particle sizes decrease postprandial glycaemia compared to smaller particle sizes (120, 180). However, a reduced glycemic response is not observed in all studies (121). In various animal studies it has been shown that food structure has an influence in gastric emptying, which reduces the rate of glucose entry into the bloodstream (180), therefore allowing circulating fatty acids to be used for energy (181). Increased circulating fatty acids are involved in the progression of peripheral and hepatic insulin resistance (182). Simply by not chewing food finely, whether the food is rice, potatoes or apples, will slow down glucose absorption, suggesting that the structure of food is more important than its composition (183). Larger particle sizes, such as wheat germ, have been shown to affect gastric emptying (184), however in this study a lower glycemic response was not observed and neither was it in other studies (185). Therefore, other explanations for the improved insulin resistance and glucose control are needed. There are data suggesting that wheat germ supplementation may reduce total lipid absorption (186) which could indirectly improve insulin resistance through decreased body fat. However, as we saw no difference in fat mass %, it is unclear how this may

have affected insulin resistance. There was no additive effect of the wheat fractions on glucose control because there was no difference between the wheat fraction groups and the whole wheat group.

Adipokines released by adipose tissue are associated with insulin resistance and fat mass. Circulating adiponectin, an adipokine that is generally inversely proportional to body fat, is generally reduced during insulin resistance (32, 187). Obese ZDF rats normally have lower concentrations of adiponectin compared to their lean counterparts (188), but in this study the obese control had significantly greater adiponectin concentrations compared to the lean rats. It is unclear why the obese control group would have greater adiponectin concentration compared to the lean and whole wheat groups, even though they have greater insulin resistance.

Resistin is released by adipose tissue (189) and has shown to be increased in animals with high body fat compared to lean animals (35). High plasma resistin levels have been associated with insulin resistance in humans (190), however other studies show no association (191). There was no significant difference in resistin levels among the ZDF groups, although there was a trend toward a decrease in the wheat groups compared to the ZDF control.

Oxidative stress can be classified as an imbalance between the formation of reactive oxygen species and the ability for the body to eliminate these reactive species that can cause damage to cellular macromolecules. These reactive species can trigger inflammatory pathways such as NFκβ and JNK, which can activate kinases that interrupt insulin signaling, causing insulin resistance and T2D (192). A buildup of free radicals can

attack lipids, particularly unsaturated fatty acids, and cause peroxidation products to accumulate. Urinary 24 h TBARS were measured as a marker of whole body lipid peroxidation. Increased urinary TBARS is typically associated with greater insulin resistance, yet in this study, all wheat diets, except for wheat germ, had greater TBARS excretion compared to the obese control. It is unclear why the groups with lower insulin resistance had greater oxidative stress. Due to urine being collected in non-fasting rats, it is possible that other compounds in the wheat may have influenced the results of the TBARS assay.

NAFLD typically accompanies obesity and T2D and is characterized by an accumulation of triglyceride lipid droplets in hepatocytes. Increased fatty acid flux from enlarged adipose tissue as well as increased de-novo lipogenesis and/or increased fatty acid oxidation during hepatic insulin resistance, contributes to the accumulation of lipids (71). There was no difference in liver weight between the obese control and wheat groups, however, all whole wheat and wheat fraction diets had lower total liver lipids compared to the obese control, indicating a reduction in fatty liver. This is supported by previous studies, where the addition of whole wheat, refined wheat or wheat bran to the diet of rats reduced liver triglycerides (88). As mentioned above, a lower glucose and insulin response, either through small amounts of soluble fiber, the structure of starch granules, or particle size, could be the explanation. There is selective insulin resistance in the liver, where insulin fails to inhibit gluconeogenesis while at the same time is able to upregulate lipogenesis (74). This is in agreement with our observation of reduced glucose control, probably in part through increased gluconeogenesis, with greater fatty liver in the obese control group compared to the wheat diets. The decrease in total liver cholesterol

concentration in wheat bran, germ, and high germ groups may be due to greater fecal bile acid excretion, which was also greater in these groups. Bile acids are synthesized from cholesterol in the liver and therefore an increase in the excretion of bile acids results in the decrease of cholesterol in the liver (193) (194). This was not the case in the whole and refined wheat groups where there was no difference in liver cholesterol yet there was greater bile acid excretion. Previous studies show that cholesterol is decreased with soluble fiber consumption (195) and wheat fiber, which is mostly insoluble, has no effect (196). Another possible explanation for the decrease in liver cholesterol in the wheat germ groups could be the relatively high concentration of plant sterols. Due to the higher affinity of plant sterols for incorporation into the micelles, compared to cholesterol, there is reduced absorption of cholesterol from the intestinal lumen and therefore greater excretion in the feces (144). It appears that neither fiber content, which was mostly insoluble and balanced among all groups, nor bile acid excretion can entirely explain the decrease in total and free liver cholesterol.

Whole wheat contains high amounts of ferulic acid, a phenolic compound that may have a beneficial role in reducing body weight (140) and slowing the progression of T2D (135). Ferulic acid is able to form a stable radical, allowing it to scavenge reactive oxygen species (ROS) produced as byproducts in the electron transport chain. ROS have been implicated in insulin resistance by interfering with normal insulin signaling pathways and triggering inflammation in the muscle and liver (139). Ferulic acid is mostly bound to the cell wall of wheat and therefore is only slightly bioavailable due to limited release by gut microflora (138). The whole wheat and wheat bran groups, which had the greatest amount of ferulic acid absorbed, based on urinary ferulic acid excretion,

showed improvements in glucose control and insulin resistance. On the other hand, this may not be due to ferulic acid absorption because the wheat germ, which showed little urinary ferulic acid excretion, also showed decreased insulin resistance. In fact, wheat bran, which is the milling fraction containing the most ferulic acid, did not cause a difference in GTT AUC or fasting blood glucose. These results do not support the suggestion that insulin resistance is altered by ferulic acid in wheat as all the wheat diets showed an improvement in glucose control.

In conclusion, the consumption of whole wheat and its milling fractions for 5 weeks improved glucose control and decrease insulin resistance and fatty liver in ZDF rats. Whole wheat, however, was no more effective at improving these parameters than its individual milling fractions. These effects do not appear to be due to changes in plasma adiponectin concentration or absorption of ferulic acid. It is possible that small amounts of soluble fiber in the whole wheat and refined wheat fraction may be responsible for the positive health benefits, but it is unclear why the bran and germ fractions were also equally effective. Thus, in this short term study, none of the wheat milling fractions stand out as responsible for the effects seen with consumption of whole wheat. The results of this study suggest that individual milling fractions of wheat are just as effective in improving insulin resistance and fatty liver as whole wheat.

Advantages and limitations of the study and future directions

This study has many advantages, such as a well-controlled study design, use of a food source in a form that is normally consumed in the population rather than a food extract, a balanced macronutrient composition, and an examination of each milling fraction in

addition to whole wheat. To our knowledge, this is the first study to do this. That said, there are also some limitations. One unavoidable limitation was the length of the study. It would have been helpful to prolong the feeding trial to see long-term effects of the diets, but due to the characteristics of the animal model, the rats would have been in an advanced state of diabetes and it would have been impossible to see changes brought on by the diet. Even in this short term study the animals may have been too diabetic and obese, making it more difficult to detect large changes between the groups. Finally, as with all animal studies, to what degree the results can be extrapolated to humans is uncertain.

Following the results acquired from this study, future research can be directed in various ways to further explore possible mechanisms of whole wheat and its milling fractions. First, the effect of particle size on postprandial glucose curve could be examined by milling whole wheat into different particle sizes and observing whether there is a difference in the parameters we studied. Another approach could be varying the ratios of amylose to amylopectin using different wheat varieties to determine its effects on insulin resistance and glucose control. Using a different animal model, such as the high fat-fed rat, and feeding for a longer period of time could also be helpful in finding small differences that may not have been detectable with the ZDF model.

Table 2-1. Diet composition

Nutrient (g/kg)	Control	Whole wheat flour	Refined wheat flour	Wheat bran	Wheat germ	High Wheat Germ
Flour	0	650	542.75	0	0	0.00
Milling fraction	0	0	0	94.25	16.25	153.75
Sucrose	40	40	40	40	40	40.00
Corn Starch	506.95	5.4	59.35	463.45	498.08	424.50
Soybean Oil	120	105.50	100.18	114.29	118.17	105.00
Casein	200	136.28	149.6	188.31	195.92	164.00
Cellulose	81.3	11.06	56.36	47.93	79.81	61.00
Mineral Mix	35	35	35	35	35	35
Vitamin Mix	10	10	10	10	10	10
L-Cystine	3	3	3	3	3	3
Cholesterol	1.25	1.25	1.25	1.25	1.25	1.25
Choline Bitartrate	2.5	2.5	2.5	2.5	2.5	2.5
BHT	0.014	0.014	0.014	0.014	0.014	0.014
Total Weight	1000.014	1000.014	1000.014	1000.014	1000.014	1000.014
% CHO	54.70	53.86	52.65	56.35	54.57	53.66
% Protein	20.00	18.90	18.78	20.00	20.00	20.26
% Fiber	8.13	6.83	7.05	8.13	8.13	7.51
% Fat	12.00	12.00	10.84	12.00	12.00	12.23

Table 2-2. Measures of body weight, food intake, tissue weights, cecum pH, glucose control

Parameters	Negative Control	Positive Control	Whole Wheat	Refined Wheat	Wheat Bran	Wheat Germ	High Wheat Germ
Weight Gain (g)	104.6 ± 5.8[a]	158.4 ± 3.1[bc]	148.7 ± 6.3[b]	153.1 ± 3.8 [bc]	158.4 ± 3.7 [bc]	161 ± 3.6[c]	160.8 ± 2.7[c]
Average food intake (g)	16.7 ± 0.6[a]	22.7 ± 1.04[b]	22.7 ± 0.41[b]	23.1 ± 0.53[b]	23.1 ± 0.7[b]	24.5 ± 0.82[b]	23.4 ± 0.33[b]
Cecal pH	6.8 ± 0.07[b]	6.8 ± 0.07 [b]	6.2 ± 0.11[a]	6.9 ± 0.04 [b]	6.8 ± 0.08 [b]	6.8 ± 0.09 [b]	6.7 ± 0.05 [b]
Cecum weight (g)	0.389 ± 0.007[bc]	0.337 ± 0.010[a]	0.525 ± 0.021[d]	0.439 ± 0.014[c]	0.374 ± 0.020[ab]	0.390 ± 0.012[b]	0.410 ± 0.018[bc]
Epididymal fat pad weight (g)	0.405 ± 0.011[a]	1.149 ± 0.055[b]	1.144 ± 0.032[bc]	1.091 ± 0.015[b]	1.154 ± 0.031[bc]	1.129 ± 0.021[bc]	1.149 ± 0.021[c]
Retroperitoneal + perirenal fat pad weight (g)	0.492 ± 0.029[a]	1.467 ± 0.027[b]	1.464 ± 0.052 [b]	1.471 ± 0.0528[b]	1.513 ± 0.049[b]	1.469 ± 0.040[b]	1.583 ± 0.023[b]
Inguinal fat pad weight (g)	0.672 ± 0.031[a]	3.443 ± 0.201[b]	3.146 ± 0.184 [b]	3.293 ± 0.162 [b]	3.236 ± 0.194 [b]	3.442 ± 0.120 [b]	3.560 ± 0.155 [b]
Body fat %[2]	18.95 ± 0.56[a]	55.15 ± 1.31[b]	51.75 ± 0.72 [b*]	53.64 ± 1.27 [b]	55.38 ± 1.95 [b]	55.7 ± 1.66 [b]	54.89 ± 2.09 [b]
Lean mass %[2]	81.027 ± 0.55[b]	44.88 ± 1.29[a]	48.20 ± 0.71 [a]	46.378 ± 1.26 [a]	44.60 ± 1.97 [a]	44.33 ± 1.69 [a]	45.13 ± 2.09 [a]
Fasting blood glucose week	98.1 ± 4.2[a]	149.6 ± 6.4[c]	125.6 ± 5.3[b]	121.3 ± 4.8[b]	136.1 ± 4.7[bc]	134.1 ± 3.2[b]	136.2± 6.4[bc]

