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The Effects of a Wild Blueberry Diet on Hepatic and Aortic Morphology in the Obese Zucker Rat, A Model of the Metabolic Syndrome

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**THE EFFECTS OF A WILD BLUEBERRY DIET ON HEPATIC AND AORTIC
MORPHOLOGY IN THE OBESE ZUCKER RAT, A MODEL OF THE
METABOLIC SYNDROME**

By

Thomas W. Merrow

B.S. Human Nutrition & Food Science, University of Maine, 2014

A THESIS

Submitted in Partial Fulfillment of the

Requirements of the Degree of

Master of Science

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December 2016

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September, 6th, 2016

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MORPHOLOGY IN THE OBESE ZUCKER RAT, A MODEL OF THE
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By Thomas W. Merrow

Thesis Advisor: Dr. Dorothy Klimis-Zacas

An Abstract of the Thesis Presented
In Partial Fulfillment of the Requirements for the
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December 2016

The goal of this study is to investigate the effect of a wild blueberry diet on pathology of the metabolic syndrome (MetS) by examining the morphological and biochemical properties of the liver and aortic tissue in the obese Zucker rat (OZR), a valid model of the MetS.

At 8-weeks of age, 16 Obese Zucker rats (OZR) and 16 lean Zucker rats (LZR) littermates were placed on either an 8% w/w wild blueberry (WB)-enriched isocaloric diet or an isocaloric control (C) diet for a duration of 8-weeks. At 16-weeks of age, the tissues of interest were harvested for the study. The morphological features for hepatic steatosis and glycogen were assessed utilizing a unique series of stains and were analyzed through image analysis and a histopathological review by a pathologist. The accumulation of hepatic triglyceride (TG) was also evaluated for the assessed. For the assessment of morphological features of the thoracic aorta, a series of unique stains were utilized and further analyzed through the use of image analysis to detect collagen and connective

tissue, the thickness of the tunica media, the number of nuclei, and the presence of glycosaminoglycans.

A significant increase in hepatic steatosis was found in the OZR compared to the LZR after image analysis and histopathological evaluation of the Hematoxylin and Eosin (H&E) stain, the Oil Red O (ORO) stain, and hepatic TG concentration. Although non-significant, image analysis of the hepatic triglycerides using the ORO stain found a trend for a decrease in hepatic TG content in the OZR-WB group compared to the OZR-C. Image analysis of the Periodic Acid-Schiff (PAS) stain revealed a significant increase in hepatic glycogen in the OZR compared to the LZR. Although non-significant, the LZR-WB and OZR-WB tended to have a greater amount of glycogen than the LZR-C and OZR-C groups respectively.

Regarding the morphology of the aortic tissues, there were no significant differences found due to rodent model or due to diet after evaluating for connective tissue, medial width, number of nuclei, and glycosaminoglycans. The LZR-WB and OZR-WB groups tended to have less medial width, and a lower percentage of glycosaminoglycans compared to the LZR-C and the OZR-C respectively. Additionally, although non-significant, there was a trend for elevated number of nuclei in the OZR-WB group compared to all other groups. In conclusion, consuming wild blueberries has the potential to alter the morphology of hepatic and aortic tissues and confirms that the OZR continues to act as a reliable model of the MetS.

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LIST OF ABBREVIATIONS

ACVD, Atherosclerotic Cardiovascular Disease

AGE, Advanced Glycation End Products

ATP III, Adult Treatment Panel III

AUC, Area Under the Curve

C, Control (Diet)

CAA, Cellular Antioxidant Activity

CAM, Complementary Alternative Medicine

CDC, Centers for Disease Control

COX-2, Cyclooxygenase-2

CRP, C-Reactive Protein

CVD, Cardiovascular Disease

EDTA, Ethylenediaminetetraacetic acid

Fa/fa, Lean Zucker Rat Genotype

fa/fa, Obese Zucker Rat Genotype

FFA, Free Fatty Acid

GLUT, Glucose Transporter

H&E, Hematoxylin and Eosin (stain)

HbA1c, Glycosylated Hemoglobin A1c

HDL-C, High Density Lipoprotein Cholesterol

HOMA-IR, Homeostatic Model Assessment of Insulin Resistance

HPLC, High Performance Liquid Chromatography

IL, Interleukin

iNOS, Inducible Nitric Oxide Synthase

LDL-C, Low Density Lipoprotein Cholesterol

LZR, Lean Zucker Rat

MetS, Metabolic Syndrome

NAFLD, Non-alcoholic Fatty Liver Disease

NASH, Non-alcoholic Steatohepatitis

NF- κ B, Nuclear Factor Kappa B

NHANES, National Health and Nutrition Examination Survey

ORAC, Oxygen Radical Absorbance Capacity

ORO, Oil-Red O (stain)

OZR, Obese Zucker Rat

PAI-1, Plasminogen Activator Inhibitor-1

PAS, Periodic-Acid Schiff (stain)

RGB, Red-Green-Blue (image)

ROI, Region of Interest

ROS, Reactive Oxygen Species

sdLDL-C, Small Dense Low Density Lipoprotein Cholesterol

T2DM, Type Two Diabetes Mellitus

TG, Triglyceride

TNF- α , Tumor Necrosis Factor Alpha

VLDL-C, Very Low Density Lipoprotein Cholesterol

w/w, Weight for Weight

WB, Wild Blueberry (Diet)

CHAPTER 1

INTRODUCTION

The metabolic syndrome (MetS) is a multifactorial condition that is an escalating world-wide health risk due to urbanization, excessive energy intake, obesity and sedentary lifestyle (Kaur, 2014). The MetS increases the risk for comorbidities, such as myocardial infarction, type 2 diabetes mellitus (T2DM), & cardiovascular disease (CVD) (Kaur, 2014) resulting in chronic inflammation (Lee et al., 2005), debilitating disease states and reduced life expectancy (Society, 2015). Components of the MetS result in drastic healthcare expenses. Obesity costs the United States an estimated \$147 to \$210 billion annually (Cawley et al., 2012) plus an additional \$245 billion per year due to diabetes medical costs (American Diabetes, 2013).

Non-alcoholic fatty liver disease (NAFLD) is the most common liver disease in the world and is associated with the MetS (Milic et al., 2014). NAFLD is represented by simple steatosis (>5% of hepatocytes infiltrated with lipid) and may progress toward non-alcoholic steatohepatitis (NASH), irreversible cirrhosis and eventually hepatocellular carcinoma (Milic et al., 2014). Eighty percent of patients with NAFLD are obese, have greater proportions of visceral adipose tissue, are often insulin resistant, present symptoms of low-grade inflammation, such as elevated levels nuclear factor Kappa B (NFκB) and Tumor Necrosis Factor alpha (TNF-α) with adipose tissue accumulation (Milic et al., 2014). NAFLD has the potential to present itself as both a manifestation of inflammation and structural change in individuals with the MetS.

It is known that the MetS may lead to vascular dysfunction which increases the chance to acquire more detrimental disease states such as atherogenic cardiovascular disease (Scott M. Grundy, 2011). High blood pressure, vascular inflammation, elevated triglycerides (TG) and small dense low-density lipoprotein cholesterol (sdLDL-C) with low levels of high density lipoprotein cholesterol (HDL-C) are symptoms of the MetS and play a part in vascular dysfunction (Scott M. Grundy, 2011). It is now thought that obesity produces an increased state of inflammation and oxidative stress which yields a cascade of pro-inflammatory cytokines that enhance endothelial dysfunction (Scott M. Grundy, 2011). Wild blueberries (WB), *vaccinium angustifolium*, are a concentrated source of dietary antioxidants, mainly in the form of anthocyanins, and are widely available in the United States. Low bush wild blueberries are unique due to their high polyphenolic content relative to their small size. Studies in our lab have shown that when the Obese Zucker rat (OZR), a model of the metabolic syndrome, consumed a diet rich in wild blueberries, the animals displayed a decrease in markers of inflammation as well as gene expression after mRNA extraction of iNOS, eNOS, and COX₂ (Vendrame et al., 2013; Vendrame et al., 2015), had a trend toward normalized serum glucose levels (Vendrame et al., 2015), improved lipid metabolism and lipid gene expression (Vendrame, Daugherty, et al., 2014), as well as improved vascular function and vasoreactivity (Vendrame, Kristo, et al., 2014).

The OZR is an appropriate model for the metabolic syndrome due to sharing similar physiological characteristics expressed in humans with the MetS (Ahima, 2011; Alexandre de Artinano et al., 2009; Frisbee et al., 2006; Scott M. Grundy, 2011). The OZR quickly progresses into an obese state (Alexandre de Artinano et al., 2009),

develops mild steatosis (Serkova et al., 2006), hyperlipidemia (Tofovic et al., 2003), increased systolic pressure, insulin resistance, mild hyperglycemia and hyperinsulinemia (Aleixandre de Artinano et al., 2009; Kucera et al., 2014; Triscari et al., 1979).

To our current knowledge there are no other studies, other than the experiments conducted in our laboratory, that have investigated how a diet rich in wild blueberries affects the morphologic properties in the liver and aorta of the obese Zucker rat. This study further explores the effects of wild blueberries on the structural and biochemical properties of aortic and hepatic tissues in the obese Zucker rat, a model of the metabolic syndrome, as well as its lean littermate, the lean Zucker rat (LZR).

The goal of this project is to identify the effect of a wild blueberry-enriched diet on morphology in hepatic and aortic tissues of the OZR. Therefore, the objectives of this project will be to investigate the role of wild blueberries in the adult male OZR and LZR evaluating the following components:

1. Morphology of the Hepatic tissue by utilizing the H&E stain to evaluate tissue structure, PAS stain to assess for glycogen content, and the ORO stain to evaluate the presence of triglycerides and;
2. Hepatic triglyceride concentration
3. Morphology of the aorta by utilizing the H&E stain to evaluate the tissue structure, Sirius Red to assess for collagen and fibrotic tissue, and Alcian Blue stain to evaluate for glycosaminoglycans.

The results from this study will aid in further characterizing the OZR while simultaneously providing results to extrapolate for public health awareness and an alternative for pharmacotherapy. This project will aid in advancing the field of

complementary alternative medicine (CAM) treatment for chronically ill patients as well as improve preventative disease treatment for an increasing population of Americans with the MetS. Additional benefits of consuming wild blueberries consist of the potential to stimulate Maine's economy and increasing job positions by expanding the export of wild blueberries to other U.S. States or internationally. Due to its unique characteristics as both a rich source of antioxidants and its powerful anti-inflammatory properties, low bush wild blueberries can be incorporated into the diet and act as an alternative method of treatment for the MetS.

CHAPTER 2

LITERATURE REVIEW

2.1. Metabolic syndrome overview

Although similar ideas had been postulated before, syndrome X was first coined in 1988 when Dr. Gerald Reaven presented the *Banting Lecture* after being honored for his work by the American Heart Association (Reaven, 1988). To this day Syndrome X is now referred to as the MetS due to its complexity and additional components that are known to cause adverse health outcomes. The MetS is mainly a quintet of coexisting factors consisting of abdominal obesity, dyslipidemia, hypertension, and impaired fasting glucose with hyperglycemia (Ahima, 2011; Scott M. Grundy, 2011; Ma et al., 2013). The National Institutes of Health, National Heart, Lung and Blood Institute, and the National Cholesterol Education Program (National Cholesterol Education Program Expert Panel on Detection et al., 2002) have defined the MetS through the creation of *the Adult Treatment Panel III* (ATP III) defined by the following criteria:

1. **Abdominal obesity** (especially visceral fat) with a waist line ≥ 35 inches for women and ≥ 40 inches for men;
2. **Dyslipidemia** with triglycerides ≥ 150 mg/dL as well as HDL ≤ 50 mg/dL for women and ≤ 40 mg/dl for men;
3. **Hypertension** with a blood pressure $\geq 130/85$ mmHg;
4. **Hyperglycemia** with a high fasting blood glucose ≥ 110 mg/dL

The coexistence of all four factors contribute toward a pro-inflammatory, pro-thrombotic state that accelerate the development of comorbidities such as atherosclerotic cardiovascular disease (ACVD), T2DM, NAFLD, sleep apnea, polycystic ovary

syndrome, and various cancers (Beck-Nielsen, 2013; Scott M. Grundy, 2011). The presence of anyone or more of these conditions has the potential to exhibit adverse social, physiological, and psychological implications that cause distress for the patient and also decrease the overall standard of living.

2.2. Prevalence of the metabolic syndrome

The prevalence of obesity and the MetS in the United States has been increasing over the past decade in adult, childhood, and adolescent populations. An article by the National Health and Nutrition Examination Survey (NHANES) program revealed that in 2003-2004, the prevalence of the MetS was at an average of 32.9% of the population (Aguilar et al., 2015). Data collected in 2011-2012 indicated the MetS had increased in prevalence to 34.7% of the population (Aguilar et al., 2015). An NHANES study (Cook et al., 2008) evaluated 1999-2002 data using cross-sectional methods from four different definitions of the MetS. The study found that the prevalence of MetS in adolescents from ages 12-19 years old varied from 2.0% to 9.4% and was increased in obese teens ranging from 12.4% to 44.2% of the population (Cook et al., 2008). These statistical results represent a severe health threat of acquiring the MetS in all age groups, especially for the youth.

2.3. Genetics and the metabolic syndrome

Genetics may play an important role in determining the expression of the MetS.

Furthermore, the MetS may place individuals at greater risk for acquiring T2DM or even insulin-dependent T2DM if healthy lifestyle adjustments are not adopted.

The *thrifty genotype hypothesis* (Neel, 1962), originally suggested in 1962 by James V. Neel, proposed that our ancestors who lived in a harsher environments were able to survive times of fasting and famine through natural selection of favoring genetic traits

that improve energy storage capabilities. A body capable of eating large quantities of food at one sitting as well as having an efficient capacity to store energy as fat would most likely survive through a period of poor food availability. The *thrifty genotype hypothesis* proposes that diabetes may be a genetic result from the natural compensation of the pancreas in times of ‘feast-or-famine’ state; when the only mode of survival is to consume large amounts of food in one sitting, your pancreas must be able to produce large amounts of insulin at a single time. Given the overabundance of food in the current society, an association can be made between the over-stimulation of the pancreas to secrete insulin in response of consistent exposure to large quantities of (refined) foods and sedentary behavior. As we observe in patients with T2DM, pancreatic beta-cells become exhausted due to over-stimulation, therefore losing the ability to secrete an adequate amount of insulin.

The *Thrifty Phenotype hypothesis* (Hales et al., 1992) suggests that environmental factors contribute to the formation of T2DM. Neonates exposed to malnutrition during pregnancy in conjunction with a low birth weight and poor nutrition in early life increases the chance of acquiring diabetes and the MetS in later years of growth (Hales et al., 1992, 2013). When a child with a low birth weight due to malnourishment is introduced to an environment rich in energy dense food (or high food availability), the researchers appear to suggest that environmental and genetic factors can cause T2DM advancement partly due to poorly developed pancreatic islets and poor ability to adapt to this new environment; the developing fetus was likely prepared to be exposed to a world devoid of food availability. Researchers of the Hertfordshire study (Barker et al., 1993) found that men who developed Syndrome X (MetS) later on in life had lower birth weights at one

year, greater 2-hour plasma glucose insulin concentrations, lower levels of HDL-C, elevated fasting pro-insulin, as well as elevated concentrations of apolipoprotein B and plasminogen activator inhibitor (Barker et al., 1993). Another study (Forsen et al., 2000) also found that the incidence of T2DM was positively associated with low birth weight and placental weight for both sexes, as well as mothers with a greater BMI.

Investigations into genetic adaptation appear to play a role in the development of the metabolic syndrome as well as progression toward T2DM. These studies show that the environment and heritability have a great influence on how the body reacts toward stress and food exposure. Although the studies presented suggest specific genetic adaptations to the environment, more research must be performed to conclude these postulations.

2.4. Economic burden

As a greater proportion of the American population acquires the MetS, healthcare costs simultaneously increase due to direct and indirect expenses. A patient who is admitted into the hospital may be treated for MetS under the ICD-10 medical coding system which identifies the MetS as E88.1, and with obesity, as E66 (Prevention, 2015), although outpatient nutritional counseling is not necessarily covered by insurance groups, such as Medicare. Also, Grundy (Scott M. Grundy, 2011) estimates that by 2025, the global prevalence of the MetS and diabetes will reach approximately 400 million. A healthcare system that can encourage treatment for MetS patients is desired although this may also result by increasing direct and indirect medical expenses. Since our current healthcare system is partially funded through federal and state tax dollars, such as Medicaid, but does not necessarily provide compensation for nutritional counseling (preventative or

lifestyle changes), an increase in people with the MetS and related comorbidities will stress the patients, tax payers, and healthcare providers.

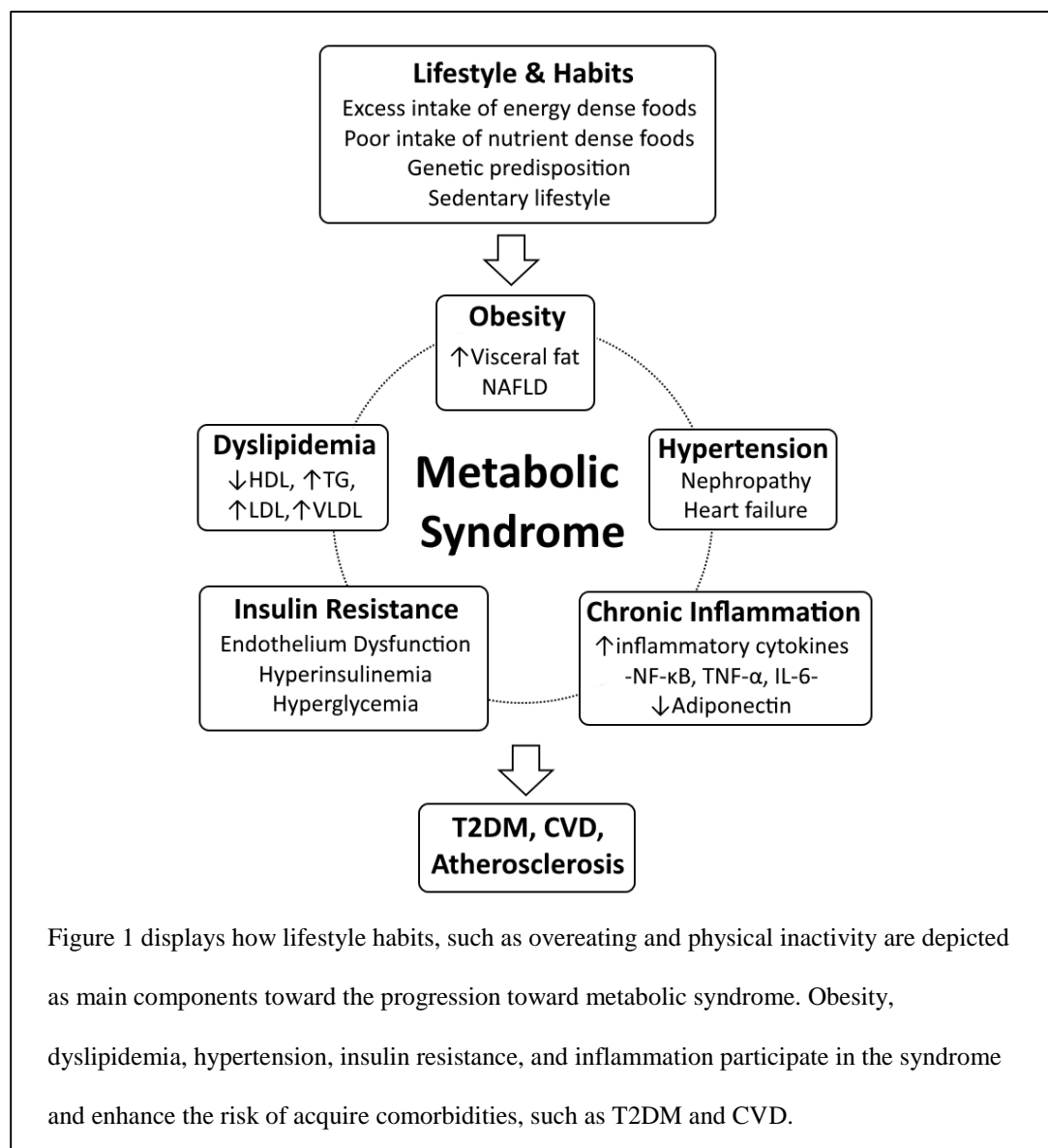
Diabetes alone costs the United States approximately \$245 billion; \$176 billion from direct costs (in the healthcare system) and \$69 billion from indirect costs (e.g. work loss and disability) (ADA, 2014). Obesity is estimated to cost \$147 billion per year in 2006 (Cawley et al., 2012) plus an expected increase in annual healthcare expenses of \$48 to \$66 billion, or approximately 16% to 18%, by 2030 (Y. C. Wang et al., 2011). The data presented displays the complexity of both estimating direct and indirect healthcare expenses as well as foreshadowing of an increase in disease prevalence, economic burden, as well as the encumbrance for future generations.