3 (mg/dL)							
AUC GTT ($x10^3$)	18.76 ± 0.33[a]	32.34 ± 1.238[c]	27.54 ± 1.45[b]	28.19 ± 0.68[b]	30.16 ± 1.28[bc]	28.62 ± 0.9[b]	29.13 ± 1.16[b]
AUC ITT ($x10^3$)	8.26 ± 0.92[a]	22.47 ± 0.8[c]	19.1 ± 0.79[b]	19.61 ± 0.64[b]	19.91 ± 0.79[b]	20.04 ± 0.55[b]	19.24 ± 0.67[b]
AUC MTT Glucose response ($x10^3$)	26.94 ± 0.34[a]	32.99 ± 0.95[bc]	30.48 ± 1.68[bc]	31.98 ± 0.91[bc]	33.74 ± 1.34 [c]	32.57 ± 0.74[bc]	29.84 ± 1.21[ab]
AUC MTT Insulin response	105.51 ± 15.67[a]	804.72 ± 114.35[bc]	758.09 ± 125.79[bc]	862.73 ± 134.07[c]	817.65 ± 132.30[bc]	863.48 ± 101.36[c]	530.98 ± 125.41[b]
Kidney weight, as a percentage of body weight (g/100g BW)	0.703 ± 0.013[b]	0.622 ± 0.059[a]	0.671 ± 0.017 [a]	0.630 ± 0.016 [a]	0.618 ± 0.019 [a]	0.606 ± 0.015 [a]	0.622 ± 0.217 [a]
TBARS (µg/24h)	3.586 ± 0.384[a]	6.002 ± 0.361[b]	12.183 ± 1.158[e]	8.367 ± 0.771[c]	8.827 ± 0.585[cd]	8.111 ± 0.622[bc]	10.810 ± 0.899de
Adiponectin (µg/ml)	28.559 ± 1.61[a]	35.668 ± 2.448[c]	29.228 ± 2.061[ab]	37.394 ± 2.506[c]	32.270 ± 2.292[abc]	36.789 ± 2.409[c]	34.518± 3.047[bc]
Resistin (ng/ml)	16.6± 1.29[a]	20.4±0.84 [b]	18.8±1.1[ab]	18.6±1.06[ab]	18.3±1.33[ab]	18.31±0.94 [ab]	18.7±1.04[ab]

[1]Values represent mean ± SEM, n=10-12, except where noted. Values within a row that do not share a common superscript are significantly different, $p<0.05$.

[2]Measured at 4 weeks.

*Significantly different from obese control by Student's t-test.

Table 2-3. Liver weights, liver lipids, liver cholesterol bile acid excretion

Parameter	Negative Control	Positive Control	Whole Wheat	Refined Wheat	Wheat Bran	Wheat Germ	High Wheat Germ
Liver weight (g)	7.421 ± 0.276^{a}	15.058 ± 0.625^{b}	15.766 ± 0.062^{b}	14.488 ± 0.437^{b}	14.527 ± 0.740^{b}	14.116 ± 0.619^{b}	14.606± 0.913^{b}
Total liver lipids (mg/liver)	527.0 ± 43.8^{a}	2583.5 ± 294.5^{c}	1844.2 ± 120.4^{b}	1694.9 ± 152.4^{b}	1491.1± 157.9^{b}	1605.7 ± 180.3^{b}	1479.9 ± 170.9^{b}
Total liver cholesterol (mg/g)	7.003 ± 0.372^{a}	13.361 ± 0.922^{d}	10.86 ± 0.733^{bcd}	11.05 ± 0.969^{cd}	8.513 ± 0.679^{ab}	9.454 ± 0.627^{abc}	6.939 ± 0.677^{a}
Free liver Cholesterol (mg/g)	2.029 ± 0.02^{b}	1.875 ± 0.05^{ab}	1.701 ± 0.04^{a}	1.862 ± 0.08^{a}	1.74 ± 0.041^{a}	1.87 ± 0.05^{ab}	1.77 ± 0.05^{a}
Bile acid excretion (mg/day)	3.262 ± 0.119^{ab}	2.915 ± 0.178^{a}	5.586 ± 0.525^{c}	3.831 ± 0.156^{b}	5.022 ± 0.406^{c}	3.970 ± 0.179^{b}	3.994 ± 0.148^{b}

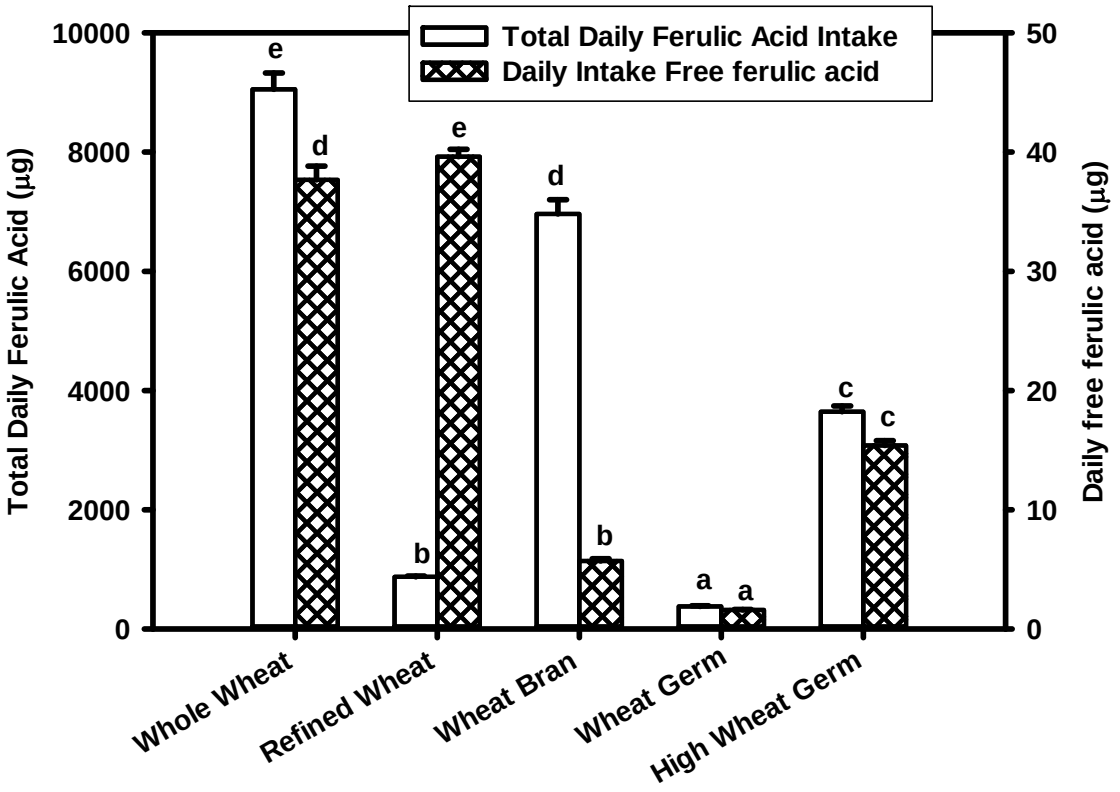

Figure 2-1. Daily total and free ferulic acid intake

Values represent mean ± SEM, n=10-12. Values that do not share a common superscript are significantly different, $p<0.05$. Measured at 4 weeks.

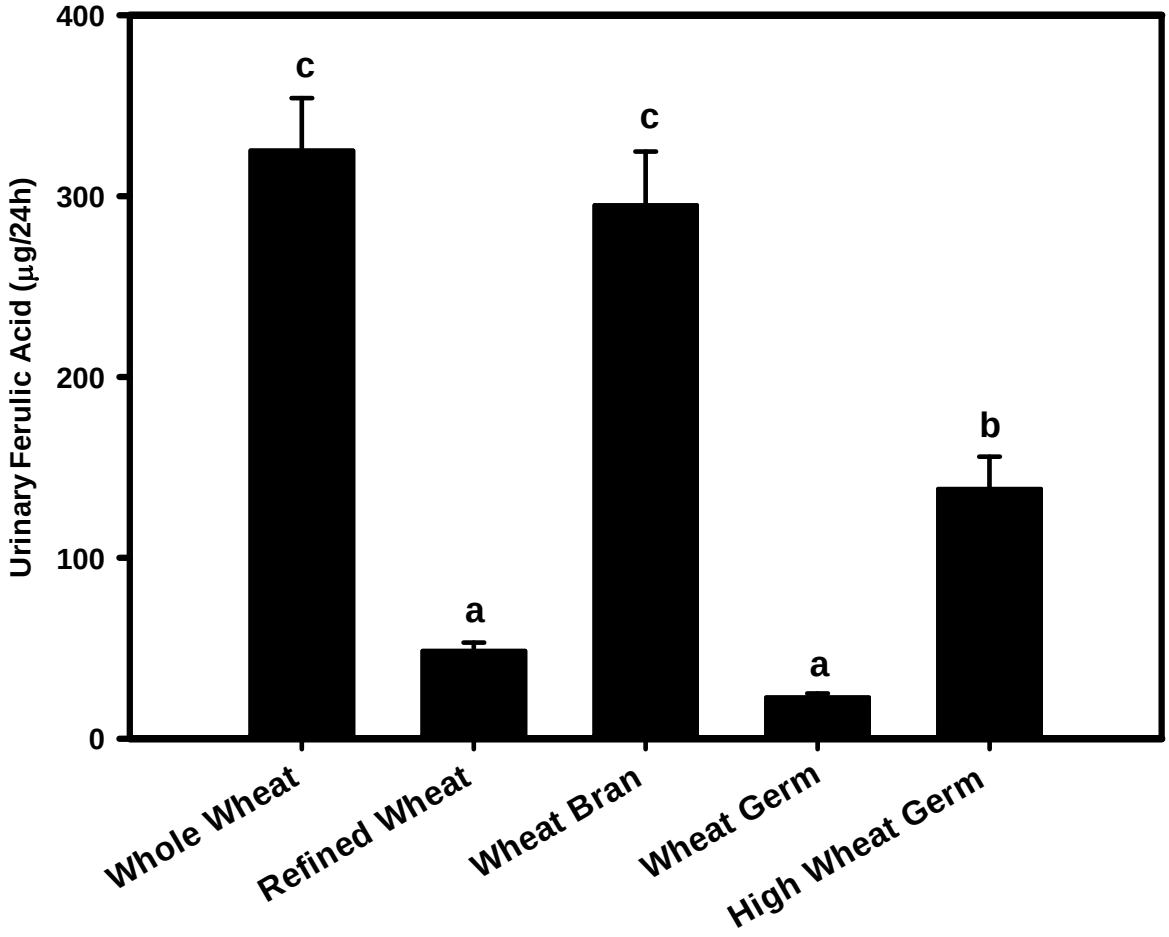

Figure 2-2. Urinary excretion of ferulic acid over 24 h

Values represent mean ± SEM, n=10-12. Values that do not share a common superscript are significantly different, $p<0.05$. Measured at 4 weeks.

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Appendices

Appendix 1: Liver Lipid Extraction

1) Weigh out ~1.00 gram of liver tissue and transfer to homogenizing tube.

2) Add 10 mL Chloroform:MeOH (2:1).

3) Homogenize for ~15-20 seconds.

4) Filter homogenate through a #1 Whatman filter paper into a glass screw top tube.

5) Rinse homogenizer with 15 ml Chloroform:MeOH (2:1) and pick out any tissue trapped on probe.

6) Pour rinse through filter paper into tube.

7) Wash filter paper with an additional 5 ml Chloroform:MeOH (2:1).

8) Add 6 ml 0.9% NaCl to filtrate. This represents a volume of 0.2 times the volume of Chloroform:MeOH used.

9) Vortex to mix aqueous and nonaqueous phases.

10) Centrifuge for 1 minutes at 750 RPM to separate phases. Be careful about placement of tubes in the centrifuge – when rotor spins, long tubes may break as they tilt. Alternatively, let the tubes stand until phases separate.

11) Remove aqueous upper phase with a transfer pipet and discard.

12) Gently layer 3 ml of MeOH:H_2O (1:1). Rinse sides of tubes while adding the MeOH:H_2O.

13) Remove this upper phase with a transfer pipet and discard.

14) Transfer the extracted lipid in Chloroform:MeOH (2:1) and transfer rinse to lipid vial.

15) Evaporate Chloroform under N_2 gas. (Gravimetric Step)

16) Reconstitute the fat with a set volume for all samples (10 ml) in Chloroform:MeOH (2:1).

Appendix 2: Enzymatic Cholesterol Assay

Reagents

1) 50 **mM Sodium Phosphate, Dibasic** (MW = 141.96). Make 1 L, pH 6.9 (7.1 g/L).

2) Stock **Phenol Reagent**: 500 ml

Dissolve 3.8 g Phenol crystals in 400 ml Phosphate Buffer. Adjust volume to 500 ml with Phosphate Buffer. Store at 4degrees? C (up to 1 month).

3) Stock **Mixed Reagent**: 500 ml

Dissolve in 400 ml Phosphate Buffer:

0.203 g 4-aminoantipyrine

1.292 g Sodium cholate

7.46 g Potassium chloride

1.0 ml Triton X

Adjust volume to 500 ml with Phosphate Buffer. Store at 4° C (up to 1 month).

4) **Working Reagent**: (shelf life: 3-4 days)

Reagent	(UI)/30mL (mL)	(UI)/100 mL (mL)	(UI)/200 mL (mL)
Stock Mixed Reagent	15	50	100
Stock Phenol	15	50	100
Cholesterol Oxidase (UI)	7.5	25	50
Cholesterol Esterase (UI)	7.5	25	50

Peroxidase (UI)	375	1250	2500

Store in ice bath until ready to use.

******NOTE**** For free cholesterol assay, omit esterase**

A) **Standard Curve:**

Use 1 mg/ml concentration of cholesterol in Chloroform:MeOH (2:1). Use 0, 5, 10, 15, 20, and 25 µl concentrations (depends on cholesterol in sample).

Add 50 µl Triton X-100/Acetone to each tube, Vortex.

Dry under N_2 gas. (Triton Sol'n = 1.5 g Triton in 10 mL acetone)

B) **Samples:** Total Cholesterol: 10 µl assayed

Free Cholesterol: 50 µl assayed

Add 50 µl Triton X-100/Acetone.

Dry solvent under N_2 gas

C) **Assay:** Add 100 µl ddH_2O to each tube, Vortex.

Add 1 ml Working Reagent/sample, Vortex.

Incubate at 37º C for 10 min. Cool to room temperature.

Read absorbance at 500 nm.

Appendix 3: Assay of Total Bile Acids

References:

1. MacDonald, Ian (1976) Clin. Biochem. 9, (3) 153-154, Enzymatic Analysis of Bile Acids.

2. Locket, P.L., Gallaher, D.D. (1989), Lipids 24 221-223

3. Bile Acid Assay III (Sheltawy, 1975, Determination of faecal bile acids by an enzymic method)

Materials:

Equipment

Spectrophotometer

Waterbath: Temp. approx. 37° C

Reagents

0.01M Cholic Acid in 100% MeOH for std. Curve

3α-Hydroxysteroid dehydrogenase; Sigma No. H-1506 from

Pseudomonas testosteroni; Need ~1.5 units enzyme/mL phosphate buffer, but could go **down to 1.25 units/mL**

- 0.01M Phosphate buffer; use sodium phosphate monobasic, pH 7.2
- 0.05M CAPS buffer, adjust pH to 10.8 w/ NaOH
- NAD (β-nicotinamide adenine dinucleotide), use 1.25mg NAD/ml CAPS buffer. Use NAD soln the day it is made
- Methanol, reagent grade

PROCEDURE

Generation of Standard Curve

A standard curve must be generated each day that sample bile acids are run. This is because the quantity of bile acids in each sample is calculated from the equation of the regression line generated by the standard curve. This equation will vary with minor changes in the concentration of NAD substrate and enzyme. Because new NAD soln must be made with each assay a new std. curve must also be run. No more than 30 samples should be run per one standard curve. **STANDARD CURVE SHOULD BE DONE IN TRIPLICATE.**

1. Label small test tubes with vol. of cholic acid std. to be added. Use std. volumes of 0, 10, 20, 30, 40, and 50ul.

2. To each tube add:

100% MeOH to give a total MeOH volume (Std. vol. + MeOH) of 100 µl. (i.e. to tubes labelled 0 add 100 µul MeOH)

Cholic acid standard in MeOH, volumes listed in step 1

1.25 ml NAD in CAPS buffer

150 µl Enzyme solution

3. Cap tubes with corks or cover the rack with parafilm tightly and vortex.

4. Incubate the tubes in a water bath at approx. 37 |C for 20 min.

5. Remove tubes from water bath and allow them to adjust to room temperature for about 5 min.

Sample Preparation

After you have determined that the bile acid assay works, (a standard curve has been run independently and proves linear) samples should be prepared for bile acid analysis. Run a standard curve each day that you run samples.

1. Resolubilize partially purified bile acids in 0.5ml 100% MeOH.

2. **Add to assay tubes**

50 µl 100% MeOH

50 µl sample

1.25ml NAD in CAPS

150 µl Enzyme soln

3. Cap samples, vortex then incubate samples with standards.

*Note: if absorbance is too high or low adjust sample volume assayed to bring absorbance to around 1.

Spectrophotometric analysis

Set up a program on the computer attached to the spec for this assay. Note the wavelength of **absorbance is 340 nm. Add a couple blank analysis samples at end for repetition if needed.**

Zero the instrument using double distilled water. Use the 0 level bile acid standard as your blank.

Run standards followed by samples. Rerun the standard blanks at end of run.

Calculation of Bile Acid Quantity

1. Convert μl Std. to nmoles B.A.

2. Run a regression of nmoles B.A. against Corrected Absorbance.

Corrected Absorbance = (Abs. 1 - Abs. 2)/2 - Ave Abs. Blank

3. Y-intercept should be close to zero, $r^2 = 0.99$

4. From the corrected absorbances of samples (ave. of 2 sample reps - blank) calculate nmoles B.A. using regression equation.

July 28, 1992, June 2004

C. Gallaher/DDG

Bile Acid Extraction and Purification

Reference: Locket, PL & Gallaher, DD (1989) Lipids 24, 221-223.

MATERIALS

Methanol

Ethanol, absolute

Chloroform

Petroleum Ether

Solid phase extraction columns, reverse phase C18

Screw-capped tubes (check to make sure they seal tight)

Bath sonicator

Heating block

Nitrogen gas

SOLUTIONS

Ethanol, 80 %

Add 20 parts of distilled water to 80 part of absolute ethanol. Store at room temperature.

Chloroform/methanol

Mix equal volumes of chloroform and methanol. Store at room temperature.

PROCEDURE

<u>Extraction</u>

1. Weigh approximately 100 mg of tissue or feces into a screw cap tube (20 X 125 mm or similar size). Record the exact weight.

2. Add 2 ml of absolute ethanol to each tube.

3. Sonicate the capped tubes in a bath sonicator for 30 min.

3. Place the tubes in a heating block and reflux at 100°C for 15 min.

4. Centrifuge the tubes at approx. 1,500 X g for 10 min. Transfer the supernatants to screw capped tubes.

5. Suspend the pellets in 2 ml of 80% ethanol, reflux for 15 min in the heating block, and centrifuge as in step 4. Again transfer the supernatants to the screw capped tubes.

6. Suspend the pellet again in 2 ml of chloroform/methanol, reflux for 15 min in the heating block, and centrifuge as in step 4. Transfer the supernatants to the screw capped tubes.

Suspend the pellet in 2 ml of chloroform/methanol, centrifuge as in step 4, and transfer the supernatants to the screw capped tubes.

8. Evaporate the pooled supernatants to dryness with nitrogen gas under low heat (<60°C). Store dried samples capped in the freezer until ready for partial purification.

Partial Purification

1. Remove the rack, if present, from inside the solid phase extraction (SPE) column processor. Insert the SPE columns into the column processor and turn on the water aspirator. Adjust the vacuum to about 5-10 mm Hg with the screw located on the vacuum arm closest to the processor (NOT the wing nut).

2. Solvate (activate) the columns by adding 4 ml of MeOH to each column. Follow with 4 ml of distilled water (DW). Do not allow the columns to dry out if possible between solutions. Stop the flow through the columns by releasing the vacuum by opening the wing nut.

3. To tubes containing the dried tissue or fecal extract, add 0.6 ml MeOH and vortex to solubilize the bile acids. Next add 2.4 ml DW to each tube and vortex again.

4. Pour the solution from above through a solvated column and apply vacuum.

5. When the level of solution in the columns drops to the frit, add 3 ml of DW.

6. When the level of DW in the columns drops to the frit, add 3 ml of hexane.

7. When the level of hexane has dropped to the frit for all columns, release the vacuum with the wing nut and carefully remove the cover of the SPE column processor. Insert the rack with labeled tubes in the correct position to collect the column eluents.

8. Add 3 ml MeOH to each column and turn on the vacuum. The flow of MeOH through the column should be 2-3 ml/min. The MeOH will elute the bile acids off the columns, which will then be collected in the tubes within the processor.

9. After the flow through the columns stops, release the vacuum, remove the tubes, and evaporate the solution to dryness with nitrogen gas at low heat.

Appendix 4: Urinary Thiobarbituric Acid Reactive Substances (TBARS) Procedure

Reference: Hye-Sung Lee, *Lipids* 27:124-128, 1992

Reagents:

- 5% (w/v) Tri-chloroacetic acid (TCA). 5g to 100 ml H_2O.
- 0.06 M Thiobarbituric acid (TBA). 0.8652g 2-thiobarbituric acid to 100 ml H_2O.

❖ Store stoppered reagents in cold room.
❖ Before use, warm TBA solution in water bath (as the bath heats up) to return all precipitate to solution, then cool to room temperature.

MDA Standard:

- 10^{-5} M Malondialdehyde (MDA)
- **Synthesis of MDA**

1. Weigh 164.2 mg (1 mmol) malondialdehyde tetramethylacetal into a 100 ml glass stoppered Erlenmeyer flask.
2. Add 100 ml 0.01 N HCl, stopper immediately and wrap the top with Parafilm.
3. Heat in 50 °C water bath for 1hr.
4. Cool to room temperature in a dark place.
5. Dilute with water to 10^{-5} M.
1 ml 10^{-2} M (as prepared) to 100 ml water = 10^{-4} M
10 ml 10^{-4} M to 100 ml water = 10^{-5} M.

❖ MDA is very sensitive to light, store in the cold room in a flask wrapped in a foil.
❖ Approximate shelf-life is one month.

Appendix 5: Ferulic Acid

Urinary ferulic acid was measured by HPLC after deconjugation using a modification of the method of Bunzel et al. Briefly, ferulic acid was deconjugated by mixing 5% of 24 h-collected rat urine with the same volume of 1 M sodium acetate buffer (pH 4.9), 300uL of sulfatase solution, and 100 μL of 0.1 mg/mL alpha coumaric acid solution (in 50% aqueous methanol). The mixture was incubated 37 °C for 3 h. The reaction was stopped with concentrated hydrochloric acid 0.3 mL and ferulic acid extracted with a total of 4mL ethyl acetate (2mL, 1mL, 1mL). The pooled ethyl acetate phases were extracted with 5ml of 5% sodium hydrogen carbonate (2mL, 2x 21.5mL) and phases separated by centrifugation. The combined sodium hydrogen carbonate phases were acidified with 0.5mL of concentrated hydrochloric acid until the pH was <2, and then extracted with 3 x 2.5mL ethyl acetate. The ethyl acetate phases were collected and dried under nitrogen. The dried extract was reconstituted in 200uL 50% methanol and 1:5 diluted for HPLC analysis.

Quantification of ferulic acid was achieved using a Shimadzu HPLC system with auto sampler and SPD-M20A PDA detector. HPLC conditions are as follows: A Luna Phenyl-Hexyl column (250 x10mm; 4.6um particle size) was used for separation at a flow rate of 1mL/min and temperature of 45 ^{o}C. Mobile phases were 0.1 mM trifluoroacetic acid (TFA) (mobile phase A), 90% methanol/10% TFA (mobile phase B) and 90% acetonitrile/10% TFA (moble phase C). The following gradient program was used (156): 87% A and 13% C for 10 min; change over 10 min to 77% A, 3% B, and 20% C; change

over 5 min to 70% A, 5% B, and 25% C; and finally change over 5 min to 25% A, 25% B, and 50% C. Detection wavelengths were 280 and 325 nm.