2.5. Risk factors of the metabolic syndrome

The metabolic syndrome is associated with dyslipidemia, abdominal obesity, inflammation, insulin resistance, and greater risk of developing cardiovascular disease (Despres et al., 2006). Environmental factors, such as physical inactivity, smoking, nutrition (energy dense foods), and high stress combined with the genetic traits have great potential to induce the MetS. This next section will discuss the MetS based upon the components of the MetS and how their development progresses to display adverse health effects. In Figure 1 (below), the origination of the MetS cascade begins with a genetic predisposition, excessive intake of energy dense foods devoid of beneficial nutrients, and sedentary lifestyle. This lifestyle results in accumulating multiple detrimental health-related issues. Obesity may result in a greater visceral fat percentage inducing NAFLD. This is often accompanied by a rise in TG's, LDL-C, VLDL-C and a decrease in HDL-C termed dyslipidemia. Hypertension may eventually result in nephropathy and heart

failure. Obesity may lead toward insulin resistance resulting in endothelium dysfunction, hyperinsulinemia, and hyperglycemia. Chronic inflammation is often seen through an increase in pro-inflammatory cytokines, such as NF- κ B, TNF- α , and IL-6 as well as a decrease in anti-inflammatory cytokines, such as adiponectin. Together these components form the metabolic syndrome and increase the risk of type 2 diabetes, cardiovascular disease, and atherosclerosis.

Figure 1: The metabolic syndrome progression, symptoms and outcomes



2.6. Obesity

The development of obesity, specifically abdominal or visceral obesity, is a risk factor for the MetS, T2DM, as well as NAFLD (Sun et al., 2012). Intra-abdominal fat area (visceral fat) is considered the most efficacious method of assessing abdominal obesity as a component of the MetS (Shuto et al., 2015). Accumulation of adipose tissue in the visceral and hepatic tissues has been shown to be associated with hyperinsulinemia, decreased β -cell function, activation of the macrophages, increased levels inflammatory factors, such as NF- κ B, TNF- α (Le et al., 2011), C-reactive protein (CRP), and IL-6 pathways in conjunction with a decrease in anti-inflammatory adiponectin leading toward a low-grade chronic inflammatory process in obese individuals (Karelis et al., 2004). The MetS is usually assessed by measuring abdominal circumference. Researchers found that a greater waist circumference was strongly associated with total abdominal fat and that abdominal subcutaneous fat and was mildly associated with visceral fat for both men and women (Grundy et al., 2013). Furthermore, it is known that as visceral fat increases, total abdominal fat increases as well, both which were also associated with an increase in serum triglycerides in both men and women (Grundy et al., 2013). Finally, there is a positive association between the homeostatic model assessment and insulin resistance (HOMA-IR) score and visceral fat (Grundy et al., 2013) suggesting a greater risk for insulin resistance and diabetes development due to visceral fat accumulation.

The MERLOT study (Nakao et al., 2012) sampled from a cohort of 25,255 Japanese subjects revealed that the intra-abdominal fat area (visceral fat), not just waist circumference, is an independent predictor for the onset of the MetS, even in individuals who were non-obese at the time. This study emphasizes that an individual may have a

normal weight or BMI but metabolically display adverse biological features that predispose the individual to the MetS. Ectopic fat accumulation in the liver and visceral areas appears to exacerbate the MetS development. The adipocyte is not only a storage site for energy in the form of triglycerides, but also a highly active endocrine organ which helps to moderate satiety through leptin signaling, generating lipoprotein lipases, as well as moderating cytokines, such as adiponectin, visfatin, and CRP, all which are used to communicate to the brain, liver, muscle, vascular system and pancreas (Scherer, 2006). Although humans with homozygous leptin mutations are rare, obese humans with features of MetS are known to have elevated levels of circulating leptin in which these elevated levels cause a dysfunction in leptin signaling and increased leptin resistance (Kelesidis et al., 2010). A review (Scherer, 2006) summarizes the effects of (anti-inflammatory) adiponectin indicating that levels are increased with less fat and improved insulin sensitivity and is often decreased with greater levels of fat, being insulin resistant, having inflammation, CVD, T2DM, as well as lipodystrophy (Scherer, 2006). Obesity is a main component of the MetS and produces detrimental effects through increases in visceral adiposity and an imbalance between pro- and anti-inflammatory cytokines

2.7. Inflammation

Low grade, chronic inflammation, such as the production of TNF- α and NF- κ B as well as increases in CRP, IL-1, IL-6, IL-10, PAI-1, and leptin (Christiana et al., 2016; Kaur, 2014), have shown to be associated symptoms of the MetS. NF- κ B is known to be induced in non-alcoholic fatty liver disease (NAFLD), such as in hepatic steatosis (Milic et al., 2014), as well as in the aortic tissue of obese rats with metabolic syndrome (Janega et al., 2014). Our studies, as well as others, have documented that wild blueberry

consumption decreased inflammation (D. Esposito et al., 2014; Riso et al., 2013; Vendrame, Daugherty, et al., 2014), such as the decrease in TNF- α and NF- κ B expression (Vendrame et al., 2013). Researchers (Welty et al., 2016) found results that suggest an increase in both TNF- α and NF- κ B may contribute to NAFLD and aortic dysfunction, in part due to increased free fatty acid (FFA) excretion and increased TG content deposited ectopically in the liver and as visceral fat resulting in altered blood lipid profile and upregulated inflammatory cytokines. TNF- α has the potential to exacerbate chronic inflammation, influence the development of insulin resistance, contributing to the metabolic syndrome (Gustafson, 2010). Obese Zucker rats have been shown to display chronic stress and inflammation which lead to the development of mild fibrotic tissue (expansion of the extracellular matrix and collagen content) and cytokines (TGF- β 1) in the liver (Cipriani et al., 2010; Deushi et al., 2007; Toblli et al., 2008), fibrotic tissue in the aorta (expansion of the extracellular matrix, increased collagen content, and increased mechanical stiffness) and cytokines (increased fibronectin, collagen type IV- α ₃, and TGF- β 1) (Kovanecz et al., 2009; Sista et al., 2005) of rodents exhibiting the presence of the MetS.

The studies in our lab have produced results that indicate NF- κ B was down-regulated in both liver and adipose tissue after obese Zucker rats consumed a diet with a blueberry diet for eight weeks (Vendrame et al., 2013) displaying the anti-inflammatory properties. On the contrary, activation of NF- κ B in endothelial cells has been shown to result in an upregulation of VCAM-1 and monocyte cell adhesion suggesting an increased risk for inflammation and atherosclerosis development (Kawakami et al., 2006) displaying morphological alterations to the aortic matrix. NF- κ B has been identified as a chronic

inflammatory transcription factor (Horrillo et al., 2010) which has been demonstrated to heighten leukocyte recruitment, stimulate MCP-1 differentiation from monocytes to macrophages, down-regulate PPAR activity, and impair insulin receptor substrate (IRS)-1 signaling (Terra et al., 2011; Verhagen et al., 2011), leading to chronic hyperglycemia and endothelial dysfunction.

2.8. Dyslipidemia

As mentioned earlier, the ATP III Guidelines (National Cholesterol Education Program Expert Panel on Detection et al., 2002) defines dyslipidemia in the MetS as elevated serum TG with low levels of HDL-C, although additional categories, such as the presence of sdLDL-C, elevated apolipoprotein B with a decrease adiponectin, have been described (Scott M Grundy, 2011).

Elevated triglycerides are another risk factor for the MetS and are commonly associated with fatty liver, obesity, and dyslipidemia. Elevated triglycerides and larger waist circumference are often referred to as the hypertriglyceridemic-waist phenotype.

Scientists examining a cohort of Puerto Rican subjects (Diaz-Santana et al., 2016) reported that the hypertriglyceridemic-waist phenotype placed a three-fold odds of males and an eight-fold odds of women to acquire pre-diabetes. Results from a European cohort (Arsenault et al., 2010) showed that, when compared to subjects with normal serum TG and waist circumferences, the hypertriglyceridemic-waist phenotype group had greater levels of apolipoprotein B, smaller LDL-C, higher blood pressure, greater levels of CPR, and lower levels of HDL-C and apolipoprotein A-I. An article (Grundy et al., 2013) based upon the Dallas Heart Study (Victor et al., 2004) found results that portrayed a higher visceral fat to abdominal subcutaneous fat ratio was associated with higher plasma

TG compared to subjects with lower ratios. The data suggests various forms of dyslipidemia increases the risk for acquiring the MetS.

2.9. Vascular dysfunction

Atherosclerotic disease progresses silently and may be caused by high levels of oxidation and inflammation. The OZR has been studied to develop fibrocellular intimal lesions in the aorta (Haudenschield et al., 1981) which corresponds to the development of atherogenic lesions formed in the arterial wall of humans (Wick et al., 2012). Endothelial vascular dysfunction related to metabolic stress in obese rats (El-Bassossy et al., 2014; Justo et al., 2013; Vendrame, Daugherty, et al., 2014) as well as oxidative stress are known symptoms to occur in obesity and have been suggested to predispose people to vascular dysfunction and the MetS (Giugliano et al., 2006). Zucker Diabetic Fatty rats display greater basal oxidative stress, hyperglycemia, poor vasodilation response to blood flow, and elevated mean blood pressure compared to lean rodents (Belin de Chantemele et al., 2009). Furthermore, the same study showed that lean Zucker rats with ‘high-flow’ arteries and obese diabetic Zuckers with ‘normal-flow’ arteries displayed significant increases in medial cross-section areas. Fibrocellular formation by the accumulation of collagen, fibronectin, and other cellular remodeling components are thought to be caused by chronic low grade chronic inflammation (Intengan et al., 2001) due to activation of NF- κ B from primary inflammatory factors, such as TNF- α , internal environmental factors, such as hyperglycemia, oxidant stress, hypoxia, as well as shear stress from a hypertensive state (De Martin et al., 2000). NF- κ B is highly active in arterial smooth muscle cells and may contribute to endothelial dysfunction resulting from dyslipidemia, advanced glycation end products (AGEs), hyperglycemia, free radicals, adhesion

molecules, as well as the conversion of LDL to oxidized LDL by monocytes (De Martin et al., 2000). Medial hypertrophic remodeling, due to chronic hypertension and inflammation, is also thought to be associated with collagenous formation in the vasculature (Intengan et al., 2001) displaying structural change of cellular matrix of the vascular wall in the MetS (Hayden et al., 2005).

2.10. Insulin resistance

Another component of the Mets syndrome is insulin resistance which is shown to be dysregulated in MetS patients in the form of hyperglycemia. Insulin resistance can be defined as having an impaired fasting glucose (IFG) of 100-125 mg/dL or impaired glucose tolerance (IGT) defined by 140-199 mg/dL after a 75-g two-hour oral glucose tolerance test (American Diabetes, 2015; Genuth et al., 2003).

Insulin itself is an anabolic hormone produced by β -cells of the pancreas (Islets of Langerhans) which facilitate the absorption of blood glucose into the body's cells using twelve monosaccharide GLUT transporters; GLUT 4 is the main glucose transported responsible for depositing glucose into adipose tissue, skeletal muscle, liver, and cardiac muscle, while GLUT-2 mainly functions to deposit hepatic glucose (Dods et al., 2013). Insulin also initiates glycogenesis and synthesis of protein and triglycerides while inhibiting lipolysis as well as glycogenolysis and gluconeogenesis in the liver (Dods et al., 2013; Sesti, 2006). When insulin homeostasis has been disrupted, insulin resistance develops, which is defined as poor glucose deposition into tissue cells due to a state of insufficient responsiveness of tissues to normal concentrations of insulin; if not addressed, this state may develop into type 2 diabetes mellitus (Sesti, 2006). One hypothesis speculated that the cascade of steps toward insulin resistance is the result of

increased levels of TG storage seen in obesity due to greater amounts of free fatty acid release into the blood stream, such as seen in a fatty liver (NAFLD). This in turn may results in an abundance of FFAs to be deposited into tissues and organs ectopically causing oversaturation as well as lipotoxicity due to the oxidation of excess FFA when left non-metabolized (Randle et al., 1963; Unger et al., 2000); this environment may also result in insulin resistance (Sesti, 2006). Recent AACE/ACE Guidelines (Handelsman et al., 2015) for diabetes further supports the idea that insulin resistance can exist as an independent factor of obesity although excess weight gain, specifically from visceral or ectopic accumulations, may exacerbate insulin resistance and other comorbidities, such as NAFLD, hypertension, T2DM, and CVD.

Insulin resistance is associated with altered biomarkers which are still being identified today. Scientists studying a cohort of 1628 northwestern Chinese subjects (768 men and 869 women) found a significant association between insulin resistance, decreased adiponectin levels, elevated IL-6 and CRP, and increased HOMA-IR score with the development of the MetS (Ding et al., 2015). In a normal physiological setting, adiponectin is associated with balanced insulin production and normal glycogen release from the liver; higher levels of adiponectin are known to be associated with improved insulin action and sensitivity (Scherer, 2006). This trend continues to support the idea that elevated glycemia in the MetS, regardless of central obesity, is a significant risk factor and gateway toward the development of T2DM (Sakashita et al., 2015). Oxidative stress induced by hyperglycemia appears to play a role in the development of T2DM (King et al., 2004). Activation of NF- κ B induced by a TNF- α and a hyperglycemic environment is observed in cell cultures (Yerneni et al., 1999) and has been suggested to

play role in the pathogenesis of T2DM due to the development of advanced glycation end products (AGEs) (Patel et al., 2009). Serum TNF- α , as well as IL-6 and IL-18, have also been shown to be elevated in hyperglycemic subjects versus control subjects (K. Esposito et al., 2002). In T2DM subjects all inflammatory scores (including TNF- α , MCP-1, and IL-6) were significantly higher when compared to normal glucose tolerant subjects; these scores were also positively correlated with elevated fasting glucose and hemoglobin A1c (HbA1c) levels (Daniele et al., 2014). Furthermore, those with T2DM and obesity will often display symptoms of impaired endothelium-dependent vasodilation which may reduce the blood flow to organs in extremities (Steinberg et al., 1996) suggesting vascular dysfunction.

The obese diabetic Zucker rat displayed a decrease in insulin resistance as well as an increase in glucose transporter-2 (GLUT-2) expression after the rodents consumed a diet enhanced with cocoa extract, which is known to contain phenolic compounds (Cordero-Herrera et al., 2015). GLUT-2 receptors are known to regulate intra and extracellular glucose equilibrium in the liver which play an important role in insulin sensitivity (Klover et al., 2004).

2.11. The obese Zucker Rat, a model of the metabolic syndrome

The Obese Zucker rat is an acceptable model for of the metabolic syndrome which will be described in detail in the following section. The Obese Zucker rat developed a recessive mutation in the 13M strain deemed the *fa* gene. Rodents carrying two genes (*fa/fa*) will display an enlargement in body fat at five weeks of age and rodents carrying the *Fa/fa* or *Fa/Fa* alleles will display normal characteristics of a lean rodent (T. F. Zucker et al., 1962). An average weight of a OZR can vary from 800g (male) and 620g

(female) and the lean litter mates as 480g (male) and 295g (female) (T. F. Zucker et al., 1962). A mutation in tissue leptin receptors (Aleixandre de Artinano et al., 2009), also known as leptin receptor deficiency, plus an up-regulation in ghrelin synthesis (Beck et al., 2004) cause a hyperphagic response, reduced ability to reach satiety, and excessive weight gain. Obese Zucker rats that develop steatosis also display mitochondrial dysfunction with low glutathione levels decreasing its ability to defend against oxidative agents (Serkova et al., 2006). The Progression of obesity in the OZR exhibits symptoms of increased systolic pressure compared to the lean Zucker rat (Kava R, 1990), insulin resistance, mild hyperglycemia and hyperinsulinemia (Aleixandre de Artinano et al., 2009; Kucera et al., 2014). Obese Zucker rats also display an increased absorption and transportation of lipids in the intestine compared to the LZR (Anzai et al., 2009). The OZR has been studied to display an overproduction of VLDL enriched with TGs up to seven times higher compared to the LZR due to hypersecretion and defective catabolism of chylomicrons (Schonfeld et al., 1974). The OZR has also shown to have decreased clearance rates of serum triglyceride and greater defective clearance rates of cholesteryl esters compared to the LZR in the chylomicron injected group (Redgrave, 1977). Furthermore, obese Zucker rats have greater liver triglyceride concentration than their lean littermates in both male and female models (Azain et al., 1985; C. A. Daubioul et al., 2000; Kasim et al., 1992; Mezei et al., 2003; Peluso et al., 2000; Raju et al., 2006; Ran et al., 2004; Redgrave, 1977).

The OZR displays symptoms of pre-diabetes (insulin resistance) in a progressing manner. At two weeks of age serum immunoreactive insulin is normal as the rat is at the beginning stages of acquiring excess fat depots. The IRI rises to a peak of 400 μ U/ml at

15 weeks (hyperinsulinemia) and subsequently declines to 200 μ U/ml in older 35-week rats (L. M. Zucker et al., 1972) displaying a decrease in insulin production. The ORZ displays a loss of glucose tolerance at 10 weeks of age, a compensatory significant increase of β -cell density until 19 weeks, and finally a significant decrease in β -cell density toward 30 weeks, although they do not develop T2DM (Augstein et al., 2009). Another study produced results that indicated a slower glucose clearance rate at 20 weeks when comparing the OZR to the LZR as well as a significantly poorer insulin response in the OZR (L. M. Zucker et al., 1972).

Hepatocytes isolated from the OZR display greater glycolysis activity than those from the LZR as evidenced by the OZR hepatocytes accumulating greater levels of pyruvate and lactate (higher ratio) in conjunction with a greater degree of net glucose utilization (glycogen used versus glycogen produced) (McCune et al., 1981). From week 6 to week 30, the OZR displays an increase in body weight, blood glucose, plasma insulin, oral glucose tolerance test, triglycerides, free fatty acids and cholesterol (Augstein et al., 2009). Liver steatosis is a common characteristic of the OZR (Kucera et al., 2014; Serkova et al., 2006) due to lipid-engrossed livers along with elevated fatty acid and triglyceride synthesis (Fukuda et al., 1982) leading to hypertrophic characteristics of adipose tissue (Kava R, 1990). The rodents also display hyperlipidemia as well as hypertriglyceridemia containing 10 times as much total fatty acids and four times as much cholesterol and lipid phosphorus with a “distinct milky appearance” of the blood serum as well as enlarged fatty livers compared to the lean litter mates (T. F. Zucker et al., 1962). It has been documented that the hepatocytes from the OZR synthesizes fatty acids 2.5-fold greater than the lean counterpart (McCune et al., 1981).

Impaired endothelium function with elevated blood pressure are apparent in the Diabetic Zucker rat and Obese Zucker rat strains (Belin de Chantemele et al., 2009; Bouvet et al., 2007) with decreased luminal enlargement in response to blood flow in the OZR.

Constriction response can partially be restored after OZR consume a wild blueberry enriched diet (Vendrame, Kristo, et al., 2014). Lean Zucker rats also display medial enlargement but not in the OZR possibly due to preexisting acquired hypertrophy in the OZR (Belin de Chantemele et al., 2009; Bouvet et al., 2007).

The OZR begins to exhibit hyperphagia in 17 days of life (Truett et al., 1991) which later leads to hepatic lipid content of approximately 40% at 14 weeks of age (Kava R, 1990) with a serum triglyceride level 4.8 times higher than the lean litter mates (Fukuda et al., 1982). Scientists discovered that the OZR on normal protein diet of casein (15% of energy) had a 2.5 times greater level of hepatic lipids and liver-body weight ratios with steatosis compared to the lean littermate on a normal protein diet (Wojcik et al., 2016). Liver steatosis in the OZR has been cited to first appear approximately eight weeks of age (Serkova et al., 2006). Advanced forms of NAFLD may lead to inflammatory non-alcoholic steatohepatitis (NASH), fibrosis, and cirrhosis (Rector et al., 2008).