Appendix 4: SAS CODE

```
data all_groups   (label = 'All diet groups')
zdf_only (label= 'ZDF Animals Only')
;
Title2 'Effect of Wheat and Wheat Fractions in ZDF Rats';
Input Rat_No Block_No Diet_Group Initial_BW Week_1_BW Week_2_BW
Week_3_BW MidWeek_4_BW Week_5_BW Total_Weight_Gain Week1_FI Week2_FI
Week3_FI Average_FI MTT_Time0 Mtt_Time15 MTT_Time30 MTT_Time60
MTT_Time90 MTT_Time120
MTT_Time180 BodyWeight_GTT Amount_Gavaged_GTT GTT_Time0 GTT_Time15
GTT_Time30 GTT_Time60 GTT_Time90 GTT_Time120 GTT_Time180 ITT_Time0
ITT_Time15 ITT_Time30 ITT_Time60 ITT_Time90 ITT_Time120
ITT_Time180 Urine_Vol Fecal_DryWeight TissuePCFat_DXA
Body_Tissue_Weight_DXA Body_Fat_DXA Lean_BW_DXA TissuePCLean_DXA
BMD_DXA BMC_DXA
KillDay_BW KillDay_Liver_Weight KillDay_Kidney_Weight Kidney_PC
      KillDay_Epi_fatpad_Weight EpifatPC Retro_perinal_fatpad
Retro_perinal_fatpadPC Killday_Inguinal_fatpad_weight
InguinalfatPC Cecum_weight CecumPH Total_LiverLipids_assayed
Lipids_1g_tissue Liverlipids_whole_liver MTT_AUC GTT_AUC ITT_AUC
Liver_total_chol_conc_true Liver_total_chol_true
Liver_free_chol_conc_true Liver_free_chol_true
fecal_wt_assayed adiponectin_conc pCA FA diOHpropA OH3MepropA
resistin_sample_ngml fecal_bile_acid TBARS_ug24hr T0_MT_insulin
T30_MT_insulin T60_MMT_insulin T120_MMT_insulin T180_MMT_insulin
AUC_MMT_Insulin

;

Label
Rat_No = 'Rat Number'
Block_No= 'Block Number'
Diet_Group= 'Diet Group'
Initial_BW = 'Initial Body Weight (g)'
Week_1_BW= 'Week 1 Body Weight (g)'
Week_2_BW=  'Week 2 Body Weight (g)'
Week_3_BW = 'Week 3 Body Weight (g)'
MidWeek_4_BW =' Mid Week 4 Body Weight (g)'
Week_5_BW ='Week 5 Body weight (g)'
Total_Weight_Gain = 'Total Weight Gain (g)'
Week1_FI= ' Food Intake Week 1 (g)'
Week2_FI = 'Food Intake Week 2 (g)'
Week3_FI = 'Food Intake Week 3 (g)'
Average_FI= 'Average Food Intake (g)'
MTT_Time0  ='Meal Tolerance Test,Blood Glucose at Time 0'
Mtt_Time15 =' Meal Tolerance Test,Blood Glucose at 15 minutes (mg/dL)'
MTT_Time30 =' Meal Tolerance Test,Blood Glucose at 30 minutes (mg/dL)'
MTT_Time60 =' Meal Tolerance Test,Blood Glucose at 60 minutes(mg/dL)'
MTT_Time90 =' Meal Tolerance Test,Blood Glucose at 90 minutes(mg/dL)'
MTT_Time120=' Meal Tolerance Test,Blood Glucose at 120 minutes(mg/dL)'
```

```
MTT_Time180=' Meal Tolerance Test,Blood Glucose at 180 minutes(mg/dL)'
BodyWeight_GTT=   'Body Weight Measurement for Glucose Tolerance Test
(g)'
Amount_Gavaged_GTT=' Amount of Glucose Gavaged During the Glucose
Tolerance Test (ml)'
GTT_Time0 = 'Glucose Tolerance Test, Blood Glucose Level at Time 0
(ml/dL)'
GTT_Time15 ='Glucose Tolerance Test, Blood Glucose Level at Time 15
(ml/dL)'
GTT_Time30='Glucose Tolerance Test, Blood Glucose Level at Time 30
(ml/dL)'
GTT_Time60 ='Glucose Tolerance Test, Blood Glucose Level at Time 60
(ml/dL)'
GTT_Time90 ='Glucose Tolerance Test, Blood Glucose Level at Time 90
(ml/dL)'
GTT_Time120 ='Glucose Tolerance Test, Blood Glucose Level at Time 120
(ml/dL)'
GTT_Time180 ='Glucose Tolerance Test, Blood Glucose Level at Time 180
(ml/dL)'
ITT_Time0=' Insulin Tolerance Test,Blood Glucose at 0 Minute (ml/dL)'
ITT_Time15=' Insulin Tolerance Test,Blood Glucose at 15 Minute (ml/dL)'
ITT_Time30=' Insulin Tolerance Test,Blood Glucose at 30 Minute (ml/dL)'
ITT_Time60 =' Insulin Tolerance Test,Blood Glucose at 60 Minute
(ml/dL)'
ITT_Time90 =' Insulin Tolerance Test,Blood Glucose at 90 Minute
(ml/dL)'
ITT_Time120=' Insulin Tolerance Test,Blood Glucose at 120 Minute
(ml/dL)'
ITT_Time180=' Insulin Tolerance Test,Blood Glucose at 180 Minute
(ml/dL)'
Urine_Vol   = 'Urine Volume over 24h Period (ml)'
Fecal_DryWeight = 'Dry Weight of 48h Fecal Collection (g)'
TissuePCFat_DXA= '% of Tissue Fat Obtained From DEXA Scanning'
Body_Tissue_Weight_DXA= 'Body Tissue Weight Obtained by DEXA (g)'
Body_Fat_DXA ='Total Rat Body Fat Calculated by DEXA (g)'
Lean_BW_DXA=' Lean Body Weight Calculated by DEXA (g)'
TissuePCLean_DXA= '% Of lean Tissue of Rat calculated by DEXA (g)'
BMD_DXA = 'Bone Mineral Density From DEXA, (g/cm2)'
BMC_DXA= 'Bone Mineral Content (g)'
KillDay_BW= 'Rat Body Weight At Kill Day (g)'
KillDay_Liver_Weight= 'Rat Liver Weight at Kill day (g)'
KillDay_Kidney_Weight= ' Rat Kidney Weight at Kill day  (g)'
Kidney_PC   = 'Kidney weight as a% of body weight'
KillDay_Epi_fatpad_Weight=    'Kill day Epididymal Fat Pad Weight(g)'
EpifatPC= 'Epididymal Fat Pad weight as a% of body weight'
Retro_perinal_fatpad = 'Kill Day Retroperitoneal and Perirenal Fat Pad
Weight (g)'
Retro_perinal_fatpadPC= ' Retroperitoneal and Perirenal weight as a% of
body weight'
Killday_Inguinal_fatpad_weight= ' Inguinal Fat Pad Weight Kill Day (g)'
InguinalfatPC = ' Inguinal Fat Pad weight as a% of body weight'
Cecum_weight = ' Cecum Weight At Kill day (g)'
CecumPH     = ' Cecum pH level at Kill day'
Total_LiverLipids_assayed='Total Liver Lipids assayed'
Lipids_1g_tissue= ' Total Liver lipids per Gram of Liver Tissue (ml/g
L)'
Liverlipids_whole_liver='Total liver lipids in whole liver (mg/liver)'
```

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MTT_AUC='Meal tolerance test AUC'
GTT_AUC='Glucose tolerance test AUC'
ITT_AUC='Insulin tolerance test AUC'
Liver_total_chol_conc_true='Liver total cholesterol conc true (mg/g)'
Liver_total_chol_true='Liver total cholesterol per liver (mg/liver)'
Liver_free_chol_conc_true='Liver free cholesterol conc true (mg/g)'
Liver_free_chol_true='Liver free cholesterol per liver (mg/liver)'
fecal_wt_assayed='Fecal wt assayed'
adiponectin_conc='Plasma fasting adiponectin (ug/mL)'
pCA='urinary total p-caffeic acid (ug/24h)'
FA='urinary total ferulic acid (ug/24h)'
diOHpropA='urinary total 3-(3,4-dihydroxy-phenyl)propA (ug/24h)'
OH3MepropA='urinary total 3-(4-hydroxy-3-methoxy-phenyl)propA (ug/24h)'
Tot_FA_ref_flour='Total ferulic acid in refined flour (ug/g flour)'
Tot_FA_WW_flour='Total ferulic acid in whole wheat flour (ug/g flour)'
Tot_FA_bran='Total ferulic acid in wheat bran (ug/g bran)'
Tot_FA_germ='Total ferulic acid in wheat germ (ug/g germ)'
Free_FA_ref_flour='Free ferulic acid in refined flour (ug/g flour)'
Free_FA_WW_flour='Free ferulic acid in whole wheat flour (ug/g flour)'
Free_FA_bran='Free ferulic acid in bran (ug/g bran)'
Free_FA_germ='Free ferulic acid in germ (ug/g germ)'
resistin_sample_ngml= 'Plasma resistin (ng/ml)'
fecal_bile_acid = 'Fecal bile acid (mg/24hr)'
TBARS_ug24hr = 'TBARS (ug/24hr)'
T0_MT_insulin = 'Insulin response to meal time 0'
T30_MT_insulin = ' Insulin response to meal time 30'
T60_MMT_insulin = 'Insulin response to meal time 60'
T120_MMT_insulin = 'Insulin response to meal time 120'
T180_MMT_insulin = 'Insulin response to meal time 180'
AUC_MMT_Insulin = ' Area under the curve for insulin response to meal
test'

;

;

*Concentration of total ferulic acid in wheat flours and fractions
(ug/g);
Tot_FA_ref_flour=67.05;
Tot_FA_WW_flour=581.50;
Tot_FA_bran=3031.28;
Tot_FA_germ=1006.54;

*Concentration of free ferulic acid in wheat flours and
fractions(ug/g);
Free_FA_ref_flour=3.04;
Free_FA_WW_flour=2.42;
Free_FA_bran=2.48;
Free_FA_germ=4.25;

*Calculation of the dietary concentration of total ferulic acid for
each diet (ug/g diet);
If Diet_Group=3 then Tot_FA_dietconc=153.7*Tot_FA_germ/1000;
If Diet_Group=4 then Tot_FA_dietconc=650*Tot_FA_WW_flour/1000;
If Diet_Group=5 then Tot_fa_dietconc=542.7*Tot_FA_ref_flour/1000;
If Diet_Group=6 then Tot_FA_dietconc=94.2*Tot_FA_bran/1000;
If Diet_Group=7 then Tot_fa_dietconc=16.2*Tot_FA_germ/1000;
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If Diet_Group=1 or Diet_group=2 then Tot_fa_dietconc=.;

*Calculation of the dietary concentration of free ferulic acid for each
diet (ug/g diet);
If Diet_Group=3 then Free_FA_dietconc=153.7*Free_FA_germ/1000;
If Diet_Group=4 then Free_FA_dietconc=650*Free_FA_WW_flour/1000;
If Diet_Group=5 then Free_FA_dietconc=542.7*Free_FA_ref_flour/1000;
If Diet_Group=6 then Free_FA_dietconc=94.2*Free_FA_bran/1000;
If Diet_Group=7 then Free_FA_dietconc=16.2*Free_FA_germ/1000;
If Diet_Group=1 or Diet_Group=2 then Free_FA_dietconc=.;

*Calculation of the daily total and free ferulic acid intake during
week 3 (ug);
Daily_Tot_FA_intake=Week3_FI*Tot_FA_dietconc;
Daily_free_FA_intake=Week3_FI*Free_FA_dietconc;