2.12. Wild blueberries

A healthy balanced diet, such as the Mediterranean diet, is historically known to reduce levels of disease, such as CVD (Menotti et al., 1999; Wirfalt et al., 2001) and has also shown to produce significant improvements of MetS components including waist circumference, HDL-C, TGs, systolic and diastolic pressure, decrease in fasting glucose levels in adult populations (Kastorini et al., 2011). Similar improvements have been observed in children and adolescents with MetS including TGs, glucose, total cholesterol,

HDL-C, and LDL-C (Velazquez-Lopez et al., 2014). The diet consists of greater levels of fruits, vegetables, whole grains, legumes, low fat dairy, low fat meats and legumes as well as high in fiber and low in refined foods. Consuming whole wild blueberries is one way to incorporate antioxidants into the diet, such as anthocyanins, a subcategory of flavonoids (Zheng et al., 2003). Access to this fruit is widely available as Maine is the largest producer of low bush wild blueberries in North America (Yarborough, 2015). With the availability of blueberries in fresh, frozen, or dried form, this fruit is now more easily integrated into the western diet. Wild blueberries contain a class of phenolic compounds, known as anthocyanins, which have been studied to provide beneficial antioxidant activity in the body. Consumption of blueberries may improve the response to oxidative stress and attenuate symptoms of the MetS (Basu et al., 2012). Anthocyanins have been shown to decrease the risk of CVD, contain anti-inflammatory and anti-carcinogenic activity, as well as improve symptoms of obesity and insulin resistance (He et al., 2010; Stull et al., 2010).

2.13. Wild blueberries and glucose metabolism

The consumption of wild blueberries may also provide an alternative for moderating glucose metabolism in humans as well as rodents. Studies in our lab demonstrated that OZR consuming a WB diet displayed a decrease in resistin, which is known to increase glucose production in the liver, plus an increase in the RBP4 mRNA (Vendrame et al., 2015), which is known to be elevated in insulin resistant individuals (Cho et al., 2006), as well as an increase in PPAR- γ (Vendrame, Daugherty, et al., 2014), a nuclear receptor which moderates glucose metabolism by promoting GLUT-4 expression in insulin sensitive tissues (Armoni et al., 2007).

Wild blueberry extract has also been noted to exhibit anti-hyperglycemic effects in other animal models as well. Scientists from one study (Grace et al., 2009) demonstrated that mice who consumed anthocyanin enriched fraction with water (prepared from whole wild blueberries) displayed a greater hypoglycemic effect (a significant 51% decrease in blood glucose) when compared to the phenolic fraction with water (33% decrease in blood glucose) and metformin (32% decrease in blood glucose).

In humans, scientists who performed a randomized, cross-over controlled trial of adult males and females found that after a mixed berry purée, serum glucose concentrations at 15 and 30 minutes post-consumption were significantly lower than the control meal (Torronen et al., 2010).

Glucose transporter-2 expression was improved in the ileum, duodenum, and jejunum of Sprague-Dawley rats after consuming a high fat diet with chlorogenic acid, a phenolic compound found in blueberries, suggesting the improvement of glucose homeostasis in the intestines (China, 2015).

Literature appears to support that the consumption of whole blueberry and blueberry extracts improves glucose metabolism in both humans and rodents.

2.14. Wild blueberries and non-alcoholic fatty liver disease

Non-alcoholic fatty liver disease (NAFLD), including simple steatosis, is “considered the hepatic manifestation of the Metabolic Syndrome” (Marra et al., 2008). Scientists found that after the OZR consumed a wild blueberry diet, a decrease in fatty acid synthesis in the liver and abdominal adipose tissue was found (Vendrame, Daugherty, et al., 2014) indicating the potential for a lesser degree of steatosis. Steatosis develops when the rate of fatty acid intake and synthesis exceeds the rate of output and oxidation for energy

(Fabbrini et al., 2010). A fatty liver, most often caused by an increased deposition of TG in the hepatocytes, is considered to be the most common morphological alteration leading to an increased risk of damage from endotoxins, free radicals and hypoxia (Kuntz et al., 2008). After OZR consumed a diet rich in high-bush blueberries, a reduction in abdominal fat was observed whereas blueberry intake in lean Zucker rats (LZR) displayed an increase in total body weight (Seymour et al., 2011). Furthermore, the same study showed that OZR displayed a decrease in plasma triglycerides, improved insulin sensitivity, improvements in GLUT-4 and insulin receptor substrate-1 expression, both which are associated with glucose metabolism displaying improved liver function and insulin sensitivity due to blueberry consumption. A study in our lab (Vendrame et al., 2013) documented that a wild blueberry diet decreases plasma levels of pro-inflammatory NF- κ B, TNF- α , IL-6, and CRP, while increasing anti-inflammatory adiponectin in liver and abdominal adipose tissue of the OZR.

Blueberry juice has been shown to reduce hepatic fibrosis as evidenced morphologically by thinner fibrous septa, a decrease in the fatty degeneration in the hepatic tissue, a decrease in fibrous bridging, as well as biologically with lower serum AST and malondialdehyde levels, a marker of oxidation, and increased levels of metallothionein (MT), a scavenger for ROS, in Sprague Dawley rats (Y. Wang et al., 2013). An *in vitro* study (Liu et al., 2011) suggested that the polyphenolic compounds found in blueberries attenuated the progression of TG synthesis toward a fatty liver in HepG2 cell yielding a maximum TG synthesis suppression value of 59.2%.

2.15. Wild blueberries and inflammation

Wild blueberries have been studied to demonstrate beneficial antioxidant characteristics that may improve inflammation. Scientists studying the antioxidant capacity of berries found that wild blueberries produced one of the highest cellular antioxidant activity (CAA), oxygen radical absorbance capacity (ORAC), and total phenolic content (Wolfe et al., 2008). Sprague-Dawley rats consuming blueberry juice for 8-weeks displayed a significant increase in superoxide dismutase and glutathione in liver indicating an increase in antioxidant activity (Ren et al., 2014). Elevated reactive oxygen (ROS) species may result in increased oxidative damage to fatty acids, DNA, mitochondria, proteins (e.g. LDL-C, HDL-C), production of advanced glycation end products (AGEs), as well as stimulation of chronic inflammatory cascades that may induce NF- κ B as well as TNF- α among many (Allen et al., 2000; Bansal et al.). HUVAC endothelial cells subjected to ROS and then treated with anthocyanins from blueberries, malvidin, malvidin-3-glucoside, and malvidin-3-galactoside showed a decrease in ROS and xanthine oxidase-1 indicating a protective effect against oxidation (W. Huang et al., 2016). An *in vitro* study (D. Esposito et al., 2014) subjected macrophage cells induced with lipopolysaccharide to polyphenols, anthocyanins, and pro-anthocyanidins fractions (derived from wild Maine blueberries) which resulted in an overall reduction of inflammation. Another study found polyphenols extracted from blueberries displayed a decrease in expression of IL-1 β , IL-6 and IL-12 (Cheng et al., 2014) as well as IL-6, TNF- α , foam cell formation (Xie et al., 2011) in lipopolysaccharide induced macrophages. Wild blueberry polyphenol extracts were associated with a decrease in

iNOS, COX2, and NF- κ B translocation in BV2 microglial cells induced with lipopolysaccharide suggesting decreased inflammation (Lau et al., 2009).

2.16. Wild blueberries and insulin resistance

The consumption of wild blueberries may also directly affect the insulin response in cells, rodents, and humans. A study (Martineau et al., 2006) that subjected C2C12 murine skeletal myoblasts cells to extracts from the root, stem, or leaf of blueberries (*V. angustifolium*) displayed a significantly improved glucose transport with and without the presence of insulin after 20 hours of incubation.

A study from our lab (Vendrame et al., 2015) indicated that obese Zucker rats fed a wild blueberries diet (8% w/w) for eight weeks displayed a significant reduction in HbA1c compared to the control group (-20% reduction).

Obese Zucker rats fed a diet containing whole blueberry powder (2% w/w) improved plasma insulin sensitivity as evidenced by a reduced fasting insulin, reduced HOMA-IR score as well as a reduction in glucose area under the curve (AUC) score after administration of a glucose bolus (Seymour et al., 2011).

In a double-blinded, randomized, placebo-controlled clinical study, scientists discovered that after consumption of 45g of whole-blueberry smoothie drink twice per day for six weeks displayed significant improvements in insulin sensitivity when compared to the control group in obese, insulin resistant adult males and females (Stull et al., 2010).

2.17. Wild blueberries and vascular dysfunction

Wild blueberries have been examined and deemed a novel alternative toward enhancing health outcomes by significantly reducing levels of oxidized DNA bases and increasing the resistance from oxidation induced DNA damage after at-risk for cardiovascular

disease male subjects consumed a blueberry drink for six weeks (Riso et al., 2013). Male and female subjects who consumed a diet supplemented with blueberries equivalent to one to two cups per day displayed an improvement in blood pressure (Johnson et al., 2015) as well as vascular function (Johnson et al., 2015; Stull et al., 2015).

As seen in humans, vascular dysfunction is also seen in the OZR. Anthocyanins from wild blueberries may promote an anti-inflammatory effect in OZR (Vendrame et al., 2013; Vendrame, Kristo, et al., 2014) and improve endothelium function in diabetic mice (Liu et al., 2014). Furthermore, the studies in our lab (Vendrame, Daugherty, et al., 2014; Vendrame, Kristo, et al., 2014) showed that OZR who consumed a wild blueberry diet for eight weeks displayed improvements in aortic vasorelaxation (Ach-induced) and constriction (Phe-induced) as well as a greater decrease in iNOS. The biomarker iNOS has been reported to be an indicator of endothelial inflammation which is over-produced in the Zucker rat and mice (Perreault et al., 2001). A decrease in vasoreactivity of iNOS as well as vascular sensitivity of tumor necrosis factor alpha (TNF- α) causes vasoconstriction (endothelin-1 over-production) which may be expressed in obesity.

CHAPTER 3

MATERIALS AND METHODS

3.1. Animal model

Male obese Zucker rats (OZR) and their lean littermates, the lean Zucker rat (LZR), were purchased from Charles River Laboratories, Wilmington, MA. They were placed on either a control diet (C) or a wild blueberry diet (WB) at 8 weeks of age and were provided water *ad libitum*. The rats were housed in individual stainless-steel cages with elevated mesh bottoms in a temperature controlled room at 20 °C with adequate ventilation. A 12-hour daylight and 12-hour darkness wake/sleep cycle was maintained. Cages were cleaned once per week by the Small Animals Facility staff and were checked upon daily to ensure adequate health, food, and water. The experiment was approved by the Animal Care and Use Committee of the University of Maine.

3.2. Animal diets

Wild blueberries were provided as a composite by Wyman's of Maine (Cherryfield, ME) and processed following standard procedures to obtain a freeze-dried powder by FutureCeuticals, Momence, IL. The C-diet was created by combining 691 g/kg of dextrose, 200 g/kg of egg white solids, 69 g/kg of corn oil, 35 g/kg of mineral mixture (AIN-93M), 10 g/kg of vitamin mix, 4 g/kg D-L-methionine, and 2 mg/kg of biotin. The diet was designed to deliver 17% protein, 6% fat, 71% carbohydrate and 403 kcals/100g. The WB diet was prepared using the same ingredients as the C-diet, although 8% weight by weight of dextrose was replaced by an equivalent amount of WB powder w/w. The final macronutrient content for the WB diet provided 17% protein, 6% fat, 68% carbohydrate, 1.5% of fiber, and 0.12% of anthocyanins yielding 393 Kcal/100g of

energy. As previously reported from a study in our lab (Del Bo et al., 2012), 21 anthocyanin profiles were comprised in the wild blueberry powder with Malvidin 3-galactoside and peonidin-3-glucoside representing the greatest concentration at 13%, or 1.6 ± 0.2 mg/100 mg, of the total anthocyanins. The WB diet is equivalent to the daily consumption of two cups of fresh wild blueberries by a human (Vendrame,Zhao, et al., 2014), a realistic portion size for human consumption.

3.3. Harvesting tissue samples

Tissue samples from the Zucker rats were harvested at 16 weeks of age after 8 weeks on the diets. The rodents were anesthetized with CO₂ (95%) and O₂ (5%) for approximately three minutes. Liver samples as well as the upper thoracic artery were harvested. Each tissue sample was preserved using 10% neutral buffered formalin solution as well as snap frozen in liquid nitrogen and stored at -80°C.

3.4. Histological overview and stain analysis

Thirty two liver samples (eight from each group) were utilized from frozen tissue (-80°C) for application of Oil-Red-O (ORO) stain and the Periodic Acid-Schiff (PAS) stain performed by Dahl Chase Pathology Associates, Bangor Maine whereas the hepatic H&E stain was performed at the University of Maine, Animal and Veterinary Sciences. The liver tissue samples were sliced at 6 µm while the aortic tissue was sliced at 4 µm. Twelve aortas (three from each group), previously preserved in paraffin, were utilized for application of the Hematoxylin and Eosin (H&E) stain, Alcian Blue stain, and Sirius Red stain, performed at the University of Maine, Animal and Veterinary Sciences. Both liver and aortic samples were observed under the Olympus BX60 light microscope using the Olympus DP71 camera to capture images.

3.4.1. Hematoxylin & Eosin stain

The Hematoxylin and Eosin (H&E) stain is a double staining technique commonly used in histopathology, cytopathology and in medical diagnostic laboratories. For the purpose of this experiment, the H&E stain was used to assess morphological and structural differences between the rodents in the liver and aortic tissues. The cytoplasm stained pink, the nuclei stained blue, and other tissue structures stained light pink or purple. All tissue was deparaffinized using xylene and hydrated through ethanol graded 100%, 95%, and 70% for 2 minutes each. Slides were rinsed in tap water and then under distilled water. Samples were hydrated in tap water for 2 minutes then placed in Harris' Hematoxylin (ThermoScientific, Waltham, MA) bath for 8 minutes with a final 2-minute tap water rinse. Samples were dipped once in acid alcohol and rinsed under tap water for 5 minutes. Samples were subjected to ammonia water for 1 minute until blue, rinsed in distilled water for 3 minutes, and then placed in Eosin-alcohol bath (ThermoScientific, Waltham, MA) for 3 minutes. Samples were then dehydrated with 95% to 100% alcohol and mounted.

3.4.2. Oil-Red-O stain

The Oil-Red-O (ORO) stain was used to determine the concentration of triglycerides in the hepatic tissue. Triglycerides stained red, the nuclei stained blue and the hepatic tissue stained pink. The protocol was performed by Dahl Chase Pathology Associates, Bangor, Maine. Frozen liver tissue was sliced using a cryostat producing 6 μm thick samples and fixed on 10% neutral buffered formaldehyde Touch Prep slides. Once rinsed in tap water, one drop of Oil-Red O solution (Poly-Scientific Research, Bay Shore, NY) was applied to each slide for 10 minutes and then rinsed with tap water. One drop of Harris

Hematoxylin (StatLab, McKinney, TX) was added to each slide and rinsed under tap water. Ammonia water (2 ml ammonium hydroxide, 40 ml tap water) was used to briefly wash the slides and then were washed in warm tap water for 5 minutes. Slides were then mounted with aqueous mounting medium (Poly-Scientific Research, Bay Shore, NY).

3.4.3. Periodic Acid-Schiff stain

The Periodic Acid-Schiff Stain (PAS) displays the exposed aldehyde sugar groups after oxidation has occurred. In this experiment, the periodic acid produced a dark magenta color for a positive stain compared to lighter pink of the tissue. The PAS stain represents glycogen, neutral mucosubstances and basement membranes. The stain was used to expose glycogen content in hepatocytes of the liver (Saxena, 2010). Once specimens (4 μ m slices mounted on glass slides) were deparaffinized and hydrated, one drop of 0.5% Periodic Acid solution was added to each slide (0.5g Periodic Acid Crystals and 100ml deionized water). Once rinsed under deionized water, the slides were placed in Schiff's Reagent (Sigma-Aldrich Co, LLC) for 15 minutes. The slides were then rinsed in two baths of Sulfurous Acid rinse (6 ml 10% sodium metabisulfite, 5 ml 1N hydrochloric acid, 100 ml deionized water) for 4 minutes each. After 10 minutes of tap water wash over the slides, one drop of Harris Hematoxylin (StatLab, McKinney, TX) was placed on each slide for 30 seconds. Ammonia water (2 ml ammonium hydroxide, 40 ml tap water) was used to briefly wash the slides and then the slides were washed in warm tap water for 5 minutes. Finally, the tissue was dehydrated and mounted with Consulmount™, mounting medium.

3.4.4. Sirius Red stain

The Sirius Red stain was used to identify collagen accumulation and fibrosis in the aortic tissues. Sirius Red stains collagen-I fibers, reticulin fibers, basement membranes and some mucins (Saxena, 2010). In the bright field microscope, the collagen appeared red, the nuclei as black or brown, and the cytoplasm as well as other background structures had a pale yellow or orange appearance. All tissue was deparaffinized using xylene and hydrated through ethanol graded 100%, 95%, and 70% for 2 minutes each, then rinsed in distilled water for two minutes. The NovaUltra™ Sirius Red Stain Kit (IHC World, Ellicott City, MD) was used for the following procedure which contains Weigert's Hematoxylin, Picro-Sirius Red Solution, and Acetic Acid Solution. Samples were placed in 250 ml of Weigert's Hematoxylin Solution for 10 minutes and then washed under tap water for 10 minutes. The samples were then stained in 250 ml of Picro-Sirius Red Solution for 60 minutes and briefly rinsed in tap water. Samples were then washed in 250 ml of Acetic Acid Solution for 1 minute and the water was removed from slides through vigorous shaking. Samples were dehydrated in two changes of 100% alcohol, 5 minutes each, and two changes of xylene, 5 minutes each. Samples were then mounted in a resinous mounting medium.

3.4.5. Alcian Blue stain

Alcian Blue stain is used to identify acid mucins, sulfated and carboxylated mucosubstances and acid mucins that may be present in aortic tissue. Sulfated mucosubstances, such as the target glycoproteins glycosaminoglycans (GAG), appeared blue. Cytoplasm and the background appeared pale pink while the nuclei stained pink to red under a bright field microscope. All tissue was deparaffinized using xylene and

hydrated through ethanol graded 100%, 95%, and 70% for 2 minutes each. Slides were rinsed in tap water and then under distilled water. The samples were then placed in 3% acetic acid for 3 minutes and then stained with Alcian Blue, pH 2.5 stain kit (American MasterTech, Lodi, CA) for 30 minutes and then rinsed in tap water for 1 minute. The samples were counterstained in Nuclear fast red for 5 minutes and rinsed with tap water for 1 minute. Finally, the samples were dehydrated through 95% and 100% ethanol to xylene for 1 minute each and mounted with resinous medium.

3.5. Image analysis overview

Histologic image analysis is a method of extrapolating qualitative and quantitative pictorial information from digital images and is commonly used in histological research. ImageJ 1.5b with Java 1.8.0 update, is an open source program developed by Wayne Rasband from the Research Services Branch, National Institute of Mental Health, Bethesda, Maryland in the 1970's. ImageJ has continued to be refined for over 25 years through the collaboration of the scientific community, NIH, and the original developer (Schneider et al., 2012). This program is loaded with pre-programmed and programmable tool sets that allow the user to freely extract the information they require for two and three dimensional objects. For this experiment, the program was specifically used to quantify the area of four unique stains as well as serve as a medium to interact and view images. Each image was taken at 4x, 10x, and 40x objectives using a resolution of 4080 x 3072 to be saved as high quality TIFF files.

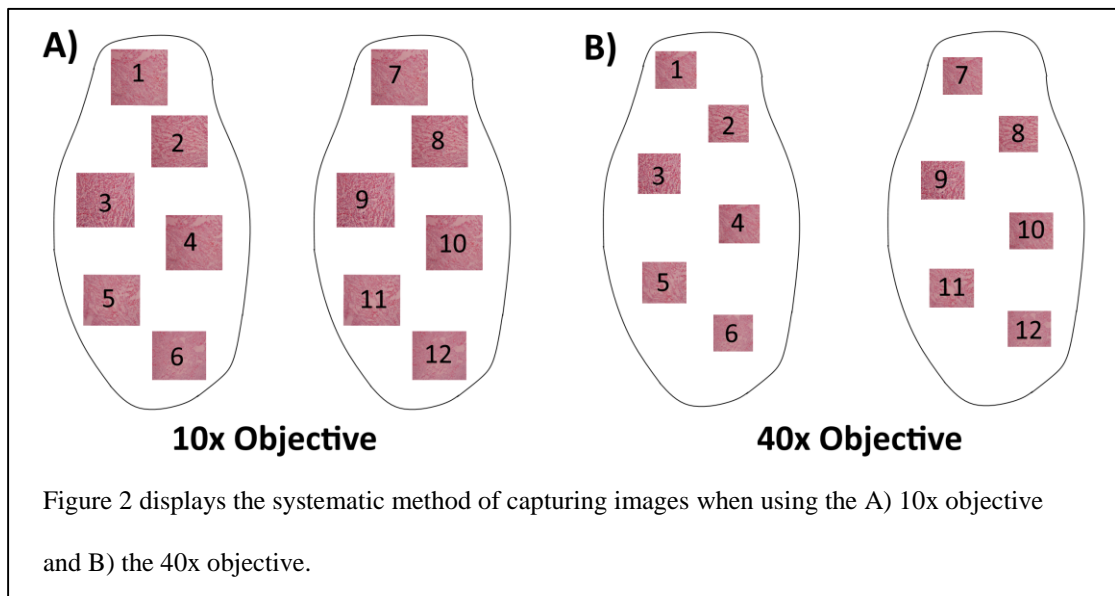
In order to assess the ultrastructure of hepatic and aortic tissue, five unique stains were utilized. The Oil-Red-O stain (hepatic tissue), Hematoxylin and Eosin Stain (aortic and

hepatic tissues), Periodic Acid-Schiff stain (hepatic tissue), Sirius Red stain (aortic tissue), and finally the Alcian blue stain (aortic tissue) were utilized.

3.5.1. Image analysis: hepatic tissue

For each rodent, two liver slices from the same animal were mounted to glass slides and stained with H&E or PAS. For each liver slice (two per animal), 6 images were taken with a total of 12 images each for each rodent. For the 10x and 40x objectives, a systematic method of sampling images was utilized as seen Figure 2. Within a single rodent 6 images (numbers 1-6) were taken from the first liver slice and then 6 more images (numbers 7-12) were taken on the second liver slice.

Figure 2: Method of capturing images from the hepatic tissue

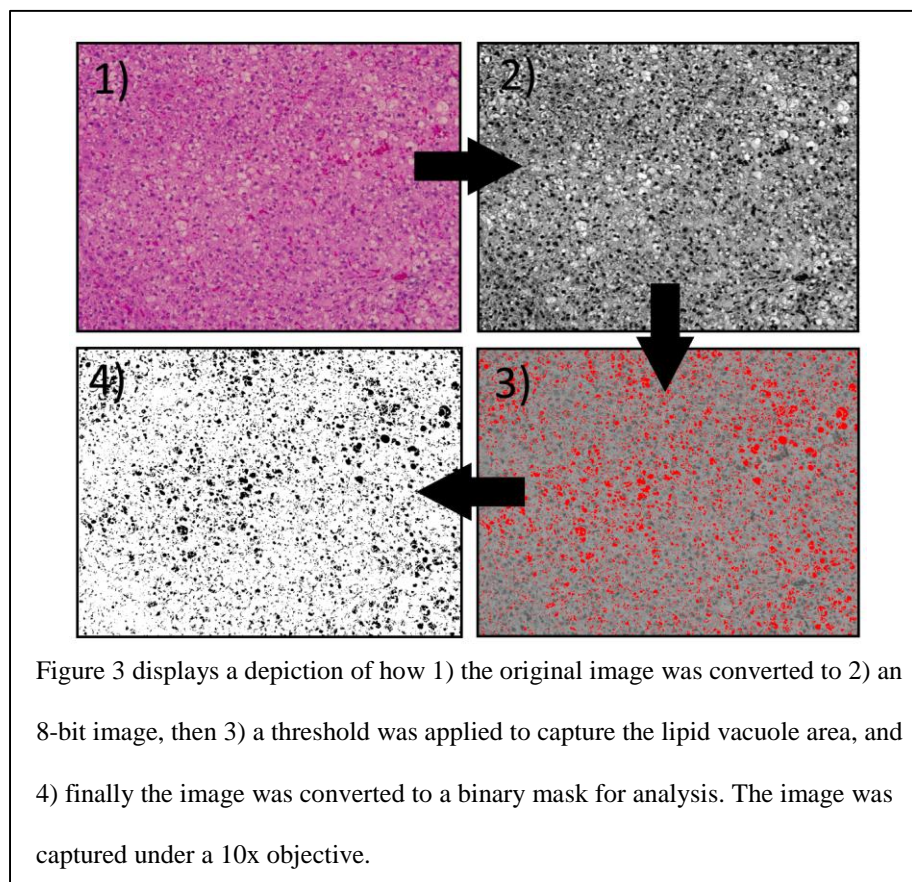


3.5.2. Digital analysis of hepatic lipid content: Hematoxylin and Eosin

To evaluate the H&E-stained hepatic tissue for lipid content, ImageJ first converted the image to an 8-bit image resulting in grey-scale. The contrast was adjusted to 100 (lower) and 190 (upper) to distinctly identify the lipid vacuoles. A threshold was then applied to

the images between 155 to 170 (lower) and 255 (upper). The images were then converted to a “mask” or binary image. The watershed algorithm was performed to separate conjoined lipid droplets. Finally, the images were analyzed for the percentage of area. This process has been visually outlined in Figure 3. To account for sinusoidal space in lean animals, a circularity factor was added to the algorithm during the analysis which excludes objects with a circularity range between 0.0 to 0.2 and included lipid droplets between 0.2 to 1.0. By performing this, the longitudinally shaped sinusoidal spaces and artifacts were excluded from the results. This the circularity function was not performed in the obese rodents since steatosis displaced the sinusoidal space.

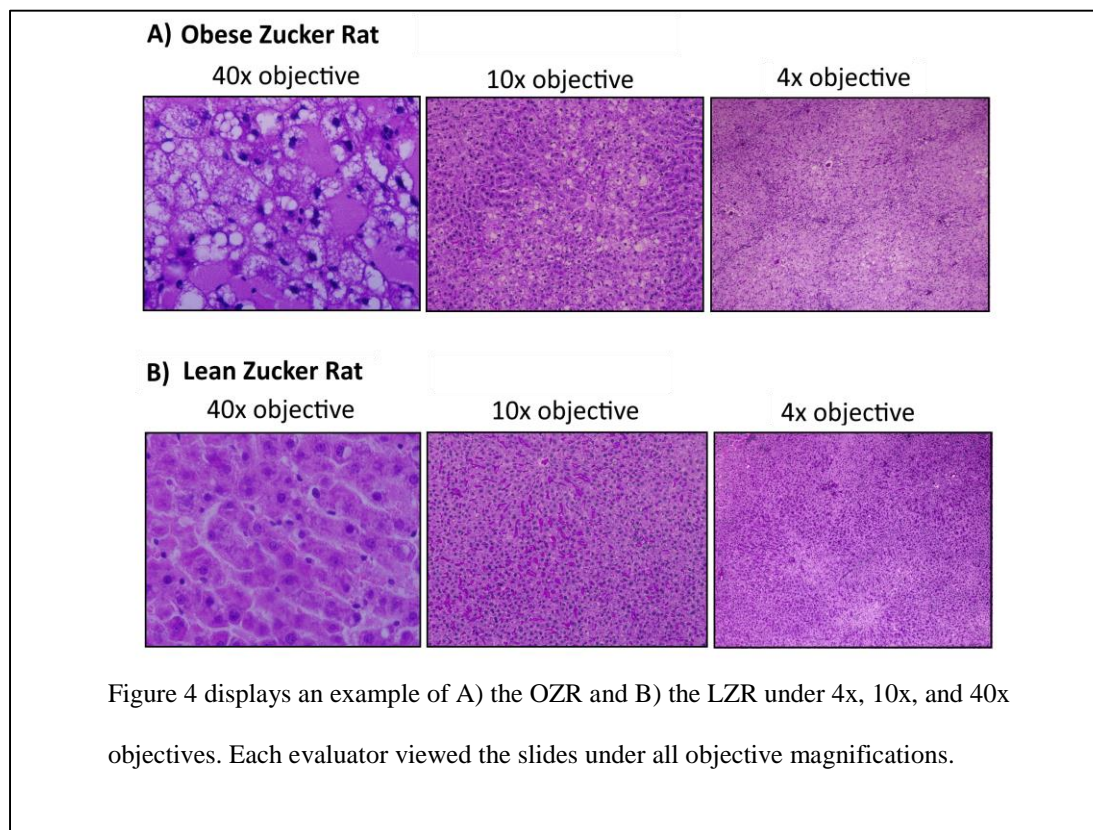
Figure 3: Sample of hepatic lipid assessment using Hematoxylin and Eosin



3.5.3. Histopathological analysis of hepatic lipid content: Hematoxylin and Eosin

The H&E stain was also used to assess for the presence of steatosis evaluated by a pathologist Dr. Jay Ye, Chief of Dahl Chase Pathology Associates, Eastern Maine Medical Center, as well as the author using a blinded randomized procedure. After 32 liver slides (n=8 LZR-C, n=8 LZR-WB, n=8 OZR-C, n=8 OZR-WB) were designated with a randomly generated number from a third party, each evaluator reviewed all 32 slides and used a template which displayed percentage choices from $\leq 5\%$ to 100% in increments of five as the percentage perceived to be lipid. The evaluator would first review all random slides under the microscope using the 4x, 10x, and 40x objectives, as seen in Figure 4 and then proceed to evaluate each individual slide.

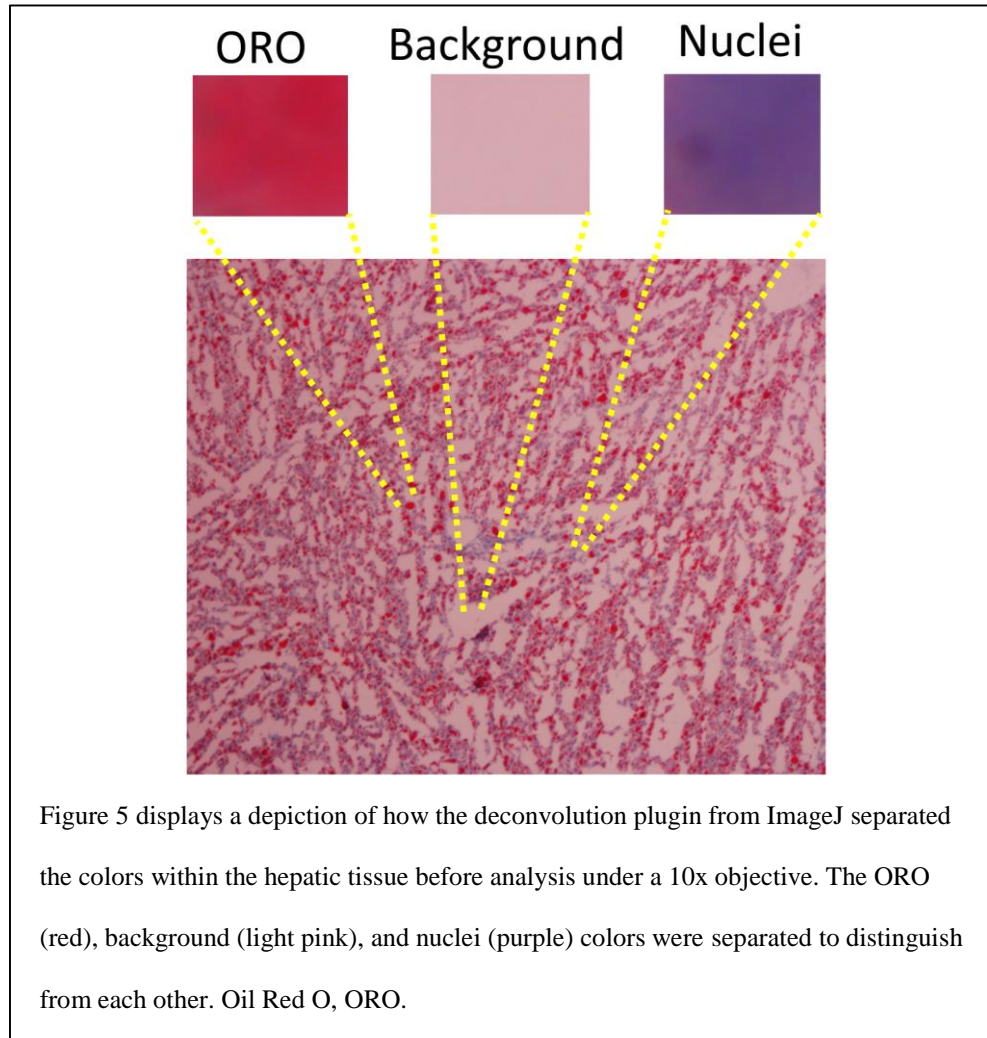
Figure 4: Sample of hepatic lipid content using Hematoxylin and Eosin



3.5.4. Digital analysis of hepatic lipid content: Oil-Red-O

To assess the lipid content in the liver, all 32 rodents were initially analyzed (n=8 LZR-C, n=8 LZR-WB, n=8 OZR-C, n=8 OZR-WB). Both the H&E and ORO stains were used for hepatic assessment of lipid content using ImageJ image analysis. For the ORO stain, each 10x objective image was converted to a 32-bit image. Contrast was adjusted and set between 100 (lower) to 190 (upper) values to enhance the contrast of the red dye. A plugin called “Color Deconvolution”, designed by A.C. Ruifrok, was used to separate image colors using “color subtraction” to yield three distinct vectors. Figure 5 displays a depiction of *color deconvolution* before the analysis. A threshold was applied to the new image (after color deconvolution) which produced a mask over the ORO stain. The images were then converted to a binary image. The watershed algorithm was used to separate lipid droplets if merged in the image. Finally, the percentage stained with ORO was analyzed.

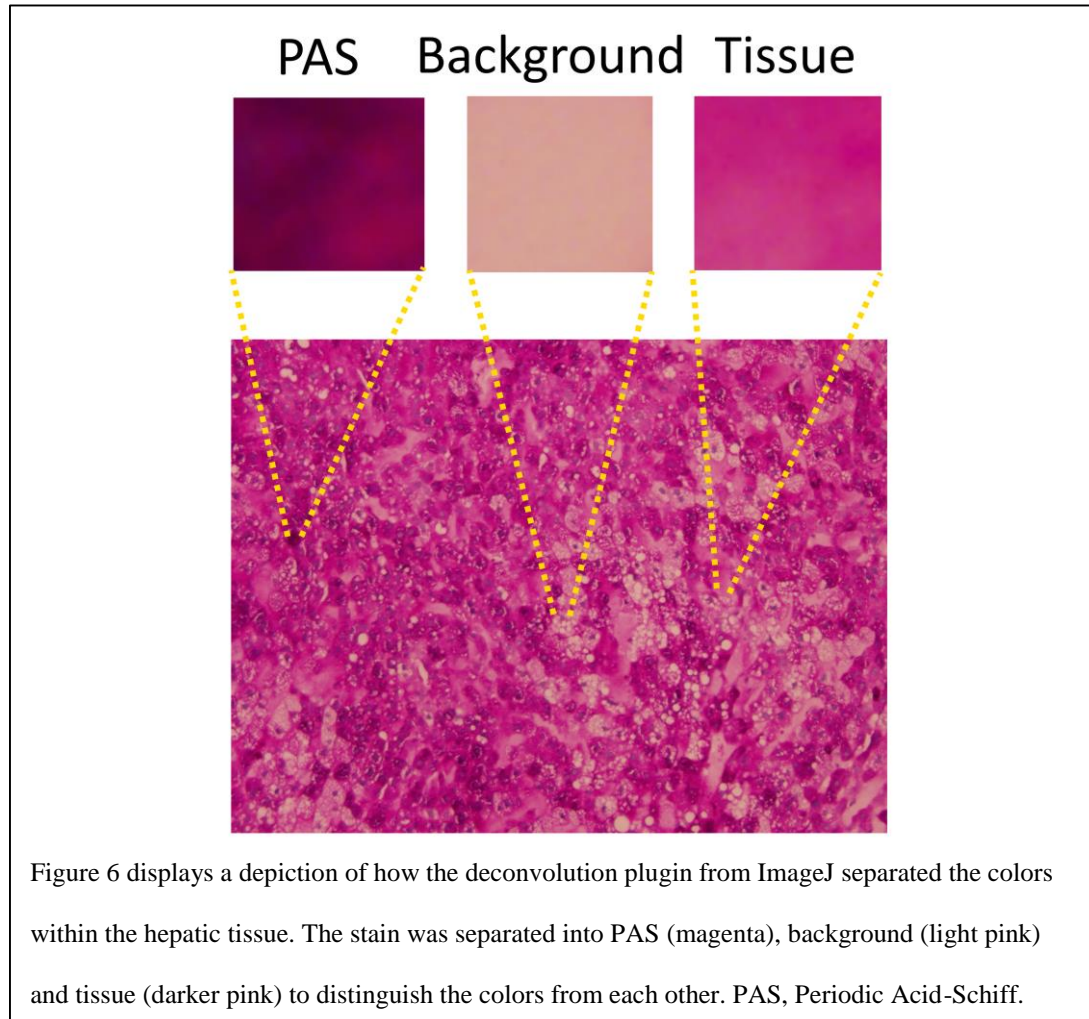
Figure 5: Sample of hepatic lipid Assessment by Oil Red O



3.5.5. Digital analysis of hepatic glycogen: Periodic Acid-Schiff

The Periodic Acid-Schiff (PAS) stain was performed by Dahl Chase Diagnostics Services in Bangor Maine. For this procedure, eight liver tissue samples from each rodent group were sliced using a cryostat at 4 μm . A total of 32 livers from the four groups were stained with PAS (n=8 LZR-C, n=8 LZR-WB, n=8 OZR-C, n=8 OZR-WB). The *color deconvolution* plugin was used in ImageJ to separate three distinct colors into background, tissue, and PAS stain, as seen in Figure 6. Once the threshold was applied to the color designated as PAS, the percentage of area stained was assessed.

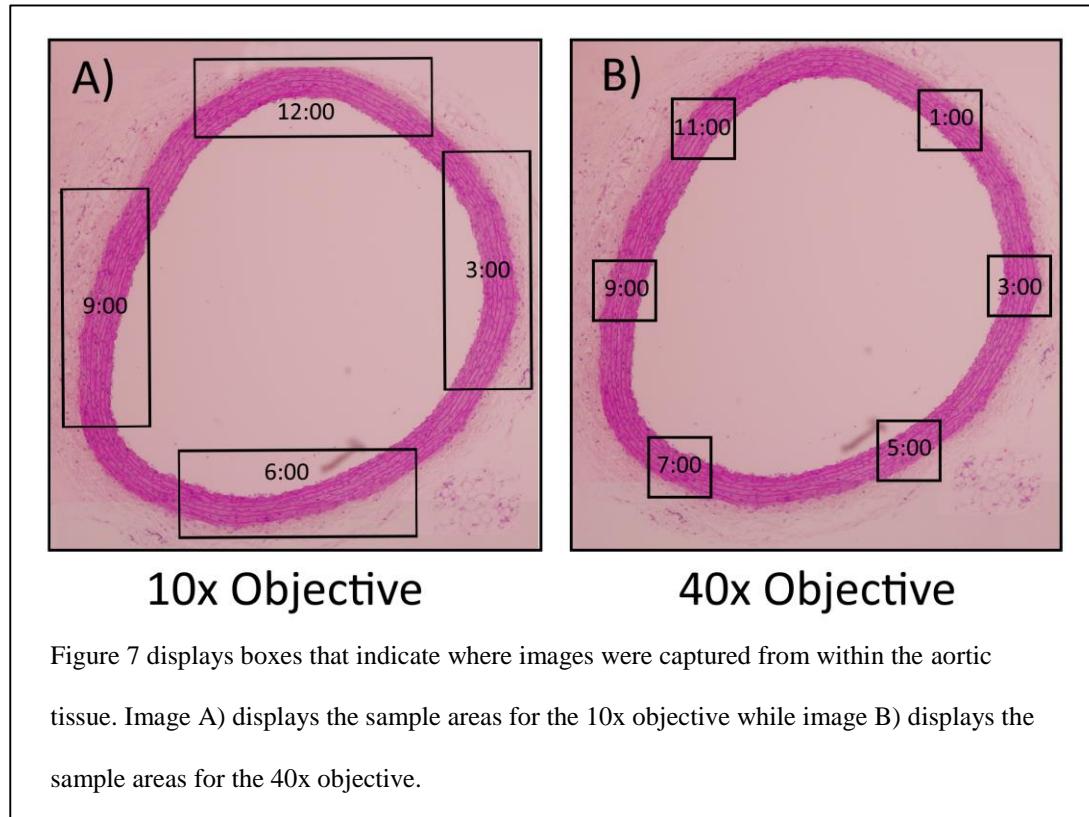
Figure 6: Sample of hepatic glycogen assessment using Periodic Acid-Schiff



3.5.6. Image analysis: aortic tissue

On the 10x objective, the image was divided into four quadrants where each section was taken using a clockwise motion from the 12:00, 3:00, 6:00, and 9:00 positions without overlap of tissue. Using the 40x objective, the same systematic method was used to capture images at the 1:00, 3:00, 5:00, 7:00, 9:00, and 11:00 positions in a clockwise motion to prevent overlapping of tissue sections. Both methods are illustrated in Figure 7. The 10x objective was used to analyze the aortic width while the 40x objective was used to analyze the nuclei count, Sirius Red Stain, and Alcian Blue stain.