*Calculation of estimate of % absorption of ferulic acid from diet,
based on total and free ferulate in flours & fractions;
Tot_FA_absorpPC=(FA/Daily_Tot_FA_intake)*100;
Free_FA_absorpPC=(FA/Daily_free_FA_intake)*100;

*check rat 59 for high value;

output all_groups;
if Diet_Group = 2 or Diet_Group=3 or Diet_Group =4 or Diet_Group=5 or
Diet_Group=6 or Diet_Group=7
then output zdf_only;

Datalines;
1     1     1     128   149   184   211   224   253   125.0       13.35
      14.80 17.70 15.28       98    143   148   165   171   142   157
      190   0.19  104   127   140   98    104   95    92          103
      35    .     .     .     .     .           1.25        2.98
      15.5  218   34    184   84.404      0.157 5.312       253
      6.6115      1.5605      0.617 0.979 0.387 1.865 0.737 2.003 0.792
      0.4053      7.02  74.41 68.20 450.89      27390 18930 .     6.752
      44.64 2.082 13.765      100.13      34.49 28.51 12.20 67.11 56.52
      23.08313842 3.5054      2.3282      0.422 0.4505      1.5517
      0.5505      2.055 184.3515
2     1     1     128   151   195   224   234   252   124.5       15.30
      15.65 17.15 15.65       81    133   178   146   147   187   152
      200   0.2   90    147   108   75    90    87    70          92
      47    35    34    30    39    75          1.35        2.95
      17.7  232   41    191   82.328      0.146 5.600       252
      8.9562      1.8718      0.743 0.812 0.322 1.077 0.428 1.579 0.627
      0.4126      6.48  67.05 63.74 570.83      28373 16275 8108  6.678
      59.81 2.107 18.872      101.55      33.52 32.45 13.29 84.27 84.88
      21.5309377  3.2318      2.8651      0.5   0.532 0.7904
      0.6471      35.316
3     1     1     111   137   171   195   206   223   112.4       15.25
      13.75 14.10 14.37       117   134   169   148   177   178   164
      179   0.179 112   134   110   102   103   98    100         97
      53    39    42    40    59    86          0.8         2.63
      21.4  204   44    160   78.431      0.139 4.839       223   7.456
      1.526 0.684 0.940 0.421 0.938 0.420 1.252 0.561 0.3349      7.1
      46.29 52.16 388.89      29370 18885 10095 5.011 37.36 1.963
      14.634      101.43      35.94 22.76 9.77  64.32 58.74 20.91431001
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      2.815 1.9379      0.2857      0.3367      1.4871      0.6087
      0.3368      127.932
4     1     1     133   159   204   225   253   255   122.3       26.60
      16.95 20.50 21.35       91    125   152   160   176   154   156
      201   0.2   111   140   113   97    103   99    97          120
      46    49    36    46    89    77          1.62        3.27
      19.5  242   47    195   80.579      0.145 5.906       255
      7.6913      1.9174      0.752 1.065 0.418 1.071 0.420 1.449 0.568
      0.4145      6.77  87.36 73.61 566.15      27668 18840 11468 6.984
      53.71 2.068 15.904      101.06      31.57 35.18 14.08 74.34 87.56
      16.87433553 3.293 3.1008      0.4   0.5116      0.887 0.3965
      0.4548      98.697
5     1     1     131   159   199   212   228   242   111.3       16.85
      18.00 17.45 17.43       97    122   178   134   151   147   156
      185   0.18  116   138   119   102   100   94    93          85
      51    22    .     .     .     .           1.8         3.37
      17.2  229   39    189   82.533      0.148 5.426       242   7.411
      1.7684      0.731 0.981 0.405 1.273 0.526 1.445 0.597 0.3827
      6.92  86.56 82.98 614.93      26408 18698 1568  9.193 68.13 2.000
      14.823      99.68 29.48 38.18 15.04 79.39 71.02 18.17137997
      3.2552      2.9015      0.3333      0.4   0.5716      0.4072
      0.3154      76.6155
6     2     3     192   226   280   315   341   360   168.0       21.25
      22.75 23.20 22.40       152   176   177   184   166   192   196
      282   0.282 136   232   224   189   144   144   147         143
      140   87    87    103   107   119         8.5         5.18
      50.4  333   168   165   49.550      0.149 7.368       360
      15.6131     2.1918      0.609 3.829 1.064 5.518 1.533 15.176
      4.215 0.4841      6.7   120.55      97.28 1518.85     32783 30420
      19215 7.974 124.50      1.668 26.037      101.36      25.70
      174.50      142.57      258.71      365.50      24.1936101
      3.8644      12.0962     2.3462      4.9593      3.3996
      2.4483      1.6447      533.193
7     2     3     184   221   275   310   334   352   168.0       24.97
      24.80 25.15 24.98       127   165   146   116   155   147   146
      278   0.278 111   204   211   183   140   137   139         137
      113   76    72    94    108   108         13.4        4.74
      63.1  324   205   120   37.037      0.160 10.707            352
      12.1462     1.9533      0.555 4.288 1.218 5.779 1.642 15.594
      4.430 0.3458      6.55  83.28 90.13 1094.74     25838 28665 17513
      7.135 86.66 1.879 22.823      100.82      38.33 152.62
      156.77      246.01      347.19      22.87354123 3.7473      6.296
      1.9257      3.6101      1.9444      0.5053      3.3504
      355.5165
8     2     3     182   216   265   296   324   340   158.0       21.25
      23.10 20.65 21.67       114   197   141   143   163   172   148
      271   0.271 161   240   213   207   145   154   158         135
      118   78    90    115   148   128         8.4         4.32
      47.7  313   150   164   52.396      0.159 7.202       340
      13.7201     2.1124      0.621 3.791 1.115 5.295 1.557 12.720
      3.741 0.3995      6.6   97.32 95.51 1310.47     28343 31830 21188
      5.965 81.84 1.871 25.676      100.95      31.84 134.20
      129.21      245.65      298.35      20.67342644 4.2732
      10.1376     1.2373      1.3277      1.3603      0.8977
      1.5704      220.578
9     2     3     205   243   293   318   340   355   150.0       22.10
      23.30 24.70 23.37       117   150   134   152   164   163   181
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      297   0.297 158   224   223   208   202   207   194         187
      143   96    91    112   165   155         17          5.59
      52.6  337   177   160   47.478      0.155 7.538       355
      18.1189     2.4789      0.698 4.090 1.152 5.734 1.615 12.848
      3.619 0.4498      6.85  166.07      126.03      2283.53     28388
      36998 23873 8.957 162.29      1.714 31.061      101.18      26.53
      191.40      141.67      226.41      353.55      15.50793954
      4.2904      12.7235     5.402 10.0446     5.917 9.4858
      5.4591      1381.554
10    2     3     193   235   282   315   342   353   160.0       24.10
      20.75 27.25 24.03       124   182   173   197   183   174   184
      294   0.294 113   165   294   158   160   126   140         138
      101   74    90    111   129   127         23.5        5.58
      49.8  338   168   170   50.296      0.161 7.520       353
      18.1068     2.6904      0.762 4.409 1.249 6.020 1.705 11.597
      3.285 0.4305      6.73  158.62      115.27      2087.13     32303
      29348 19860 8.518 154.23      1.643 29.755      101.43      21.66
      197.65      127.90      161.71      221.74      19.90816912
      5.0544      12.092      3.9801      2.0971      3.8738
      4.5053      .     432.0945
11    1     2     175   216   269   287   310   337   161.8       24.50
      22.75 15.35 20.87       164   235   229   159   204   219   200
      268   0.26  147   202   191   182   149   146   134         189
      140   81    82    124   156   158         4.8         4.95
      51.7  307   159   149   48.534      0.161 6.720       337
      16.5345     1.7864      0.530 3.639 1.080 4.503 1.336 9.832 2.918
      0.3356      7.01  245.63      216.97      3587.47     36653 28950
      23280 12.156      200.99      1.607 26.573      100.62      41.93
      47.76 12.84 185.52      182.65      22.21135445 3.5814
      6.1267      0.4536      1.4617      7.7185      0.3336
      5.1693      573.0825
12    1     2     160   190   245   275   291   318   158.3       20.75
      22.05 .     31.58       108   149   155   136   273   161   196
      247   0.24  137   194   221   172   156   153   139         137
      112   97    77    130   137   156         5.4         4.58
      63.2  284   180   105   36.972      0.157 9.072       318
      12.6854     1.678 0.528 3.645 1.146 4.398 1.383 8.270 2.601
      0.3391      7.31  125.09      107.74      1366.77     31928 29805
      21945 9.168 116.30      1.792 22.728      101.36      41.22 43.68
      11.52 123.72      182.52      20.08505209 2.6786      6.778 1.482
      2.237 2.668 0.8015      0.7082      278.736
13    1     2     155   184   240   263   282   302   146.8       18.90
      21.20 20.00 20.03       117   171   113   156   194   199   182
      235   0.23  124   230   219   183   183   179   141         124
      133   73    77    118   133   142         3.8         4.15
      62.3  275   172   104   37.818      0.155 9.031       302
      11.553      1.6504      0.546 3.373 1.117 4.339 1.437 10.492
      3.474 0.3192      6.9   137.81      111.03      1282.73     30900
      32573 20663 2.217 25.61 1.754 20.260      101.33      39.63 48.05
      13.24 130.16      157.15      22.38145864 2.0875      6.8051
      3.445 5.4643      1.4537      1.6598      1.212 416.9685
14    1     2     169   201   256   284   308   330   161.5       20.85
      21.95 20.40 21.07       139   196   124   157   155   195   210
      263   0.26  160   248   233   215   177   151   121         145
      131   60    75    100   145   129         5           4.19
      53.2  300   160   140   46.667      0.165 7.380       330
      14.1342     1.7737      0.537 4.072 1.234 4.464 1.353 9.577 2.902
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      0.2552      6.97  163.18      138.88      1962.91     31208 32348
      20048 11.587      163.78      1.833 25.903      101.25      48.24
      64.31 19.97 164.99      183.41      23.18945354 2.8865
      5.7858      1.388 5.0043      11.789      2.2418      6.9265
      1043.757
15    1     2     175   207   162   290   317   352   177.5       22.75
      23.35 19.95 22.02       139   193   152   152   155   166   207
      270   0.27  187   247   233   210   192   173   138         140
      130   59    88    113   138   132         4.1         4.74
      51.8  312   162   150   48.077      0.157 6.600       352
      15.642      1.994 0.566 4.227 1.201 5.315 1.510 13.647      3.877
      0.3435      6.6   199.9 161.30      2523.07     30248 34335 20528
      11.760      183.95      2.024 31.655      101.30      39.04
      509.51      5.88  149.37      165.67      20.63789071 3.382
      4.6863      4.2735      29.801      13.9682     .     9.0356
      1167.6555
16    2     2     178   209   267   288   315   327   149.0       21.95
      21.60 21.15 21.57       172   250   157   163   183   192   188
      265   0.265 152   195   201   185   160   164   165         137
      137   74    72    137   125   130         8.2         4.13
      54.6  308   168   140   45.455      0.147 6.984       327
      15.134      1.8494      0.566 3.653 1.117 4.873 1.490 15.191
      4.645 0.3373      6.93  203.35      158.84      2403.92     33233
      31268 20543 14.427      218.34      1.814 27.458      101.20
      31.19 48.92 15.09 155.34      136.08      23.52400995 3.1642
      7.8335      5.332 10.875      3.2972      4.8635      5.5578
      1013.148
17    2     2     186   218   284   297   319   335   149.0       20.75
      20.60 22.55 21.30       168   218   190   229   221   216   200
      272   0.272 144   277   254   224   186   162   165         160
      152   97    79    130   153   162         58.5        4.42
      54.5  314   171   143   45.541      0.156 7.240       335
      15.8483     1.9274      0.575 3.859 1.152 5.122 1.529 14.068
      4.199 0.3804      6.73  149.47      136.61      2165.11     38025
      35490 23678 12.882      204.16      1.819 28.828      101.16
      25.70 35.64 0.04  119.30      118.37      15.62272814 3.0837
      4.9257      4.7369      2.4158      15.1639     6.1575
      2.9553      1284.012
18    2     2     193   224   254   267   314   342   149.0       21.95
      23.25 .     22.60       140   194   130   165   148   157   176
      262   0.262 142   225   196   152   108   128   121         176
      159   129   130   158   164   157         6.9         4.94
      52.4  311   163   148   47.588      0.155 7.108       342
      15.9663     2.0456      0.598 3.820 1.117 5.505 1.610 11.814
      3.454 0.3607      6.65  256.95      220.60      3522.10     28620
      26040 27338 13.237      211.35      1.762 28.139      101.08
      27.80 68.97 25.55 139.17      184.29      16.56016836 3.6798
      6.3764      3.4606      4.6655      3.791 12.2871     0.9113
      1127.034
19    2     2     168   205   285   285   312   331   163.0       24.20
      21.80 21.40 22.47       105   243   196   164   207   186   168
      265   0.265 158   252   258   235   220   215   211         152
      137   101   82    127   165   167         6.8         3.65
      54.6  306   167   139   45.425      0.153 6.837       331
      14.5412     1.8183      0.549 3.704 1.119 5.069 1.532 10.589
      3.199 0.3657      6.52  267.63      209.53      3046.79     33383
      40425 24173 18.028      262.15      2.272 33.033      101.29
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      36.38 46.38 35.06 151.91      200.60      19.35335757 2.3507
      4.7006      3.7102      5.7922      2.9553      2.4484
      5.5296      675.1995
20    2     2     196   233   284   317   346   365   169.0       24.50
      23.05 25.15 24.23       118   227   163   202   201   201   221
      297   0.297 129   229   209   187   161   168   168         136
      122   74    108   123   152   152         20.05       5.16
      53.2  340   181   159   46.765      0.159 7.791       365
      18.5417     4.1639      1.141 2.231 0.611 5.445 1.492 11.531
      3.159 0.3355      6.58  238.45      214.32      3973.78     35723
      32145 .     17.001      315.22      2.074 38.463      101.93
      25.56 34.75 41.02 150.77      153.02      21.38128946 2.2515
      .     4.3413      9.5966      7.6407      .     3.1633
      467.628
21    1     4     164   194   245   273   285   298   134.3       20.25
      21.95 21.10 21.10       144   200   177   208   202   218   211
      247   0.24  113   213   194   153   118   110   116         120
      131   91    85    99    114   123         16          3.59
      56.2  280   158   123   43.929      0.153 5.981       298
      13.8018     2.092 0.702 3.853 1.293 4.352 1.461 8.349 2.802
      0.4743      6.17  120.08      118.26      1632.18     36503 24968
      19253 11.608      160.21      1.952 26.939      100.48      29.33
      15.92 227.05      348.37      198.17      16.00255156 6.5087
      9.7504      1.218 0.864 3.2061      1.0269      2.5994
      328.0605
22    1     4     157   185   240   268   286   306   149.3       19.00
      21.30 24.20 21.50       103   174   184   232   197   176   188
      245   0.24  116   187   157   132   167   108   .           117
      72    67    73    109   107   125         8           3.71
      50.4  283   142   140   49.470      0.144 5.743       306
      12.545      1.8591      0.608 3.231 1.056 3.209 1.049 7.698 2.516
      0.5232      6.01  109.35      94.67 1187.60     33953 17798 17490
      6.879 86.29 1.590 19.944      101.39      38.71 11.37 196.59
      497.97      154.36      20.85052094 7.0962      7.4624
      0.4969      0.7758      1.2261      0.7609      0.8155
      156.021
23    1     4     172   201   244   273   283   306   133.8       20.45
      22.25 23.80 21.35       99    146   132   114   170   125   167
      245   0.24  122   220   289   190   219   139   152         117
      100   64    95    112   124   121         29.5        3.79
      54.8  283   155   128   45.230      0.153 5.949       306
      15.048      2.0146      0.658 3.788 1.238 4.700 1.536 7.829 2.558
      0.5818      6.17  137.38      132.47      1993.34     25058 33803
      19238 11.481      172.77      1.634 24.590      100.19      34.49
      22.57 279.28      339.44      237.08      15.21581969 6.9954
      12.5215     4.604 10.6724     11.7723     1.4209      3.9063
      1121.4285
24    1     4     182   213   265   293   316   336   153.6       20.40
      25.85 22.00 23.13       121   136   132   119   130   143   170
      275   0.27  115   187   155   118   105   108   131         113
      90    51    42    83    92    132         21          3.33
      52.7  312   164   147   47.115      0.141 6.756       336
      15.0468     2.237 0.666 3.662 1.090 4.733 1.409 8.088 2.407
      0.5536      5.66  140.84      112.90      1698.75     24923 22635
      15195 11.754      176.86      1.857 27.938      101.10      36.77
      25.27 375.08      450.24      292.08      22.19009143 6.7331
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      11.0995     0.8121      2.388 6.6264      2.1184      1
      539.1135
25    1     4     187   209   253   283   311   325   138.1       23.45
      24.75 23.90 24.03       156   131   116   109   124   137   165
      260   0.26  134   215   198   164   157   159   132         120
      79    67    69    127   132   129         18.5        3.78
      52.3  311   163   148   47.588      0.152 6.964       325
      17.0682     2.225 0.685 4.117 1.267 4.953 1.524 11.950      3.677
      0.5362      5.94  114.54      120.68      2059.84     23850 29430
      19283 9.883 168.69      1.802 30.752      101.39      23.02 14.37
      275.68      485.33      263.84      14.25898363 7.4601
      11.0121     2.1935      8.4388      10.1971     1.4516
      1.8893      888.711
26    2     4     195   230   282   325   359   372   177.0       20.70
      23.95 25.95 23.53       140   233   187   232   216   248   206
      293   0.293 123   223   210   161   110   147   148         121
      118   74    90    111   104   132         15          4
      49.6  351   174   177   50.427      0.156 7.989       372
      17.2865     2.3062      0.620 3.606 0.969 5.720 1.538 11.064
      2.974 0.6004      6.66  133.48      130.10      2248.93     39533
      28178 19013 12.703      219.59      1.623 28.053      101.11
      27.15 46.23 470.50      522.15      393.47      19.56380333
      3.3955      12.2089     3.5634      5.0598      12.352
      3.2939      1.5913      1006.458
27    2     4     160   195   255   295   325   340   180.0       20.90
      23.60 20.25 21.58       121   183   131   172   196   178   193
      262   0.262 138   198   204   180   133   149   128         135
      93    67    80    108   118   116         8.85        3.72
      50.1  320   160   160   50.000      0.140 6.442       340
      14.7619     2.3704      0.697 3.974 1.169 5.107 1.502 13.127
      3.861 0.5631      6.68  96.03 100.50      1483.61     31440 28530
      18345 8.785 129.69      1.723 25.439      100.33      29.60 22.78
      256.19      549.23      198.27      21.62999809 2.9936
      10.3587     2.0265      1.8558      4.1965      0.7505
      2.2724      388.116
28    2     4     226   252   295   332   359   364   138.0       23.40
      23.00 24.65 23.68       120   178   117   133   130   162   203
      303   0.303 121   208   272   148   126   135   156         119
      108   83    73    100   107   124         27          4.18
      50.7  354   179   174   49.153      0.153 7.618       364
      18.1672     2.2281      0.612 4.229 1.162 6.040 1.659 14.293
      3.927 0.571 6.76  137.26      115.64      2100.78     27473 29123
      18105 12.799      232.53      1.791 32.545      101.19      23.93
      21.40 361.30      521.40      357.41      20.04208915 5.9583
      16.2505     2.6894      .     16.252      5.7815      3.0028
      924.534
29    2     4     188   223   270   305   330   353   165.0       20.40
      21.55 26.00 22.65       113   171   130   201   226   183   188
      285   0.285 119   213   224   161   145   149   163         124
      113   71    66    105   136   150         11.5        4.07
      51.5  326   168   158   48.466      0.158 7.407       353
      15.6103     2.3973      0.679 3.752 1.063 4.911 1.391 11.123
      3.151 0.4662      6.43  106.45      103.73      1619.29     33023
      29903 19973 8.392 130.99      1.589 24.798      100.66      31.51
      50.10 434.17      658.71      364.94      24.09795294 4.6911
      10.7469     2.0107      3.0558      6.2158      3.761 5.6988
      798.1695
```

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30    2     4     196   232   276   292   306   314   118.0       21.50
      25.30 27.65 24.82       144   186   140   154   153   167   178
      275   0.275 140   201   224   181   153   158   161         170
      156   81    98    119   155   205         55.5        3.99
      49.2  296   146   150   50.676      0.157 7.055       314
      18.3242     2.4717      0.787 3.553 1.131 4.931 1.570 11.280
      3.592 0.3816      6.2   130.51      131.95      2417.85     29085
      31065 25073 14.357      263.09      1.446 26.505      101.24
      17.77 25.05 377.65      484.50      332.11      14.93399656
      4.0293      20.4231     5.904 16.9536     5.4591      6.3788
      6.8262      1430.3415
31    1     5     136   170   224   258   275   300   164.1       20.20
      21.40 22.70 20.80       151   197   190   196   219   201   206
      231   0.23  125   199   186   171   151   133   132         139
      108   68    97    117   121   116         9.8         3.88
      50.7  271   138   134   49.446      0.160 6.374       300
      13.5883     1.9948      0.665 3.263 1.088 3.750 1.250 7.317 2.439
      0.4126      6.88  117.1 98.14 1333.55     36038 27713 19538 8.841
      120.13      1.696 23.049      100.59      40.81 39.97 74.50
      198.74      202.69      23.4233468  2.9337      8.2698      2.111
      10.3992     4.0298      1.2786      3.237 698.808
32    1     5     159   187   243   277   291   319   160.0       21.25
      22.05 23.40 22.23       117   131   155   200   170   212   201
      247   0.24  192   238   252   196   160   137   146         125
      116   81    111   126   140   121         6.4         3.63
      53.7  290   156   134   46.207      0.146 6.092       319
      12.544      1.8376      0.576 3.530 1.106 5.293 1.659 9.063 2.841
      0.465 6.8   107.19      94.42 1184.35     33000 31905 21540 7.647
      95.93 1.669 20.939      100.72      54.38 21.58 38.02 143.54
      224.57      21.8924091  3.3182      4.8057      0.5627
      2.9056      3.2594      0.7195      1.2025      321.5265
33    1     5     183   215   265   290   303   318   135.0       20.45
      26.05 23.60 23.37       152   202   155   179   153   182   219
      260   0.26  133   233   189   187   138   125   114         93
      82    52    47    79    112   128         28.5        5.67
      51.9  300   155   144   48.000      0.159 6.848       318
      17.0641     2.3688      0.745 3.726 1.172 5.064 1.592 9.179 2.886
      0.474 6.57  151.53      161.03      2747.85     32378 27540 15758
      17.816      304.01      2.604 44.434      101.52      23.82 45.72
      64.64 199.14      227.99      12.04762917 4.3861      13.8252
      5.0982      9.8751      1.1707      1.0766      3.0306
      580.9215
34    1     5     176   215   273   307   330   353   176.7       24.00
      26.50 25.25 25.25       132   174   112   133   160   174   205
      282   0.282 154   216   206   176   148   153   138         117
      103   41    71    106   123   153         9.4         4.33
      63.3  322   204   118   36.646      0.159 10.681            353
      16.2914     2.1317      0.604 3.867 1.095 5.447 1.543 10.657
      3.019 0.4131      6.8   108.62      112.01      1824.87     28890
      29775 18780 9.405 153.22      1.679 27.357      101.52      37.89
      31.66 46.23 185.74      223.08      17.78864555 3.6029
      9.6327      3.6044      6.793 4.479 1.4633      4.4792
      681.585
35    1     5     192   219   261   289   305   335   142.7       28.65
      26.05 25.05 26.58       144   196   153   175   147   159   189
      265   0.26  116   171   176   145   145   132   131         120