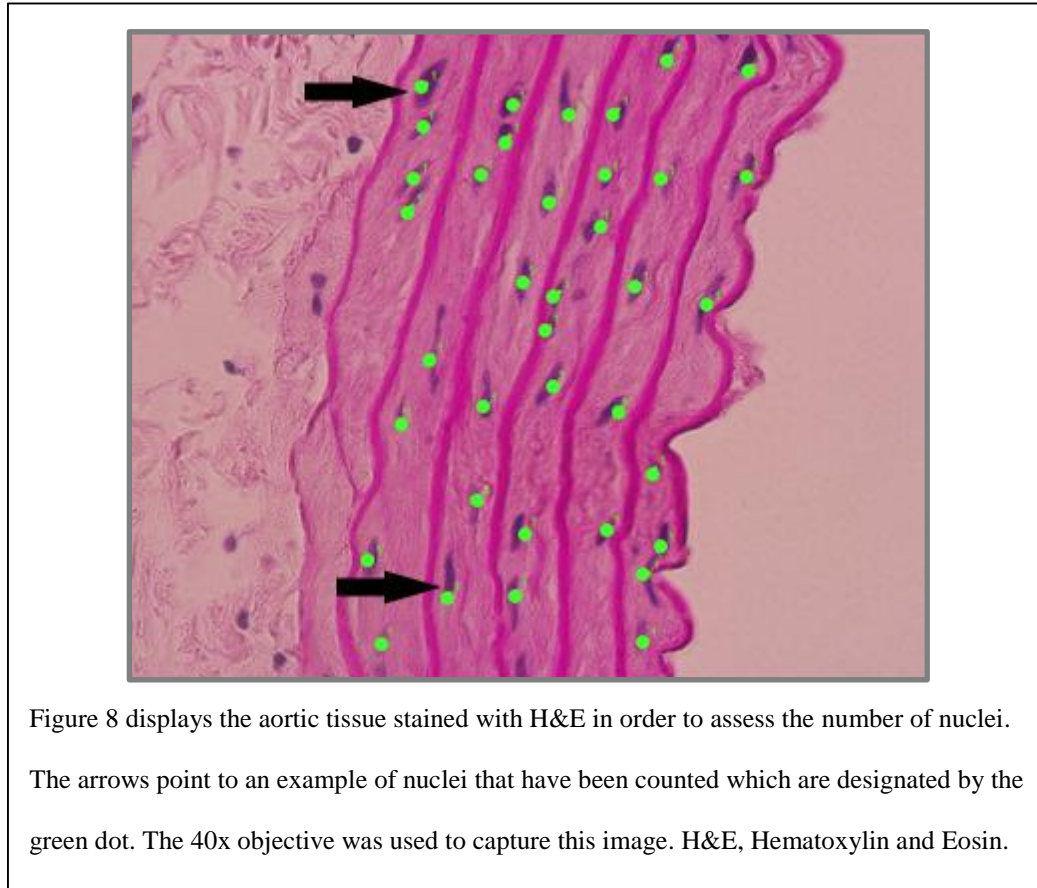
Figure 7: Method of capturing images from the aortic tissue



3.5.7. Digital analysis of aortic nuclei: Hematoxylin and Eosin

To assess the number of nuclei in the aorta (n=3 LZR control, n=3 LZR WB, n= 3 OZR control, n= 3 OZR WB), each rat aorta was viewed upon the 40x objective to capture six images taken from the 1:00, 3:00, 5:00, 7:00, 9:00, and 11:00 positions to avoid overlapping tissue sections for a total of 6 images per rodent. Each image was opened separately on ImageJ as a normal red, green, blue (RGB) file as seen in Figure 8. A plugin called “Cell Counter” designed by Kurt De Vos was used to mark the nuclei in the medial layer. The intimal layer and adventitial layer containing collagen was excluded from the count. Also, if nuclei fell off the border of the image they were excluded from the count.

Figure 8: Sample of nuclei assessment of aortic tissue using Hematoxylin and Eosin

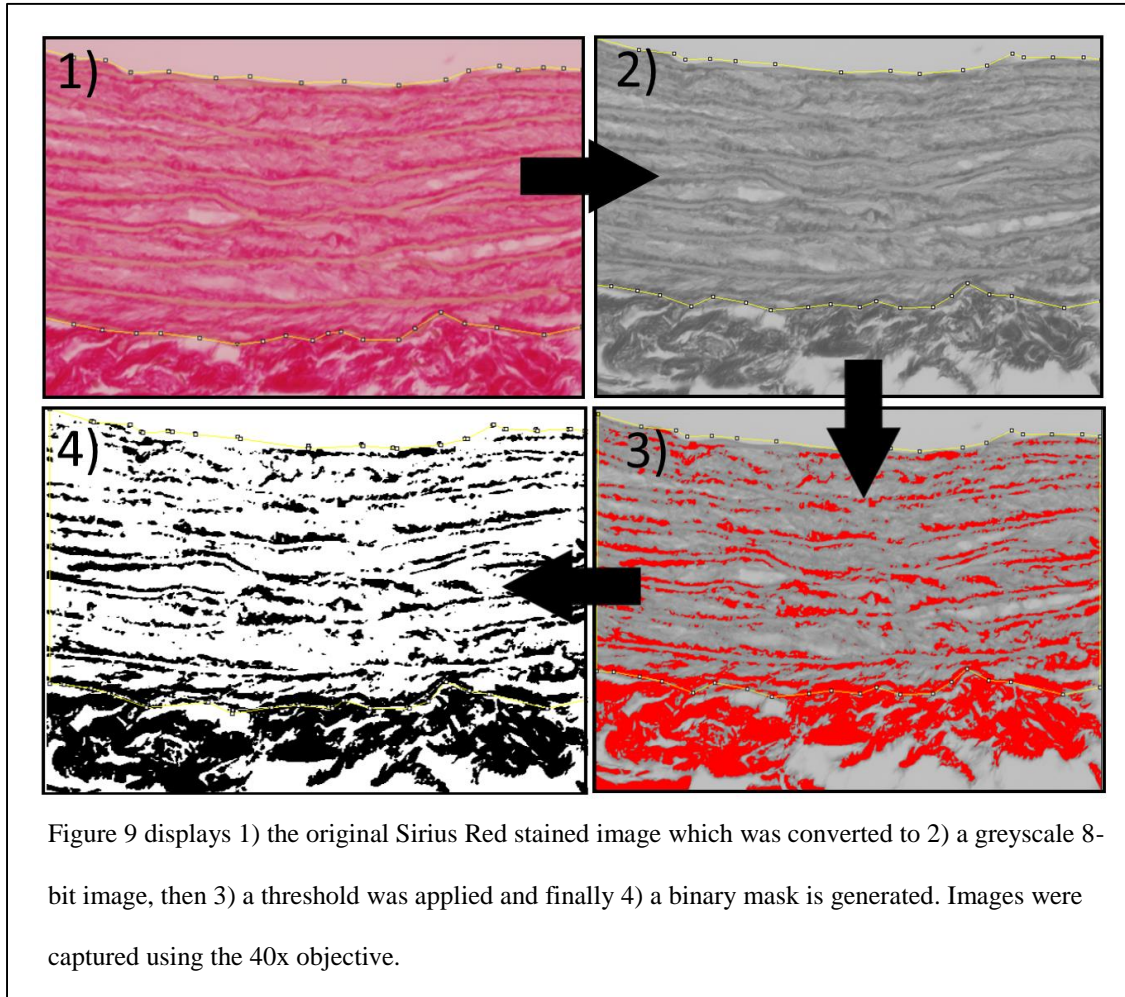


3.5.8. Digital analysis of aortic collagen and connective tissue: Sirius Red

The percentage area of the collagen and connective tissue in the tunica media was assessed using ImageJ software and the Sirius Red stain for all rodents (n= 3 LZR-C, n=3 LZR-WB, n=3 OZR-C, n=3 OZR-WB). Once the images were captured, each image was opened and converted to a red, green, blue (RGB) image. Before applying the threshold mask, the region of interest (ROS) was outline with the polygon tool. This was performed as a RGB image to capture only the area that was to be measured and avoid including the luminal (white) space, intimal layer, and adventitial layer, which is dense with collagenous tissue. Therefore, the total area captured consisted of the tunica media. Once the polygon tool selected the region of interest (ROI), the image was converted to an 8-bit

grey image. A threshold was then applied and adjusted to an appropriate mask depending on the intensity of the Sirius Red dye. The threshold was adjusted by examining the 8-bit color image next to the RGB image to ensure correct representation of the threshold mask over the collagen sections was applied. This process is visually outlined in Figure 9.

Figure 9: Sample of aortic collagen and connective tissue using Sirius Red

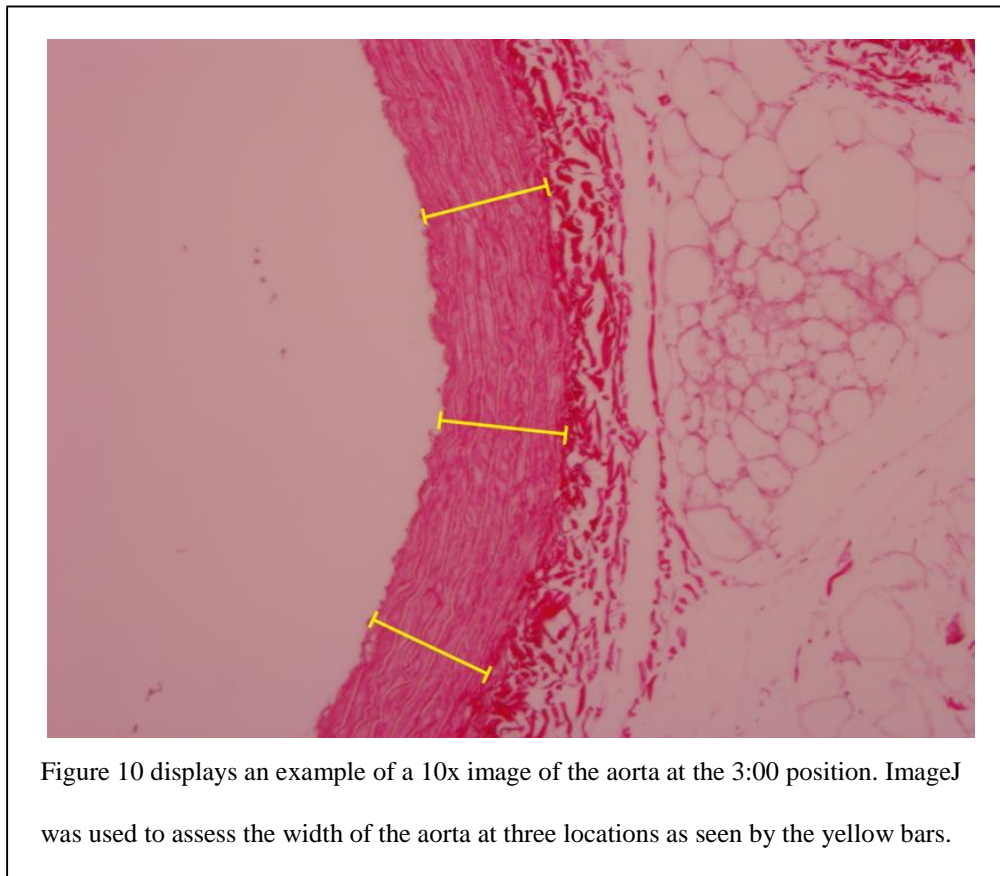


3.5.9. Digital analysis of aortic width: Sirius Red

The Sirius Red stain was used to analyze the tissue morphology of the aortic width for each rodent (n= 3 LZR-C, n=3 LZR-WB, n=3 OZR-C, n=3 OZR-WB). This procedure required the use of 10x objectives which produced four images per aorta, or per rodent.

Each image was opened in ImageJ software and three measurements per aortic image were taken. For each aorta, four images were taken on the 10x objective at the 3:00, 6:00, 9:00, and 12:00 positions. The “line tool” from ImageJ was used to measure the width from the external elastic lamina to the internal elastic lamina of the aorta, as seen in Figure 10. This method excluded the natural presence of collagen in the adventitia.

Figure 10: Width assessment of the tunica media using Sirius Red

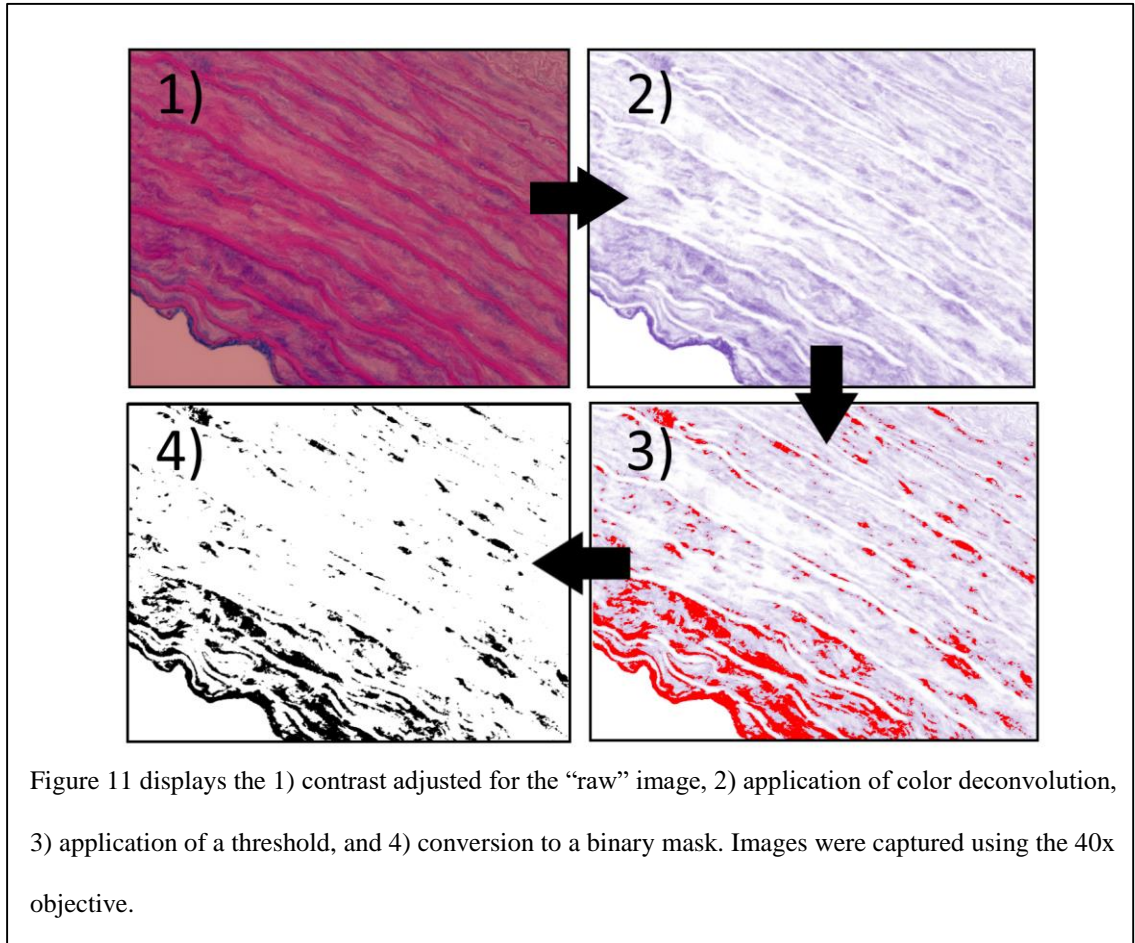


3.5.10. Digital analysis of aortic glycosaminoglycans: Alcian Blue

The Alcian Blue stain was used to analyze the visual presence of glycosaminoglycans in the tunica media for each rodent (n= 3 LZR-C, n=3 LZR-WB, n=3 OZR-C, n=3 OZR-WB). The procedure utilized the 40x objective for visual acuity of the Alcian Blue stain. For each rodent, one aorta slice was sampled and six images were taken of the aortic

medial layer at the 1:00, 3:00, 5:00, 7:00, 9:00 and 11:00 positions. ImageJ was used to adjust the contrast, perform a *color deconvolution*, apply an appropriate threshold, generate a binary mask and finally take measurements of the percent stained with Alcian Blue. This process has been outlined in Figure 11.

Figure 11: Sample of aortic glycosaminoglycans using Alcian Blue



3.6. Biochemical assessment: hepatic triglycerides

Triglycerides were extracted and quantified by the use of a commercially available Triglyceride Colorimetric Assay kit (Cayman Chemical, Item No. 10010303). The kit provided the Standard Diluent Assay Reagent (5X) which was then diluted with HPLC-grade water and powdered EDTA, acting as a protease inhibitor, to achieve the proper

concentration. The Sodium Phosphate Assay Buffer was diluted with HPLC-grade water in a 50 ml centrifuge tube and stored at room temperature. The Triglyceride Enzyme powder was reconstituted with HPLC-grade water in a 15 ml centrifuge tube and then wrapped in aluminum foil to prevent degradation of enzymes. After the liver tissue was thawed, 360 ± 2 mg of each tissue sample was weighed and homogenized with 2 ml of Standard Diluent using a Tenbroeck glass tissue homogenizer. The homogenates were then centrifuged at $10,000 \times g$ for 10 minutes at 4°C . Avoiding the pellet on the bottom, the supernatant was transferred to a separate centrifuge. A vortex mixer aided in dissolving the fatty mixture in the supernatant. To create the Triglyceride Standard, the contents of the Standard Diluent and Triglyceride Enzyme were serially diluted between each of the Eppendorf tubes as directed starting from tube one and ending at tube seven, leaving tube eight as a blank. The supernatants were then diluted with Standard Diluent at a 1:5 ratio for the lean rodents and 1:20 ratio for the obese rodents to account for high fat content; each sample was vortexed again before pipetting. From each diluted supernatant, 10 μl of each animal were pipetted into the 96-well plate in duplicates. The Triglyceride Standard was also pipetted on the 96-well plate in duplicate. The Sodium Phosphate Assay Buffer (enzyme buffer) was added to each well to initiate the reaction. The 96-well plate was mixed using a microtiter plate and then incubated at room temperature for 15 minutes. Finally, the BioTek Synergy 2™ was used to assess absorbance at 540 nm and expressed as optical density. After standardizing the curve, the optical density was used to express the amount of triglycerides as mg of TG per gram of tissue (mg/g).

3.7. Statistical analysis

A two-way Analysis of Variance (ANOVA) was used to compare the diet and the rodent type as the two dependent variables. For the hepatic tissue, the analyses performed included the percentage of total lipid, the concentration of triglycerides, and the percentage of glycogen. The two-way ANOVA was followed by a Tukey Test if significance was found. The Kruskal-Wallis statistical analysis is a conservative test utilized to evaluate data from aortic morphology due to a small sample size. This statistical test evaluated aortic data derived from the number of nuclei, aortic width, percentage of glycosaminoglycans, and the percentage of collagen and connective tissue. The Kruskal-Wallis test was followed by Dunn's test to correct for multiple comparisons. The statistical software, GraphPad Prism 6 (GraphPad Software inc, La Jolla, CA, United States) was used to for the analyses and the results were presented as mean \pm SEM (standard error of the mean). Observed differences were considered statistically significantly if $p \leq 0.05$.