      96    62    71    100   113   121         7.8         4.06
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      49.6  300   149   151   50.333      0.160 7.120       335
      14.9641     2.1642      0.646 3.387 1.011 5.536 1.653 11.056
      3.300 0.4848      6.89  117.87      122.15      1827.79     29948
      25965 17580 11.747      175.78      1.928 28.852      101.15
      40.01 28.56 47.15 204.25      173.55      20.04252605 3.9073
      7.8512      2.4203      12.1587     2.3733      4.3324
      5.3642      928.734
36    2     5     191   226   274   309   334   348   157.0       21.33
      23.75 22.25 22.44       131   194   115   125   167   164   161
      282   0.282 133   212   196   169   156   138   140         132
      109   91    89    104   115   117         5.3         4.19
      54.3  325   176   149   45.846      0.160 7.687       348
      13.7559     2.0398      0.586 3.991 1.147 4.376 1.257 14.398
      4.137 0.4319      7.08  115.01      95.92 1319.49     27450 28748
      19148 10.357      142.47      1.800 24.762      101.32      35.41
      35.81 34.95 202.40      225.35      18.26286589 4.5971
      6.5065      4.22  5.3984      3.1272      .     4.8345
      272.16
37    2     5     186   217   266   295   320   337   151.0       20.95
      20.30 24.30 21.85       146   175   116   142   166   195   211
      265   0.265 125   195   191   158   120   122   137         140
      107   71    86    107   118   129         15          3.83
      49.3  316   156   160   50.633      0.163 7.658       337
      14.6512     2.2483      0.667 3.605 1.070 4.960 1.472 11.403
      3.384 0.5094      7.03  110.34      111.16      1628.67     30675
      26100 19223 11.062      162.08      1.757 25.736      100.27
      38.00 37.68 62.11 221.68      214.77      22.01262675 4.1836
      9.8233      4.9711      7.4978      8.0948      5.0681
      2.7453      1050.2115
38    2     5     175   207   261   289   313   330   155.0       21.05
      23.20 22.95 22.40       130   191   126   180   230   199   156
      260   0.26  138   245   219   178   163   134   158         124
      120   83    82    124   154   136         5           3.9
      55.7  306   171   136   44.444      0.147 6.674       330
      13.1756     1.9671      0.596 3.372 1.022 4.039 1.224 12.260
      3.715 0.437 7.06  105.42      89.12 1174.21     32610 30638 21788
      7.707 101.54      1.741 22.933      101.31      36.85 36.81 49.59
      188.18      250.47      18.26286589 3.7731      6.8763
      6.6442      5.0404      10.255      9.7871      2.2435
      1366.881
39    2     5     189   220   261   293   311   329   140.0       21.40
      22.60 25.60 23.20       123   243   169   199   243   193   204
      270   0.27  110   169   155   138   132   135   138         100
      98    69    75    121   152   131         15          4.36
      54.1  302   163   139   46.026      0.162 6.886       329
      14.7276     1.9718      0.599 3.671 1.116 4.966 1.510 12.595
      3.828 0.3584      6.99  157.83      129.85      1912.35     36435
      25163 20423 13.284      195.64      1.882 27.712      101.41
      29.10 58.14 34.73 256.66      468.51      16.15840826 3.9256
      7.3129      1.978 16.0877     6.1786      10.6476     2.6485
      1508.649
40    2     5     196   234   276   308   333   346   150.0       23.40
      21.65 25.02 23.36       135   193   156   170   159   195   210
      291   0.291 101   169   185   189   147   143   147         123
      118   85    85    126   139   171         7.5         3.56
      53.8  329   177   152   46.201      0.154 7.632       346
      14.1223     2.1216      0.613 3.752 1.084 5.352 1.547 11.709
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3.384 0.4023 6.93 155.66 141.38 1996.62 32363
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37.68 41.83 34.95 197.37 240.31 16.63669409 3.6839
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6.3969 1217.8215
41 1 6 187 228 282 316 331 342 155.2 23.70
24.95 27.25 24.33 159 248 173 178 221 201 175
286 0.28 134 181 170 166 153 135 125 162
125 85 92 99 133 164 22 7.1
61.8 327 202 125 38.226 0.162 10.878 342
18.505 2.4252 0.709 3.954 1.156 5.257 1.537 11.025
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26940 21638 8.500 157.29 1.687 31.218 101.04 22.69
61.08 299.24 336.67 357.83 15.74739528 7.149
12.3396 0.895 5.482 4.1319 3.2184 2.462 630.7845
42 1 6 153 190 244 279 298 323 169.7 21.65
23.00 24.20 22.95 125 162 142 161 221 193 177
250 0.25 160 225 247 184 158 146 144 121
118 64 84 100 113 125 6 4.29
50 291 145 146 50.172 0.160 7.044 323
13.6405 2.013 0.623 3.741 1.158 4.186 1.296 8.347 2.584
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73.28 1.581 21.562 101.90 40.95 30.69 275.79
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0.5175 7.12 153.35 142.68 2617.64 32648 25290
16148 13.016 238.80 2.009 36.858 101.49 25.61
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240 0.24 151 213 212 143 157 143 136 130
117 67 71 105 116 127 6.4 5
46.1 291 134 157 53.952 0.166 7.485 322
14.225 2.0515 0.637 2.986 0.927 4.501 1.398 8.717 2.707
0.3766 6.55 122.2 101.91 1449.67 34215 28613 18548
8.707 123.86 1.585 22.542 101.27 39.04 68.92
234.53 446.67 257.87 24.91175845 3.7326

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      7.4446      0.4194      2.0813      1.1843      1.1775
      2.6997      273.6645
46    2     6     192   231   283   320   352   368   176.0       23.15
      23.85 25.75 24.25       157   234   175   177   181   203   176
      292   0.292 148   244   230   192   159   128   154         143
      144   102   80    118   121   128       8.2       4.99
      51    347   177   170   48.991      0.156 7.545       368
      14.3895     2.1335      0.580 4.194 1.140 5.174 1.406 12.032
      3.270 0.4487      6.76  107.8 104.91      1509.67     33780 30855
      20753 8.457 121.69      1.700 24.469      101.32      28.45 45.44
      250.29      500.43      306.44      20.04208915 5.0499
      9.7173      4.0479      12.1379     12.1243     4.0373
      4.8206      1357.305
47    2     6     152   182   230   266   290   302   150.0       17.75
      20.60 22.30 20.22       108   214   157   146   162   168   175
      235   0.235 153   276   245   187   167   165   146         143
      104   71    89    117   122   117       6.8       4.57
      54.6  280   153   127   45.357      0.159 6.896       302
      10.7227     1.6363      0.542 3.544 1.174 4.381 1.451 12.524
      4.147 0.3387      6.93  92.62 87.97 943.33      29603 33225 19410
      6.035 64.72 1.672 17.933      101.05      45.44 39.66 251.41
      584.49      327.00      19.64032906 4.7678      7.2665      1.311
      3.1485      2.2365      2.24  1.357 389.8725
48    2     6     194   224   267   297   318   334   140.0       23.10
      20.80 24.50 22.80       137   228   201   180   202   199   176
      278   0.278 120   184   192   223   200   165   162         147
      121   63    87    122   131   171       6.8       4.34
      57.9  312   181   131   41.987      0.149 6.895       334
      12.8906     2.0541      0.615 4.018 1.203 5.495 1.645 13.449
      4.027 0.3553      6.75  91.34 100.16      1291.18     34665 32955
      21630 8.992 115.91      1.799 23.190      100.79      29.42 56.18
      265.94      624.32      390.88      10.70594988 3.9526
      6.8495      0.8703      9.8262      2.3488      7.191 4.1102
      968.3025
49    2     6     196   230   276   305   328   348   152.0       21.75
      21.00 25.00 22.58       164   287   212   223   250   185   224
      288   0.288 142   168   167   140   122   126   122         128
      108   54    78    100   137   120       8.6       5.01
      53.5  321   172   149   46.417      0.151 7.116       348
      13.6027     2.1557      0.619 3.692 1.061 4.785 1.375 9.975 2.866
      0.3165      6.9   99.2  104.45      1420.86     39540 24533 18900
      10.491      142.71      1.771 24.095      100.39      29.42 56.14
      266.33      539.50      288.68      19.18117467 4.5533
      8.5464      3.2585      4.7735      9.3786      12.5523
      2.5875      1444.8825
50    2     6     198   238   269   310   340   354   156.0       25.00
      13.45 19.40 19.28       178   251   195   267   242   246   205
      294   0.294 143   193   236   249   226   190   193         149
      153   94    94    130   170   175       6.4       4.54
      63.2  326   206   120   36.810      0.167 11.429            354
      14.5118     1.9674      0.556 4.279 1.209 5.435 1.535 14.208
      4.014 0.3054      6.75  86.92 86.60 1256.72     41978 37868 25148
      7.732 112.20      1.711 24.836      101.09      28.95 30.23
      184.77      484.71      221.05      20.36732351 3.8393      8.662
      3.4651      5.7276      7.6407      7.3589      4.3142
      1138.596
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51    1     7     159   195   242   272   288   314   155.2       23.60
      23.00 21.58 22.73       127   185   190   180   280   206   198
      249   0.24  128   194   177   167   157   131   129         149
      112   68    78    109   132   120         5.7         5.18
      56.3  289   163   126   43.599      0.150 6.046       314
      14.4887     2.2266      0.709 3.444 1.097 3.737 1.190 10.055
      3.202 0.3494      6.94  148.36      125.40      1816.87     37013
      27338 19478 9.474 137.27      2.111 30.585      101.74      36.59
      41.40 17.50 97.24 143.84      20.91431001 4.0012      5.8123
      4.9067      3.5123      1.2577      0.677 3.4654      380.148
52    1     7     142   176   234   270   290   324   181.9       21.65
      22.40 23.05 22.37       129   174   173   156   185   178   206
      241   0.241 153   227   199   173   147   134   130         128
      101   68    101   131   142   115         7.4         4.48
      66.7  281   187   94    33.452      0.158 9.841       324
      13.2501     1.8464      0.570 3.571 1.102 4.906 1.514 10.788
      3.329 0.3071      6.66  107.09      101.95      1350.87     31890
      28560 20805 8.326 110.32      1.734 22.971      100.56      45.65
      103.87      35.11 199.88      303.49      18.25643206 3.2892
      8.2189      0.6894      10.0089     0.814 2.8873      3.1557
      615.147
53    1     7     192   228   283   317   346   366   174.4       24.70
      25.95 24.65 25.10       144   181   215   177   207   180   178
      291   0.29  119   188   183   148   134   127   127         128
      86    75    62    81    131   115         10.25       5.35
      64.1  334   214   120   35.928      0.154 10.359            366
      16.6927     2.1533      0.588 4.190 1.145 5.353 1.463 10.318
      2.819 0.4032      6.49  155.82      155.46      2595.09     33593
      25815 17573 10.632      177.48      1.911 31.902      101.18
      46.47 53.83 17.88 138.72      196.95      23.91239634 3.077
      7.1268      2.9712      6.4573      2.9727      5.0512
      5.8171      849.6435
54    1     7     180   217   270   293   320   334   154.2       21.35
      24.45 20.60 22.13       113   126   126   170   176   201   217
      272   0.27  158   224   195   177   157   131   133         134
      85    68    81    107   131   140         5.6         4.33
      52.7  315   166   149   47.302      0.156 7.281       334
      11.6951     1.9605      0.587 4.191 1.255 5.551 1.662 13.460
      4.030 0.4183      6.9   101.95      99.10 1158.94     31508 28838
      19545 7.834 91.62 1.789 20.919      100.68      47.27 50.21 23.40
      134.35      213.32      18.41591735 3.8665      6.8819
      3.2284      5.4085      5.082 6.9092      4.8576      999.651
55    1     7     173   198   241   273   301   319   145.9       21.25
      22.40 22.75 22.13       157   218   184   174   222   208   196
      250   0.25  139   224   140   152   131   133   130         138
      105   61    82    116   114   133         5           4.49
      50.5  294   148   146   49.660      0.176 7.645       319
      12.1531     1.9277      0.604 3.653 1.145 4.783 1.499 9.992 3.132