CHAPTER 4

RESULTS

4.1. Animal weight

The averaged, final weight for the obese Zucker rats ($454.95 \pm 11.34\text{g}$) was significantly higher than the weight for the lean rodents ($163.97 \pm 9.94\text{g}$) (Table 1) starting at 8 weeks of age until 16 weeks of age. No statistically significant differences were found between the OZR-C and the OZR-WB groups.

4.2. Food intake

Significantly higher food intake in the obese Zucker rats ($29.80 \pm 1.30 \text{ g/day}$) compared to the lean rodents ($23.45 \pm 1.44 \text{ g/day}$) was observed. Within each rodent type, the food consumption was homogeneous between rodent types (LZR-C versus LZR-WB as well as OZR-C versus OZR-WB) as seen in Table 1.

Table 1: The effect of a wild blueberry diet on the average final weight, weight gain, and food intake in the Zucker rat

| | LZR-C | LZR-WB | OZR-C | OZR-WB |
|---------------------------------|------------------|------------------|-------------------------------|-------------------------------|
| Average Final Weight (g) | 311.3 \pm 18.4 | 302 \pm 18.6 | 447.7 \pm 23.2 [#] | 462.2 \pm 22.1 [*] |
| Average Weight gain (g) | 166.5 \pm 17.5 | 161.4 \pm 22.3 | 276.6 \pm 24.8 [#] | 274.6 \pm 35.0 [*] |
| Average food intake (g) | 23.8 \pm 2.3 | 23.1 \pm 1.9 | 30.2 \pm 2.9 [#] | 29.4 \pm 2.3 [*] |

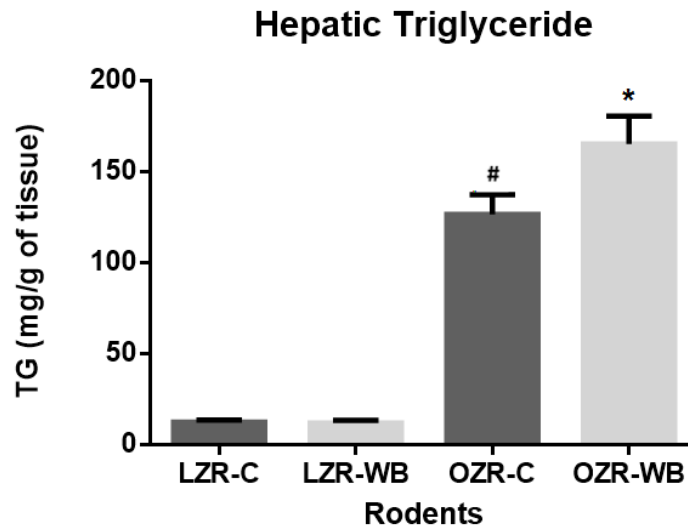
Values are means \pm SEM; LZR-C (n=8), LZR-WB (n=8), OZR-C (n=8), OZR-WB (n=8); statistical significance ($p \leq 0.05$) due to the rodent type consuming the WB-diet^{*} and the C-diet[#]; Abbreviations: LZR, Lean Zucker Rat; OZR, Obese Zucker Rat; C, Control diet; WB, Wild Blueberry diet.

4.3. Hepatic triglyceride concentration in the Zucker rat

As seen in Figure 12 and Table 2, there were significant differences due to rodent type between the LZR-WB versus the OZR-WB as well as the LZR-C versus the OZR-C.

Triglyceride (TG) content did not differ significantly between the LZR-C (12.46 ± 1.08 mg/g) and the LZR-WB (11.84 ± 1.58 mg/g). There was also no significant difference between the OZR-C (116.30 ± 13.53 mg/g) and the OZR-WB (165.20 ± 15.34 mg/g).

Figure 12: The effect of a wild blueberry diet on hepatic triglyceride concentration in the Zucker rat



Values are means \pm SEM; LZR-C (n=8), LZR-WB (n=8), OZR-C (n=5), OZR-WB (n=8); statistical significance ($p \leq 0.05$) due to the rodent type consuming the WB-diet* and the C-diet[#]; LZR, Lean Zucker Rat; OZR, Obese Zucker Rat; C, Control diet; WB, Wild Blueberry diet.

Table 2: The effect of a wild blueberry diet on hepatic triglyceride concentration in the Zucker rat

| | LZR-C | LZR-WB | OZR-C | OZR-WB |
|----------------------|--------------|---------------|-----------------------------|-----------------------------|
| Ave TG (mg/g) | 12.46 ± 1.08 | 11.84 ± 1.58 | 116.30 ± 13.53 [#] | 165.20 ± 15.34 [*] |

Values are means ± SEM; LZR-C (n=8), LZR-WB (n=8), OZR-C (n=5), OZR-WB (n=8); statistical significance ($p \leq 0.05$) due to the rodent type consuming the WB-diet* and the C-diet[#]; LZR, Lean Zucker Rat; OZR, Obese Zucker Rat; C, Control diet; WB, Wild Blueberry diet.

4.4. Percentage of hepatic triglyceride using Oil-Red O in the Zucker rat

Figure 13 displays an image analysis example of hepatic TG content using the ORO stain. Figure 14 and Table 3 show significant differences for the average percentage of lipid area between the LZR-WB (0.90 ± 0.49 %) versus the OZR-WB (20.58 ± 3.33 %) as well as the LZR-C (1.23 ± 0.56 %) versus the OZR-C (23.68 ± 1.97 %). Although no significant differences were observed, there appeared to be a trend for lower total lipid percentage in the OZR-WB compared to the OZR-C as well as in the LZR-WB compared to the LZR-C. Results were based upon analysis using a 10x objective.

Figure 13: Hepatic tissue stained with Oil-Red-O to display total triglycerides in the Zucker rat

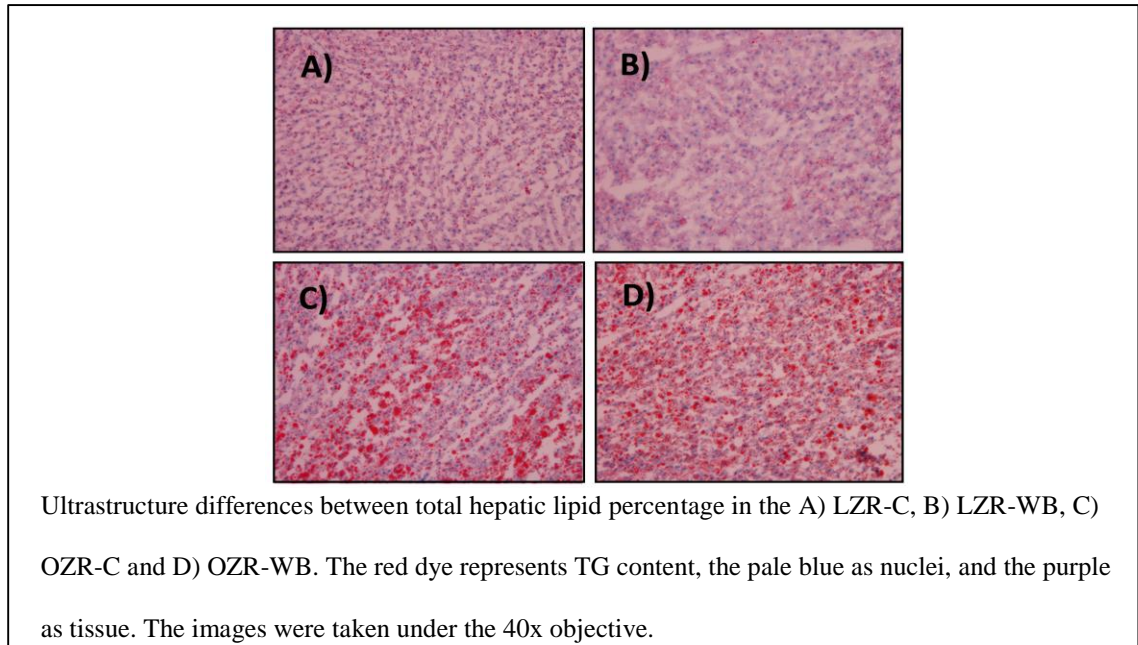
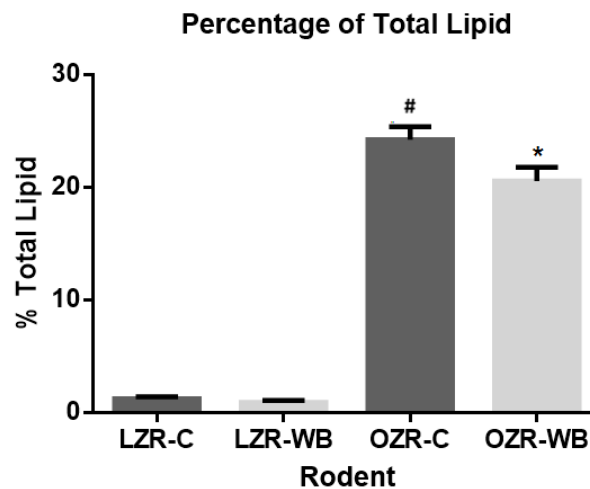


Figure 14: The effect of a wild blueberry diet on the percentage of hepatic triglycerides using Oil-Red O in the Zucker rat



Values are means \pm SEM; LZR-C (n=8), LZR-WB (n=8), OZR-C (n=6), OZR-WB (n=8); statistical significance ($p \leq 0.05$) due to the rodent type consuming the WB-diet* and the C-diet[#]; LZR, Lean Zucker Rat; OZR, Obese Zucker Rat; C, Control diet; WB, Wild Blueberry diet.

Table 3: The effect of a wild blueberry diet on the percentage of hepatic triglyceride using Oil-Red O in the Zucker rat

| | LZR-C | LZR-WB | OZR-C | OZR-WB |
|------------------|--------------|---------------|---------------------------|---------------------------|
| Ave % ORO | 1.23 ± 0.56 | 0.90 ± 0.49 | 23.68 ± 1.97 [#] | 20.58 ± 3.33 [*] |

Values are means ± SEM; LZR-C (n=8), LZR-WB (n=8), OZR-C (n=6), OZR-WB (n=8); statistical significance ($p \leq 0.05$) due to the rodent type consuming the WB-diet* and the C-diet[#]; LZR, Lean Zucker Rat; OZR, Obese Zucker Rat; C, Control diet; WB, Wild Blueberry diet.

4.5. Percentage of hepatic lipid using Hematoxylin and Eosin in the Zucker rat

Figure 15 displays an image analysis example of hepatic lipid content using the H&E stain. Figure 16 and Table 4 show significant differences between the percentage of total hepatic lipid in the LZR-WB ($3.61 \pm 0.57\%$) versus the OZR-WB ($30.73 \pm 3.13\%$) as well as the LZR-C ($2.14 \pm 0.27\%$) versus the OZR-C ($28.24 \pm 2.89\%$). There were no statistically significant results due to the effect of diet.

Figure 15: Hepatic tissue stained with Hematoxylin and Eosin to display lipid content in the Zucker rat

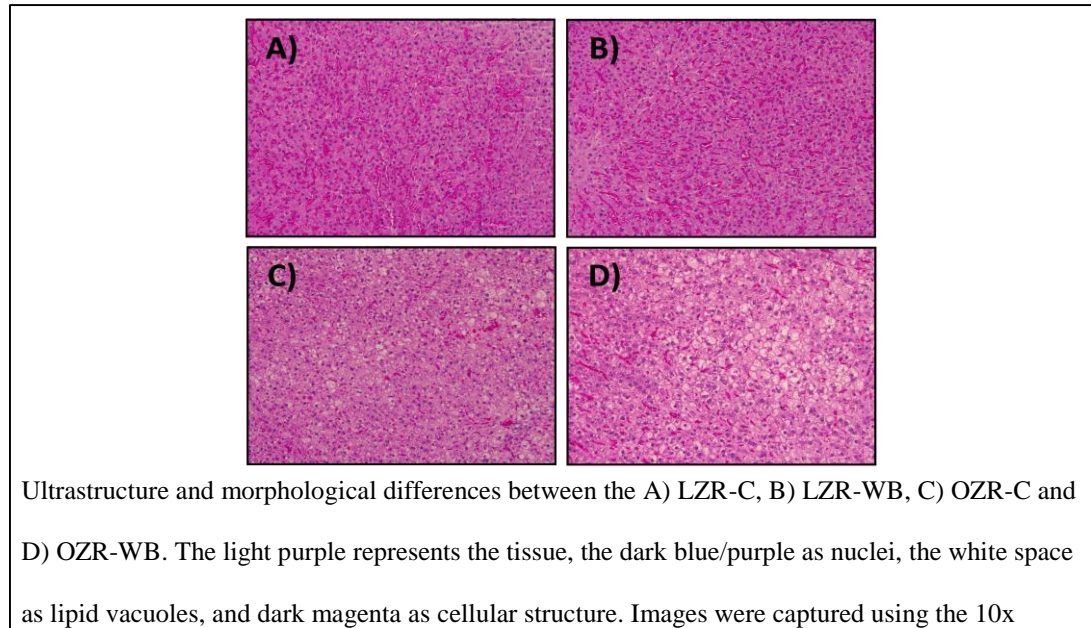
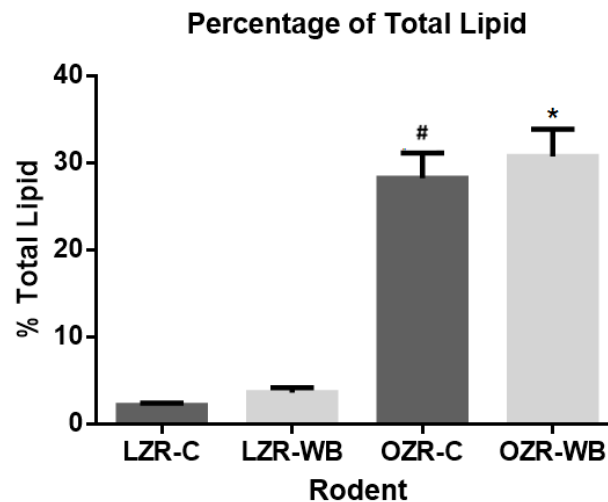


Figure 16: The effect of a wild blueberry diet on the percentage of hepatic lipid using Hematoxylin and Eosin in the Zucker rat



Values are means \pm SEM; LZR-C (n=8), LZR-WB (n=8), OZR-C (n=8), OZR-WB (n=8); statistical significance ($p \leq 0.05$) due to the rodent type consuming the WB-diet* and the C-diet[#]; LZR, Lean Zucker Rat; OZR, Obese Zucker Rat; C, Control diet; WB, Wild Blueberry diet.

Table 4: The effect of a wild blueberry diet on the percentage of hepatic lipid using Hematoxylin and Eosin in the Zucker rat

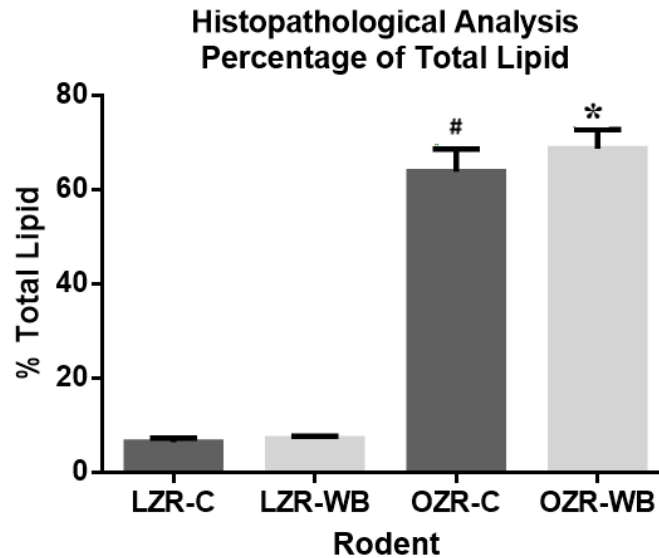
| | LZR-C | LZR-WB | OZR-C | OZR-WB |
|----------------------|--------------|---------------|---------------------------|---------------------------|
| Ave % H&E | 2.14 ± 0.27 | 3.61 ± 0.57 | 28.24 ± 2.89 [#] | 30.73 ± 3.13 [*] |

Values are means ± SEM; LZR-C (n=8), LZR-WB (n=8), OZR-C (n=8), OZR-WB (n=8); statistical significance ($p \leq 0.05$) due to the rodent type consuming the WB-diet* and the C-diet[#]; LZR, Lean Zucker Rat; OZR, Obese Zucker Rat; C, Control diet; WB, Wild Blueberry diet.

4.6. Analysis by a pathologist for the percentage of hepatic lipid stained with Hematoxylin and Eosin in the Zucker rat

Figure 17 and Table 5 show multiple evaluations of the liver sections using the H&E stain displayed significant differences between the percentage of lipid content between the LZR-WB ($7.18 \pm 0.52\%$) versus the OZR-WB ($68.71 \pm 4.05\%$) as well as the LZR-C ($6.45 \pm 0.85\%$) versus the OZR-C ($63.85 \pm 4.79\%$). Results were based upon a blinded, randomized controlled analysis.

Figure 17: The effect of a wild blueberry diet after histopathological analysis by a pathologist for the percentage of hepatic lipid stained with Hematoxylin Eosin in the Zucker rat



Values are means \pm SEM; LZR-C (n=8), LZR-WB (n=8), OZR-C (n=8), OZR-WB (n=8); statistical significance ($p \leq 0.05$) due to the rodent type consuming the WB-diet* and the C-diet#; LZR, Lean Zucker Rat; OZR, Obese Zucker Rat; C, Control diet; WB, Wild Blueberry diet.

Table 5: The effect of a wild blueberry diet after histopathological analysis by a pathologist for the percentage of hepatic lipid stained with Hematoxylin Eosin in the Zucker rat

| | LZR-C | LZR-WB | OZR-C | OZR-WB |
|-----------|-----------------|-----------------|-------------------------------|-------------------------------|
| Ave % H&E | 6.45 \pm 0.85 | 7.18 \pm 0.52 | 63.85 \pm 4.79 [#] | 68.71 \pm 4.05 [*] |

Values are means \pm SEM; LZR-C (n=8), LZR-WB (n=8), OZR-C (n=8), OZR-WB (n=8); statistical significance ($p \leq 0.05$) due to the rodent type consuming the WB-diet* and the C-diet#; LZR, Lean Zucker Rat; OZR, Obese Zucker Rat; C, Control diet; WB, Wild Blueberry diet.

4.7. Percentage of hepatic glycogen using Periodic Acid-Schiff in the Zucker rat

Figure 18 displays an example of hepatic glycogen content using Periodic Acid-Schiff stain. Figure 19 and Table 6 show significant differences between rodent type as evidenced by the percentage of area stained in the LZR-WB ($2.92 \pm 0.18\%$) group compared to the OZR-WB ($27.98 \pm 5.55\%$) group as well as the LZR-C ($0.94 \pm 0.75\%$) group compared to the OZR-C ($25.22 \pm 5.08\%$).

Figure 18: Hepatic tissue stained with Periodic Acid-Schiff to display glycogen content in the Zucker rat

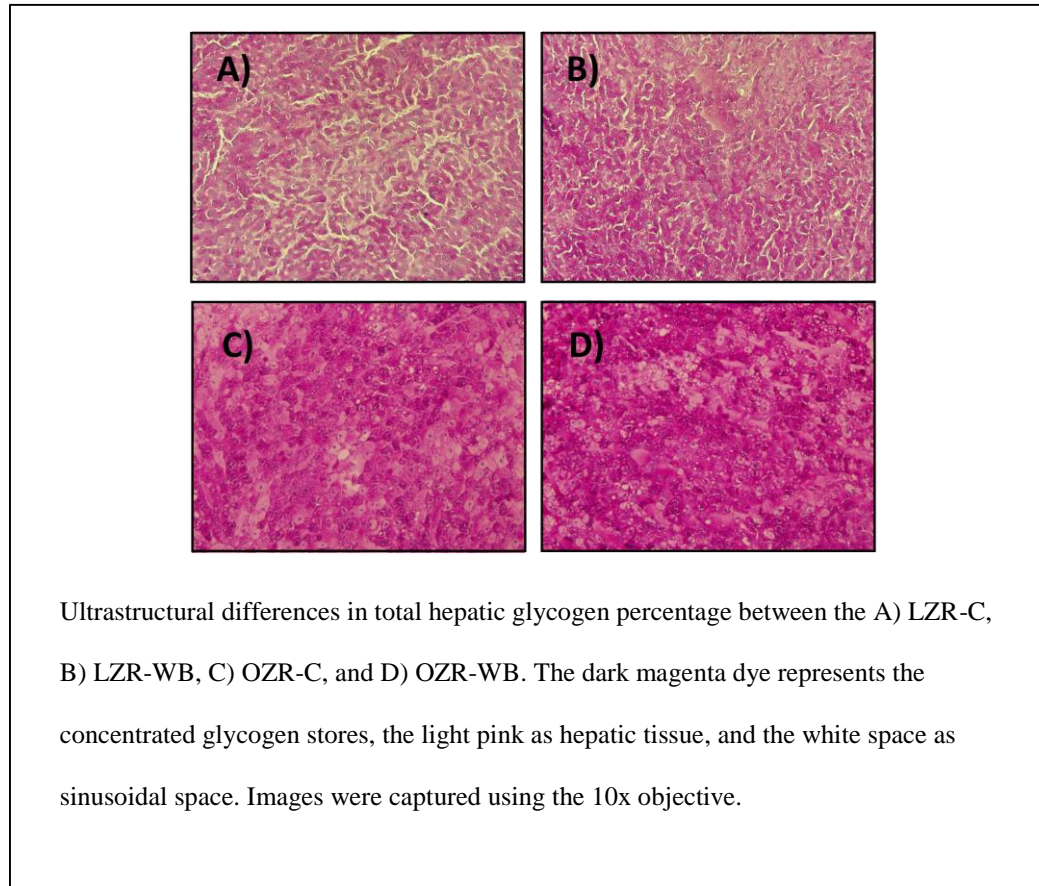
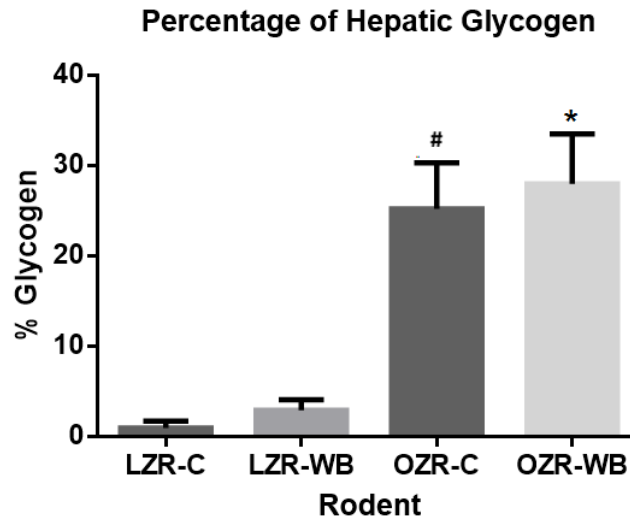


Figure 19: The effect of a wild blueberry diet on the percentage of hepatic glycogen using Periodic Acid-Schiff in the Zucker rat



Values are means \pm SEM; LZR-C (n=8), LZR-WB (n=8), OZR-C (n=8), OZR-WB (n=8); statistical significance ($p \leq 0.05$) due to the rodent type consuming the WB-diet* and the C-diet[#]; LZR, Lean Zucker Rat; OZR, Obese Zucker Rat; C, Control diet; WB, Wild Blueberry diet.

Table 6: The effect of a wild blueberry diet on the percentage of hepatic glycogen using Periodic Acid-Schiff in the Zucker rat

| | LZR-C | LZR-WB | OZR-C | OZR-WB |
|-----------|-----------------|-----------------|-------------------------------|-------------------------------|
| Ave % PAS | 0.94 \pm 0.75 | 2.92 \pm 0.18 | 25.22 \pm 5.08 [#] | 27.98 \pm 5.55 [*] |

Values are means \pm SEM; LZR-C (n=8), LZR-WB (n=8), OZR-C (n=8), OZR-WB (n=8); statistical significance ($p \leq 0.05$) due to the rodent type consuming the WB-diet* and the C-diet[#]; LZR, Lean Zucker Rat; OZR, Obese Zucker Rat; C, Control diet; WB, Wild Blueberry diet.

4.8. Percentage of collagen and connective tissue using Sirius Red in the Zucker rat

Figure 20 displays an example of aortic tissue stained with Sirius Red to detect collagen content. As seen in Figure 21 and Table 7, there were no significant differences due to

diet or animal model between the OZR-WB and the OZR-C nor were there significant differences between LZR-WB and LZR-C groups. However, there appeared to be a trend for less visual presence of Sirius Red stain in the LZR-WB ($11.74 \pm 1.68\%$) group compared to the LZR-C ($16.31 \pm 3.91\%$) group as well as the OZR-WB ($17.90 \pm 1.14\%$) group compared to the OZR-C ($18.05 \pm 1.57\%$).

Figure 20: Hepatic tissue stained with Sirius Red to display collagen and connective tissue in the Zucker rat

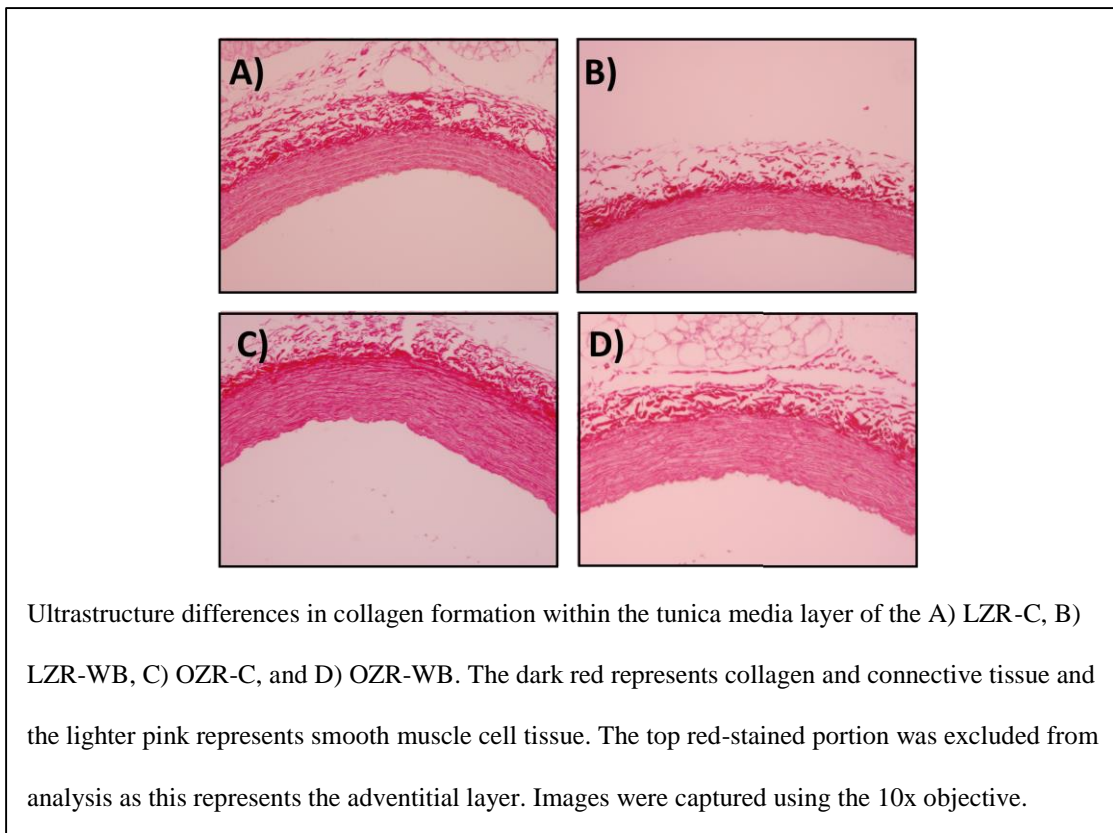
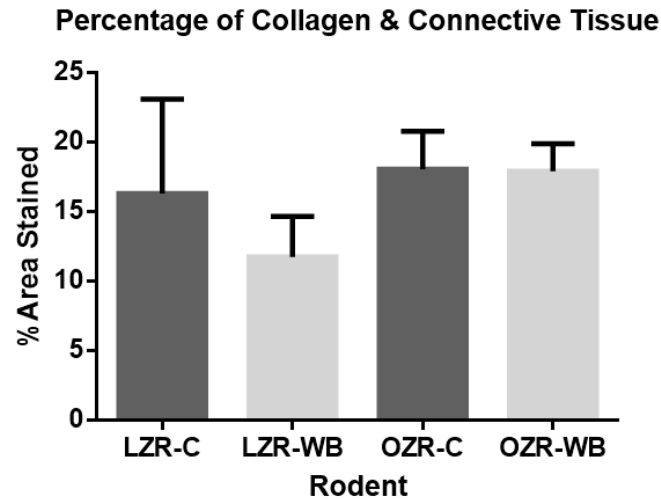


Figure 21: The effect of a wild blueberry diet on the percentage of collagen and connective tissue using Sirius Red in the Zucker rat



Values are means \pm SEM; LZR-C (n=3), LZR-WB (n=3), OZR-C (n=3), OZR-WB (n=3); LZR, Lean Zucker Rat; OZR, Obese Zucker Rat; C, Control diet; WB, Wild Blueberry diet.

Table 7: The effect of a wild blueberry diet on the percentage of collagen and connective tissue using Sirius Red in the Zucker rat

| | LZR-C | LZR-WB | OZR-C | OZR-WB |
|--------------------|------------------|------------------|------------------|------------------|
| Ave. Area % | 16.31 \pm 3.91 | 11.74 \pm 1.68 | 18.05 \pm 1.57 | 17.90 \pm 1.14 |

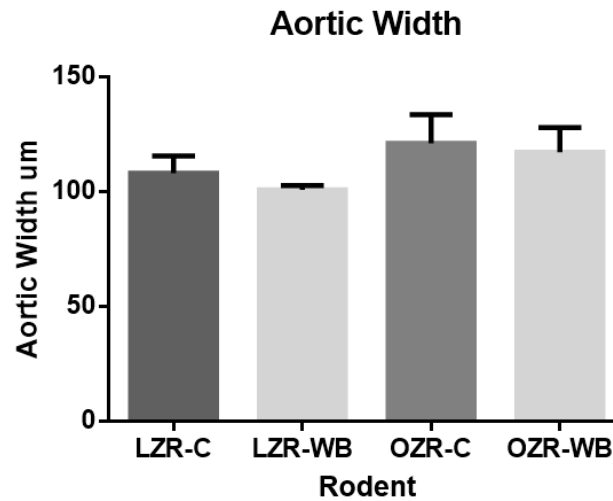
Values are means \pm SEM; LZR-C (n=3), LZR-WB (n=3), OZR-C (n=3), OZR-WB (n=3); LZR, Lean Zucker Rat; OZR, Obese Zucker Rat; C, Control diet; WB, Wild Blueberry diet.

4.9. Width of the tunica media using Sirius Red in the Zucker rat

As seen in Figure 22 and Table 8, there were no significant differences observed due to diet between the OZR-WB and the OZR-C as well as the LZR-WB and the LZR-C. There were also no significant differences observed due to rodent type between the LZR-WB and the OZR-WB as well as the LZR-C and the OZR-WB. Although not significant, there

appeared to be a trend toward smaller width size in the LZR-WB ($100.80 \pm 1.94 \mu\text{m}$) compared to the LZR-C ($108.10 \pm 7.48 \mu\text{m}$) as well as smaller width size in the OZR-WB ($117.20 \pm 10.76 \mu\text{m}$) compared to the OZR-C ($121.10 \pm 12.44 \mu\text{m}$).

Figure 22: The effect of a wild blueberry diet on the width of the tunica media using Sirius Red in the Zucker rat



Values are means \pm SEM; LZR-C (n=3), LZR-WB (n=3), OZR-C (n=3), OZR-WB (n=3); LZR, Lean Zucker Rat; OZR, Obese Zucker Rat; C, Control diet; WB, Wild Blueberry diet.

Table 8: The effect of a wild blueberry diet on the width of the tunica media using Sirius Red in the Zucker rat

| | LZR-C | LZR-WB | OZR-C | OZR-WB |
|--|-------------------|-------------------|--------------------|--------------------|
| Ave. Width μm | 108.10 \pm 7.48 | 100.80 \pm 1.94 | 121.10 \pm 12.44 | 117.20 \pm 10.76 |

Values are means \pm SEM; LZR-C (n=3), LZR-WB (n=3), OZR-C (n=3), OZR-WB (n=3); LZR, Lean Zucker Rat; OZR, Obese Zucker Rat; C, Control diet; WB, Wild Blueberry diet.

4.10. Average number of nuclei in the tunica media using Hematoxylin and Eosin in the Zucker rat

Figure 23 displays an example of aortic nuclei in the tunica media using the H&E stain.

As seen in Figure 24 and Table 9, there were no significant differences between diet (OZR-C versus OZR-WB) nor were there significant differences between the rodent group (LZR-C versus OZR-C and LZR-WB versus OZR-WB). However, there was a non-significant trend for a greater number of nuclei in the LZR-WB (53.17 ± 2.55) compared to the LZR-C (50.10 ± 5.42) as well as the OZR-WB (77.00 ± 12.35) compared to the OZR-C (56.13 ± 9.47).

Figure 23: Aortic tissue of the tunica medial stained with Hematoxylin and Eosin to display the nuclei in the Zucker rat

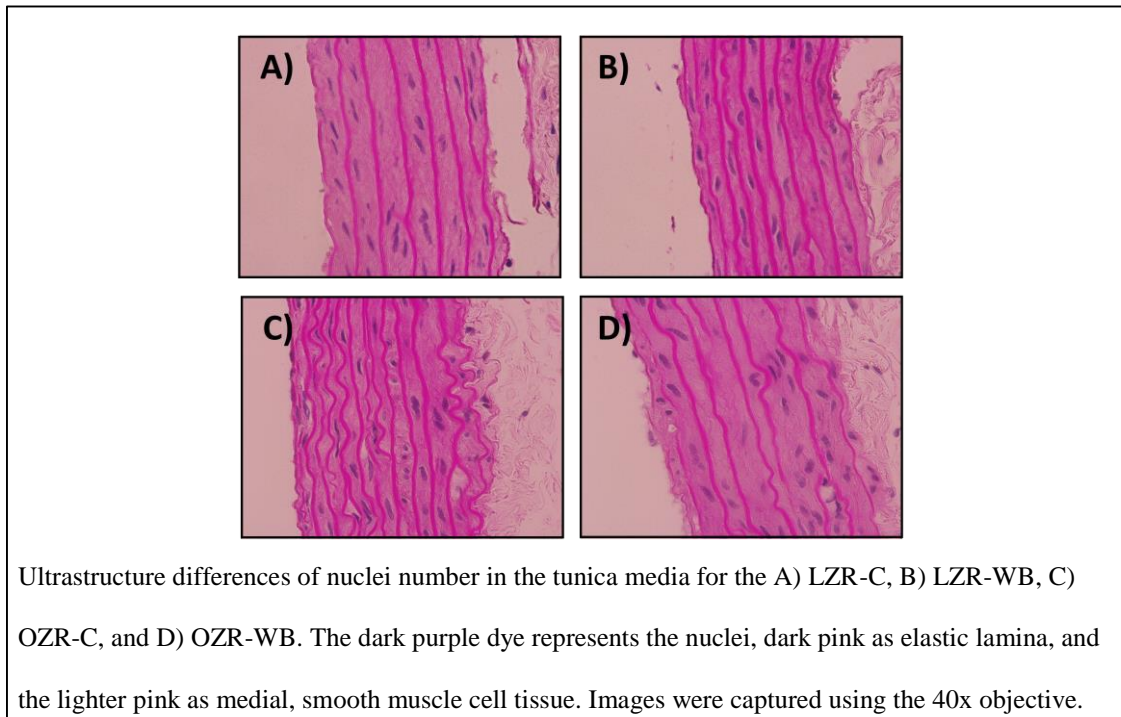
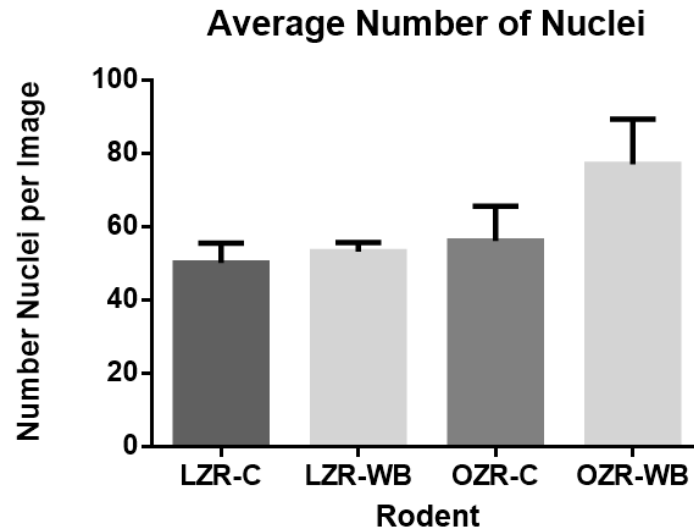


Figure 24: The effect of a wild blueberry diet on the average number of nuclei in the tunica media using Hematoxylin and Eosin in the Zucker rat



Values are means \pm SEM; LZR-C (n=3), LZR-WB (n=3), OZR-C (n=3), OZR-WB (n=3); LZR, Lean Zucker Rat; OZR, Obese Zucker Rat; C, Control diet; WB, Wild Blueberry diet

Table 9: The effect of a wild blueberry diet on the average number of nuclei in the tunica media using Hematoxylin and Eosin in the Zucker rat

| | LZR-C | LZR-WB | OZR-C | OZR-WB |
|----------------------|------------------|------------------|------------------|-------------------|
| Ave. # Nuclei | 50.10 \pm 5.42 | 53.17 \pm 2.55 | 56.13 \pm 9.47 | 77.00 \pm 12.35 |

Values are means \pm SEM; LZR-C (n=3), LZR-WB (n=3), OZR-C (n=3), OZR-WB (n=3); LZR, Lean Zucker Rat; OZR, Obese Zucker Rat; C, Control diet; WB, Wild Blueberry diet.

4.11. Percentage of glycosaminoglycans in the tunic media using Alcian Blue in the Zucker rat

Figure 25 displays an example of aortic tissue stained with Alcian Blue to detect the presence of glycosaminoglycans. As observed in Figure 26 and Table 10, there were no significant differences between the diet groups (OZR-WB versus OZR-C and LZR-WB

and LZR-C) nor were there significant differences between the rodent types (LZR-WB versus OZR-WB and LZR-C versus OZR-C). Although non-significant, there appeared to be a trend displaying less visual Alcian Blue stain in the LZR-WB ($3.83 \pm 0.83\%$) compared to the LZR-C ($4.39 \pm 0.89\%$) as well as in the OZR-WB ($5.95 \pm 1.51\%$) compared to the OZR-C ($8.02 \pm 0.91\%$).