      0.3766      6.37  81.44 88.50 1075.58     35708 25928 19043 8.434
      102.50      1.979 24.051      101.50      26.35 49.37 16.50
      135.78      183.41      16.80887698 3.4469      5.7554      3.575
      3.4236      1.6856      3.832 7.0558      673.779
56    2     7     185   222   272   297   316   344   159.0       23.15
      24.40 20.20 22.58       159   289   165   164   173   180   160
      279   0.279 145   225   225   179   161   164   169         136
      122   69    75    111   148   149         7.6         5.27
      53.8  312   168   144   46.154      0.157 7.472       344
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      13.1861     1.9067      0.554 4.009 1.166 5.027 1.461 12.309
      3.578 0.41  7.18  95.6  85.87 1132.30     32250 32175 21113 6.108
      80.54 1.577 20.796      100.57      34.61 92.57 22.25 182.24
      279.96      14.3217907  4.6778      10.6969     1.9447
      5.8251      5.7826      5.3258      6.074 965.9085
57    2     7     184   213   261   298   324   342   158.0       23.10
      27.15 24.65 24.97       126   209   155   139   185   162   193
      270   0.27  163   249   214   166   179   162   163         140
      125   74    77    111   127   138         6.5         4.64
      53.1  317   168   149   47.003      0.149 6.802       342
      13.0817     1.9279      0.564 3.440 1.006 4.622 1.351 13.274
      3.881 0.4154      7.11  89.06 94.94 1241.93     30368 32303 20085
      9.396 122.92      1.927 25.203      101.34      39.45 70.57 23.99
      198.48      298.81      21.34302659 4.5959      8.9865
      2.4283      8.1406      1.3845      1.2232      4.4729
      550.524
58    2     7     200   236   278   308   329   347   147.0       25.60
      .     26.35 25.98       128   238   179   171   157   137   164
      298   0.298 112   218   186   163   141   140   158         148
      154   121   104   125   142   159         19          5.58
      54.1  321   173   147   45.794      0.159 7.569       347
      17.964      2.2392      0.645 4.017 1.158 5.262 1.516 13.039
      3.758 0.3814      6.62  204.23      144.85      2602.16     29483
      28455 24173 12.183      218.85      1.973 35.441      101.93
      29.74 98.36 30.32 169.13      219.84      15.14444232 4.2017
      10.9418     0.8975      16.2811     1.2411      6.2621
      2.3428      1003.755
59    2     7     183   219   269   304   323   350   167.0       21.95
      39.60 24.35 28.63       155   231   171   188   194   165   187
      287   0.287 135   206   215   181   150   135   132         119
      116   63    69    100   132   140         6.25        4.76
      52    314   164   151   48.089      0.165 7.613       350
      14.749      2.2024      0.629 4.005 1.144 5.484 1.567 11.050
      3.157 0.3886      7.2   143.38      104.12      1535.74     32970
      28905 19260 9.474 139.73      1.712 25.246      101.43      31.19
      42.80 17.07 132.74      184.51      17.84197436 3.941 6.6268
      4.6053      10.9859     8.7639      7.7188      7.5754
      1483.422
60    2     7     197   237   283   317   340   365   168.0       24.60
      37.55 24.10 28.75       142   203   182   151   153   182   179
      297   0.297 118   204   192   181   161   124   126         121
      103   79    88    111   120   1258        8.4         4.91
      53.7  330   177   153   46.364      0.164 8.018       365
      13.9027     2.2346      0.612 3.921 1.074 5.332 1.461 12.894
      3.533 0.4488      7.1   140.81      111.36      1548.15     30885
      27885 19350 12.680      176.29      2.069 28.761      100.96
      30.57 82.10 26.26 268.46      331.07      16.21580256 4.606
      10.059      2.9693      8.6367      0.6136      8.6997      8.656
      1112.9145
61    1     3     187   224   280   315   326   346   159.1       20.50
      24.30 24.05 22.95       181   245   181   173   180   216   189
      283   0.28  123   180   175   153   146   142   125         141
      130   87    85    79    97    141         29          4.5
      61.5  320   197   123   38.438      0.163 10.433            346
      17.0218     2.1772      0.629 4.110 1.188 5.508 1.592 10.330
      2.986 0.3712      6.49  140.32      125.27      2132.40     35085
      26670 18480 10.143      172.65      2.119 36.072      101.89
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      29.15 531.71      252.13      339.07      432.75      13.92003061
      4.0369      16.7054     1.8194      1.8385      2.3184
      2.3709      2.1556      393.696
62    1     3     175   209   264   287   309   333   157.7       22.60
      24.30 21.35 22.75       136   180   169   166   232   208   196
      263   0.26  126   160   165   143   131   133   113         118
      114   81    85    114   124   128         7.5         4.5
      60.9  295   179   115   38.983      0.159 9.562       333
      12.0112     1.8762      0.563 3.704 1.112 5.185 1.557 11.326
      3.401 0.4459      7.14  91.46 96.85 1163.34     34703 24653 19808
      4.541 54.55 1.889 22.693      101.17      39.18 375.93
      142.69      258.46      35.14 19.04725464 3.301 10.1945
      0.7126      2.7943      0.5   1.0367      1.47  223.32
63    1     3     181   220   266   300   323   339   157.9       25.50
      25.45 22.45 24.47       142   154   153   166   191   195   147
      271   0.27  133   203   224   191   168   130   137         125
      110   71    86    87    115   126         9.2         4.2
      63.8  314   201   114   36.306      0.148 8.979       339
      11.0424     1.7954      0.530 3.938 1.162 5.597 1.651 12.716
      3.751 0.2929      6.76  95.12 74.14 818.67      30713 29813 18330
      4.119 45.49 1.833 20.243      100.69      54.88 81.79 25.09
      247.69      443.94      16.77061412 3.7563      9.4278      4.563
      4.9714      2.2111      1.5371      1.0425      440.5875
64    1     3     194   232   285   317   343   373   178.8       23.70
      25.55 24.60 24.62       119   129   129   119   130   142   172
      293   0.29  142   168   200   154   153   156   142         118
      112   63    69    89    113   119         12.5        5.27
      51.1  337   172   165   48.961      0.162 7.610       373
      17.0418     2.3746      0.637 4.479 1.201 5.571 1.494 11.937
      3.200 0.4407      6.69  90.74 93.23 1588.79     24750 28575 17378
      7.889 134.45      1.579 26.902      101.10      37.74 290.71
      159.40      237.80      522.99      15.71838531 3.859 10.3967
      5.482 4.8746      3.4302      9.8686      4.5216      1110.591
65    1     3     164   192   237   263   293   315   151.5       22.35
      25.70 22.35 23.47       136   152   140   119   130   142   172
      238   0.23  107   179   185   137   135   112   115         120
      68    67    66    128   100   99          6.4         5.01
      48    283   136   147   51.943      0.157 6.488       315
      11.2358     1.9365      0.615 3.249 1.031 4.665 1.481 9.360 2.971
      0.4405      6.7   65.94 71.35 801.65      25470 24300 16718 4.152
      46.65 1.593 17.903      100.13      40.16 60.87 105.49
      257.76      345.56      18.76028315 3.7534      8.0267
      0.6757      3.4893      1.2089      1.6502      .     218.721
66    2     1     160   180   206   221   236   242   82.0        15.00
      12.65 17.40 15.02       100   203   151   140   160   147   138
      206   0.206 100   150   109   105   99    101   97          81
      55    32    31    38    45    88          3.5         3.27
      20.7  228   47    181   79.386      0.145 6.165       242   6.582
      1.6187      0.669 0.950 0.393 1.139 0.471 1.394 0.576 0.3653
      7.2   86.83 78.07 513.86      26948 19028 8888  6.599 43.44 2.103
      13.840      101.00      27.00 40.91 23.81 149.60      101.08
      13.38435049 4.134 3.1907      0.5945      0.653 0.5907
      0.7067      0.6266      116.289
67    2     1     151   178   215   240   256   265   114.0       17.60
      14.60 19.40 17.20       96    153   176   143   175   144   138
      219   0.219 110   156   133   117   100   109   105         115
      61    44    32    25    37    78          3           2.98
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      18.5  256   47    209   81.641      0.156 6.414       265
      8.8707      1.9356      0.730 1.170 0.442 1.288 0.486 2.185 0.824
      0.3828      6.57  107.48      96.19 853.25      27135 20723 8483
      7.658 67.93 1.885 16.725      100.40      23.43 45.73 24.07
      182.08      193.20      13.99655634 2.9266      5.1642      0.556
      1.3848      0.7253      1.0817      0.6846      167.9625
68    2     1     158   187   211   226   238   247   89.0        18.05
      16.05 17.60 17.23       112   129   154   153   141   146   145
      212   0.212 96    121   111   99    99    100   105         103
      50    25    33    38    66    82          3           3.2
      19.6  230   45    185   80.435      0.146 6.091       247
      7.0619      1.7721      0.717 1.060 0.429 1.132 0.458 1.671 0.677
      0.4005      6.92  86.18 69.54 491.08      25980 18623 9645  7.184
      50.73 2.002 14.137      101.04      23.28 41.05 31.55 111.34
      103.97      11.85383585 3.4164      4.9709      0.4863
      0.6528      .     0.6543      1.1868      72.3195
69    2     1     152   182   208   216   232   240   88.0        18.60
      13.80 17.65 16.68       115   151   160   131   188   146   136
      201   0.201 89    110   124   105   103   106   94          83
      35    24    36    40    39    83          2.9         2.72
      19    226   43    183   80.973      0.149 6.048       240
      6.6562      1.6228      0.676 1.051 0.438 1.233 0.514 1.782 0.742
      0.3904      6.77  68.13 65.08 433.17      26948 18938 8213  8.180
      54.45 2.074 13.808      101.46      23.43 46.43 24.24 102.49
      149.72      14.03481921 2.9921      5.3026      0.5245
      0.7603      0.5253      0.6595      1.0332      124.881
70    2     1     166   193   215   228   236   244   78.0        18.60
      13.65 18.10 16.78       122   153   147   145   148   142   142
      213   0.213 95    129   122   104   83    103   101         102
      33    34    36    30    32    83          3           2.8
      20.4  230   47    183   79.565      0.153 6.364       244
      6.9172      1.7411      0.714 0.971 0.398 1.113 0.456 1.833 0.751
      0.4021      6.63  52.42 55.98 387.23      25958 18668 7935  5.787
      40.03 2.007 13.886      100.89      23.43 35.01 19.62 127.84
      114.42      12.54256744 3.0464      4.1008      0.5012
      0.9109      0.7475      0.8305      0.7   .

;

proc sort data = all_groups; by Diet_Group;

proc format;
      value dietfmt
            1='Lean Control'
            2='Obese Control'
            3='High Germ'
            4='Whole Wheat'
            5='Refined Wheat'
            6='Bran'
            7='Germ';

proc print data= all_groups; by Diet_Group; format Diet_Group dietfmt.;

proc means data=all_groups n mean stderr min max; by Diet_Group; format
Diet_Group dietfmt.;
var Tot_FA_absorpPC Free_FA_absorpPC Daily_Tot_FA_intake
Daily_free_FA_intake FA;
```

```
run;

proc glm data= all_groups;
title 'One Way Analysis of Variance in all rats';
format Diet_Group dietfmt.;
class Diet_Group Block_No;
model Initial_BW--AUC_MMT_Insulin Daily_Tot_FA_intake--
Free_FA_absorpPC=Diet_Group Block_No;
Means Diet_Group / Duncan;
LSMeans Diet_Group / STDERR pdiff;
run;
proc glm data= zdf_only;
title 'One Way Analysis of Variance in ZDF rats';
format Diet_Group dietfmt.;
class Diet_Group Block_No;
model Initial_BW--AUC_MMT_Insulin Daily_Tot_FA_intake--
Free_FA_absorpPC=Diet_Group Block_No;
Means Diet_Group / Duncan;
LSMeans Diet_Group / STDERR pdiff;
run;
proc corr data=all_groups;
title 'Correlation of Fecal Bile Acids and Liver Cholesterol in all
rats';
format Diet_Group dietfmt.;
var Liver_total_chol_conc_true resistin_sample_ngml;
with fecal_bile_acid Body_Fat_DXA;
run;
proc corr data=zdf_only;
title 'Correlation of Fecal Bile Acids and Liver Cholesterol in zdf
rats';
format Diet_Group dietfmt.;
var Liver_total_chol_conc_true resistin_sample_ngml;
with fecal_bile_acid Body_Fat_DXA;
run;
quit;
```