Figure 25: Aortic tissue of the tunica media using Alcian Blue to display glycosaminoglycans in the Zucker rat

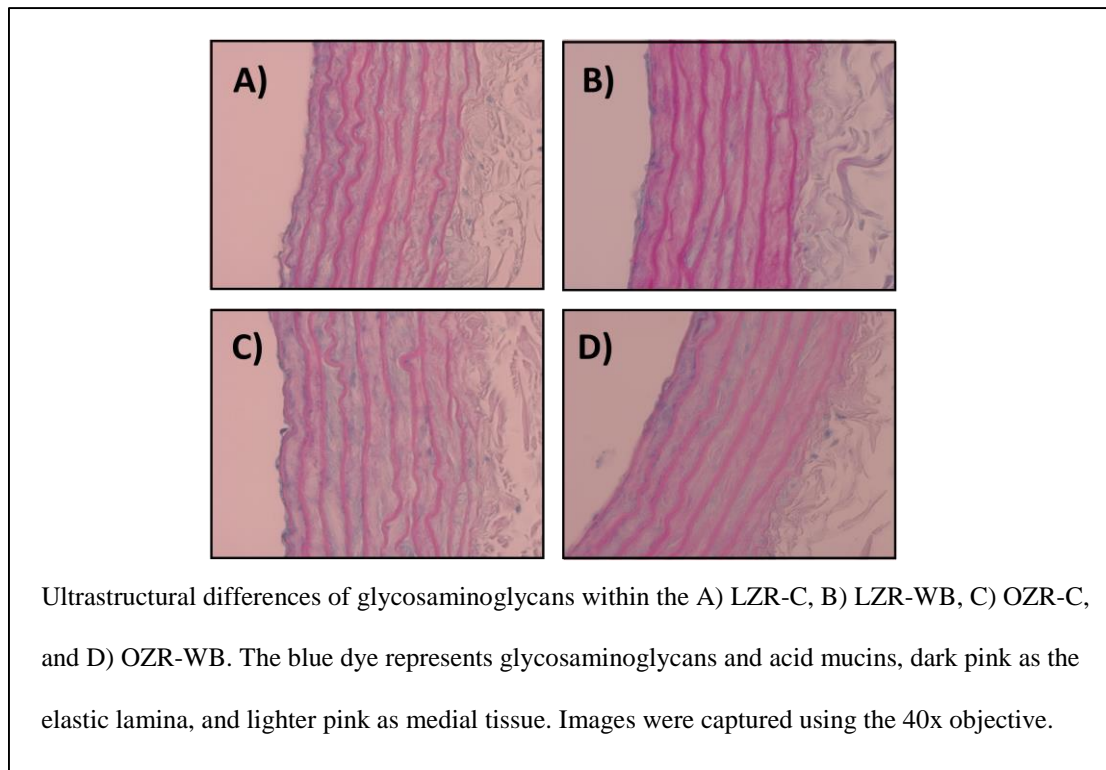
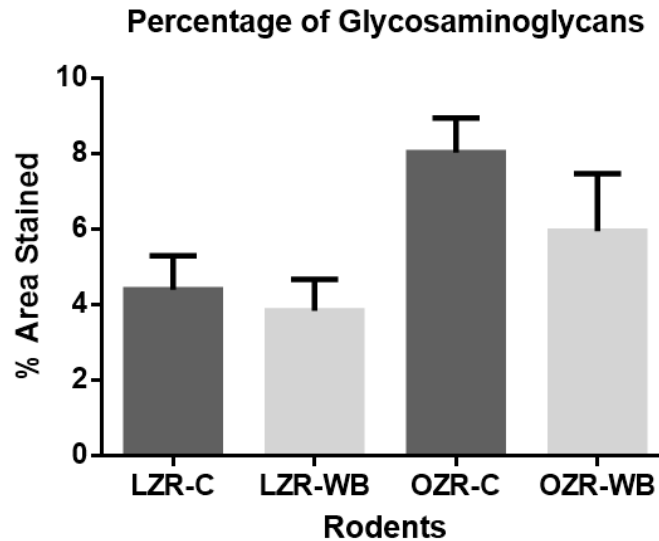


Figure 26: The effect of wild blueberry diet on the percentage of aortic glycosaminoglycans using Alcian Blue in the Zucker rat



Values are means \pm SEM; LZR-C (n=3), LZR-WB (n=3), OZR-C (n=3), OZR-WB (n=3); LZR, Lean Zucker Rat; OZR, Obese Zucker Rat; C, Control diet; WB, Wild Blueberry diet.

Table 10: The effect of a wild blueberry diet on the percentage of aortic glycosaminoglycans using Alcian Blue in the Zucker rat

| | LZR-C | LZR-WB | OZR-C | OZR-WB |
|-----------------------|-----------------|-----------------|-----------------|-----------------|
| Ave. % Stained | 4.39 \pm 0.89 | 3.83 \pm 0.83 | 8.02 \pm 0.91 | 5.95 \pm 1.51 |

Values are means \pm SEM; LZR-C (n=3), LZR-WB (n=3), OZR-C (n=3), OZR-WB (n=3); LZR, Lean Zucker Rat; OZR, Obese Zucker Rat; C, Control diet; WB, Wild Blueberry diet.

CHAPTER 5

DISCUSSION

Four methods were utilized to compare and validate total hepatic lipid and TG content.

Results revealed a statistically significant increase in hepatic steatosis in the OZR

compared to the LZR after image analysis of the H&E and ORO stains, after evaluation of the H&E stain by a pathologist as well as from hepatic TG concentration results.

Although non-significant, image analysis of hepatic triglycerides using the ORO stain found a decreasing trend in TG content in the OZR-WB group compared to the OZR-C. Image analysis of the Periodic Acid-Schiff (PAS) stain revealed a significant increase in hepatic glycogen content in the OZR compared to the LZR. Although non-significant, the LZR-WB and OZR-WB tended to have a greater amount of glycogen than the LZR-C and OZR-C groups respectively.

To evaluate the morphology of aortic tissue, four methods were utilized to assess structural changes and validate the potential for fibrosis in the medial layer. There were no statistically significant differences found due to rodent model or due to diet after evaluating for connective tissue, medial width, number of nuclei, and glycosaminoglycans. Compared to the LZR-C and LZR-WB groups, the OZR-C and OZR-WB groups tended to have a greater percentage of glycosaminoglycans in the aortic tissue, although non-significant. In addition, the LZR-WB and OZR-WB groups tended to have less medial width, and a lower percentage of glycosaminoglycans compared to the LZR-C and the OZR-C respectively.

5.1. The effect of wild blueberry consumption on total hepatic lipid and triglyceride content in the Zucker rat

This study has documented the morphological and biochemical effects of how consuming a diet enriched with wild blueberries for 8-weeks influences the hepatic lipid content in lean and obese Zucker rats.

Previous studies support the OZR as an appropriate model for the MetS as the rodent shares similar physiological characteristics found in humans (Ahima, 2011; Aleixandre de Artinano et al., 2009; Frisbee et al., 2006; Scott M. Grundy, 2011), such as obesity (Aleixandre de Artinano et al., 2009), hepatic steatosis (Serkova et al., 2006), hyperlipidemia (Tofovic et al., 2003), as well as increased systolic pressure, insulin resistance, as well as mild hyperglycemia and hyperinsulinemia (Aleixandre de Artinano et al., 2009; Kucera et al., 2014). In the present study, significant morphological differences were found between the obese and lean Zucker rats, however, the effect of diet produced non-significant results.

Hepatic Triglyceride Concentration: Hypertriglyceridemia is associated with NAFLD and the MetS in human subjects (Marchesini et al., 2001) as well as in the OZR (T. F. Zucker et al., 1962). In the current study, results indicate a significantly greater concentration of hepatic TG in the OZR-C compared to the LZR-C independent of diet as seen in Figure 12 and Table 2.

These concentrations are found at similar levels in the livers of OZR and LZR groups from other studies (Raju et al., 2006) (Jasinski et al., 2013). Other studies (Vendrame, Daugherty, et al., 2014) (Noratto et al., 2015) have found a significant increase in serum TG in the OZR-C compared to the LZR-C group.

To the authors knowledge, there are currently no studies assessing the effect of a WB diet on hepatic triglycerides in the OZR compared to the LZR. Results from the current study found no significant differences in hepatic TG concentration between the OZR-WB and the OZR-C groups. Comparatively, obese Zucker rats consuming a diet containing anti-oxidant rich resveratrol displayed a significant reduction in hepatic TG concentration compared to the OZR-C group (Rivera et al., 2009). Similar to Rivera et al, scientists from another study (Elks et al., 2015) observed that obese female mice consuming a high fat diet enriched with 4% w/w wild blueberry powder displayed a significant reduction in total hepatic triglyceride content compared to the obese control group. After assessing serum TG content, obese Zucker rats consuming a wild blueberry enriched diet (Seymour et al., 2011; Vendrame, Daugherty, et al., 2014), obese-induced male Sprague-Dawley rats consuming a diet enriched with 3% blueberry leaf extract (Yuji et al., 2013) as well as obese-induced male Sprague-Dawley rats consuming a high-fat diet with blueberry juice and Bifidobacterium (Ren et al., 2014) displayed a significant reduction in serum triglycerides compared to the obese control groups. Since the current results between the OZR-WB and OZR-C were not congruent with previous studies as referenced, one explanation could be due to sample size. Limitations in sample size of the current study may have not captured the true range of hepatic TG content leading to non-significant differences. Other factors which may have contributed the discrepancy may have been due to the variables encountered while performing the TG assay.

In the current study, there were no significant differences between the LZR-WB and LZR-C groups. Compared to the LZR-C group, there were no differences in hepatic TG content when Lean Zucker rats consumed a diet enriched with resveratrol (Rivera et al.,

2009). Additionally, a previous study in our lab (Vendrame, Daugherty, et al., 2014) found no significant differences in serum TG levels when comparing the LZR-WB to the LZR-C group which confirms the results from the current study.

Histological analysis of the percentage of total hepatic lipid content utilizing image analysis and histopathological analysis of the H&E stain (C. Daubioul et al., 2002; Kleiner et al., 2005; Nativ et al., 2014) as well as image analysis of the ORO stain (Levene et al., 2010) are common methods for assessing total fat and morphological features in the liver. To the author's knowledge, there are currently no studies that evaluated the morphology of hepatic parenchymal cells in OZR and LZR after wild blueberry consumption in terms of evaluating total percentage of fat utilizing image analysis of the H&E stain, ORO stain and histopathological analysis.

Total hepatic lipid content as assessed by Oil Red O: Histological analysis utilizing the ORO stain and image analysis indicated a significantly greater percentage of total hepatic lipid in the OZR-C compared to the LZR-C as seen in Figure 14 and Table 3. One study evaluated the differences in hepatic lipid content between lean and obese Zucker rats and found a significant increase in the OZR after staining with Oil Red O which was later confirmed after a hepatic TG assay (Cipriani et al., 2010) in concordance to the current study.

In the current study there were no significant differences between the OZR-WB and the OZR-C groups, however there appeared to be a trend for a decrease in the percentage of total hepatic lipid content in OZR-WB compared to the OZR-C group. In one study (A. R. Tovar et al., 2005) scientists fed obese Zucker rats soy protein, which contains a subgroup of flavonoids called isoflavones, and found that liver tissue stained with ORO

displayed a lesser degree of fat depots compared to the casein fed control OZR group; this study indicates that flavonoids, which are also present in wild blueberries, have the potential to attenuate lipogenesis. Although the current study did not find significant differences from the diet, the results suggested that there was a trend for a decrease in the percentage of hepatic lipid content in the OZR-WB group compared to the OZR-C group in accordance the literature. The ORO stain is indicative of TG content and appears to be most effective confirming the presence of fat content in the liver. Since the percentages of hepatic lipid content utilizing the ORO and H&E stains produced non-significant results, there may have been factors that influenced the outcomes such as issues with a smaller sample size, hepatic artifacts, damage to the tissue from storage or from processing, the staining technique itself, quality of the images captured, as well as potential errors in writing the algorithm with ImageJ used to process the tissue images. In the current study there were no significant differences in the percentage of TG content between the LZR-WB and the LZR-C groups. Studies (T. H. Huang et al., 2006) reported that no differences in hepatic lipid content were observed between the LZR experimental group fed anti-obesogenic *Salacia oblonga* root compared to the LZR-C group after the ORO stain had been applied to liver tissue and image analysis estimated the percentage of fatty droplet area (TG content). Although the *Salacia* species is not a berry, the root is known to exhibit antioxidant effects (T. H. Huang et al., 2006). The results from the literature and the current study support the idea that lean rodents are able to maintain a level of hepatic lipid homeostasis regardless of treatment after consuming a naturally occurring substance rich in antioxidants.

Total hepatic lipid content as assessed by Hematoxylin and Eosin: In the current study, both image analysis (Figure 16 and Table 4) and histopathological analysis (Figure 17 and Table 5) found a significant increase in the percentage of total hepatic lipid content in the OZR-C compared to the LZR-C group. One study confirmed hepatic steatosis in the obese Zucker rat with significantly greater levels of total fatty acid and hepatic TG compared to the LZR (Serkova et al., 2006). Results from another study (Wojcik et al., 2016) found that there was a significant increase in steatosis rating in the OZR compared to the LZR group consuming both normal and high-casein diets.

No significant differences between the OZR-WB and OZR-C groups were observed after assessing the percentage of total hepatic lipid content with image analysis and histopathological analysis. The previously mentioned study (Wojcik et al., 2016) also found that the OZR consuming a high soy diet, which is rich in polyphenols (Pratt et al., 1979), significantly decreased the steatosis rating compared to the high-casein diet indicating a potential effect due to diet and its antioxidant properties from polyphenols.

Although not reported as significant, a histopathological assessment of the H&E stain on hepatic lipid concentration by two pathologists (Gomez-Zorita et al., 2012) found that the OZR group consuming a diet enriched with resveratrol reported less hepatic lipid accumulation compared to the OZR-C group based upon a point scale system. Tissue quality, the process of capturing images, and applying an algorithm specific enough to capture all lipid vacuoles may have been factors for the observed results of this study.

Also, bias from researchers may be a factor during histological assessment of the tissue. Results from the current study did not observe any significant differences in lipid content between the LZR-WB and the LZR-C group. One study (El-Rashedy et al., 2011)

confirmed the results from the current study in which scientists did not find significant differences in hepatic fat content between lean Zucker rats fed anthocyanin-rich pomegranate extract and the LZR-C group after analyzing H&E stained tissue.

5.2. The effect of wild blueberry consumption on hepatic glycogen content in the Zucker rat

This section of the study has documented the morphological effects of how an 8-week consumption of a diet enriched with wild blueberries influences the percentage of hepatic glycogen content in the LZR group as well as the OZR group, a model of the MetS.

Impaired fasting glucose (IFG) is one of the primary features seen in the metabolic syndrome (Ma et al., 2013) and is strongly associated with NAFLD as IFG advances toward insulin resistance (Samuel et al., 2016). Insulin resistance occurs due to chronically IFG when a surplus of energy is consumed causing ectopic lipid accumulation in the muscles and organs, such as the liver, resulting in the expression of *de novo* lipogenesis and hyperlipidemia (Samuel et al., 2016).

Results from the current study indicate a significantly greater percentage of hepatic glycogen content in the OZR-C compared to LZR-C groups (Figure 19 and Table 6). The results from the current study are also historically supported (Triscari et al., 1979) when comparing the lean to obese Zucker rats. Triscari and colleagues (Triscari et al., 1979) found that phosphoenolpyruvate carboxykinase (PEPCK) activity, a rate-controlling substrate that acts as a catalyzer in gluconeogenesis, was in part responsible for the production of excess glycogen stores in the OZR compared to the LZR which may explain the significant differences observed.

The current study did not observe any significant differences between the OZR-WB group and the OZR-C group. A prior study in our lab (Vendrame et al., 2015) found a significant down-regulation of resistin, a hormone located in the liver which both increases glucose production and release into the blood stream (thus promoting insulin resistance) as well as a decrease in plasma HbA1c and plasma glucose in the OZR-WB compared to the OZR-C group. Although Vendrame et al (Vendrame et al., 2015) did not specify the effects on hepatic glycogen content directly, it suggests improved glucose control and a decrease in hepatic glucose release. Others have documented (Adeyemi et al., 2014) that diabetic Wistar rats consuming anthocyanin-rich Hibiscus extract displayed a decrease in hepatic glycogen stores toward normal values compared to the diabetic control group in tissue samples stained with PAS; this appears to signify that insulin resistance in diabetes may be a contributing factor toward the decrease in hepatic glycogen stores. The current study did not find significant differences between the OZR-WB and the OZR-C groups, however, the literature appears to suggest that anthocyanins, which are present in wild blueberries, have the potential to improve glycogen stores as well as serum glucose control. Factors which may have contributed to the non-significant results portrayed in this current study could be due to the tissue quality and preservation techniques, method of capturing images, PAS staining technique, and application of the ImageJ algorithm. Staining with PAS portrays glycogen content as an intense magenta color which may have been underrepresented due to the algorithm written to process all of the images in one batch. The PAS stain is also somewhat difficult to interpret when other tissue components are stained a lighter pink color. Defining the intensity of colors between the pink tissue and magenta glycogen may have been misrepresented in some

tissue slides. This could have been due to the brightness of lighting used while capturing images with the microscope as well as through the processes of evaluating the intensity of various colors through image analysis.

The current study did not find significant differences in glycogen content between the LZR-WB and the LZR-C groups. This appears to be congruent with a past study (Popovic et al., 2016) in which scientists discovered that there were no significant differences in glycogen content between lean Wistar rats consuming anthocyanin-rich bilberry extract and lean control rats after assessing the hepatic PAS stain (score) as well as the concentration of glycogen in hepatic tissue.

5.3. The effect of wild blueberry consumption on the aortic morphology in the Zucker rat

This section of the study documents the morphological effects in the aorta after the consumption of a wild blueberry diet in lean and obese Zucker rats. Four experiments were performed to evaluate the potential presence of aortic fibrosis and hyperplasia of smooth muscle cells. Vascular dysfunction and oxidative stress are associated with the MetS (Giugliano et al., 2006) which is characterized specifically by endothelial dysfunction in obese rats (El-Bassossy et al., 2014; Justo et al., 2013;

Vendrame, Daugherty, et al., 2014), as well as a general pro-thrombotic state and atherosclerotic disease (Teran-Garcia et al., 2007). Previous studies in our lab suggest that obese Zucker rats (Vendrame, Kristo, et al., 2014) consuming a wild blueberry enriched diet displayed improvements in endothelial function.

Aortic collagen and connective tissue as assessed by Sirius Red: Studies show that in normal to overweight women who consume a higher intake of anthocyanins and

flavonoids mainly from wine and berries, a significant decrease arterial stiffness and thickening are observed (Jennings et al., 2012) suggesting an anti-atherogenic, anti-hypertensive, and cardio-protective role of anthocyanins on the elasticity of the aorta. Results from the current study did not find significant differences in the percentage of collagenous tissue in the tunica media between OZR-C and LZR-C groups (Figure 21 and Table 7). A past study reported that obese Zucker rats displayed greater levels of aortic hyperplasia as evidenced by an increase of intimal:medial thickness ratio compared to lean rodents at baseline levels (Orozco et al., 2012) suggesting the development of elastins and collagenous fibers in response to the stress from obesity and the Mets. Obese Zucker rats were found to have twice the amount of fibrocellular lesions in the aorta compared to the LZR indicating a fibrotic response to stress, however the ratios of cellular to extracellular material, consisting of collagenous fibers and elastin, were similar between the OZR and LZR groups (Haudenschild et al., 1981). The same study (Haudenschild et al., 1981) did not find any statistically significant differences in the intimal or medial thickness nor in fibrocellular material between diabetic, obese Wistar rats compared to the leaner Wistar rats (Haudenschild et al., 1981). Finally, scientists (Wolinsky, 1970) found significantly elevated absolute weights of collagen and elastin as well as greater aortic diameters in hypertensive, overweight/obese Carworth rats compared to lean, normotensive rodents. It is apparent that there are conflicting results amongst the literature as well as the current results depending on diet, the rodent type as well as the environment they are exposed to. The literature appears to suggest the presence of a compensatory increase from collagenous materials in response to vascular stress. However, one factor which may have contributed to non-significant findings in the

current study was a smaller sample size as well as the location in which the aortic ring was sampled from. Other contributing factors is the duration of time it takes for collagenous materials of form in the aorta as well as the “second hit theory” which is thought to stimulate aggressive fibrosis in tissue.

In the current study there were no statistically significant differences between the percentage of collagen and connective tissue content in the aortas of the OZR-WB group compared to the OZR-C group. Obese and diabetic Wistar rodents consuming polyphenol-rich green tea extract, mainly known to be in the form of catechins which are also extracted in monomeric forms from wild berries in the *Vaccinium* family (Maatta-Riihinen et al., 2005), displayed a significant decrease in collagen content, collagenous cross-linking, as well as systolic blood pressure, compared to obese and diabetic Wistar control rodents (Babu et al., 2006). Scientists observed that diabetic Sprague-Dawley rats treated with antioxidant-rich resveratrol displayed a significant decrease in total collagen content in the aorta compared to diabetic control rats (Jing et al., 2010). Literature suggests the antioxidants promote the improvement of collagenous content in the aorta by preventing or slowing collagen synthesis, however the current study did not produce similar results. The literature presented administered extracts to the rodents which are highly concentrated forms of antioxidants. Since the current study provided whole wild blueberries rather than extracts, the results may have not produced similar findings. There may also be the potential for error when capturing tissue images due to microscope lighting adjustments or lighting techniques. Applying the correct image analysis algorithm that can process all images at once to detect collagen consistently may have also caused results dissimilar from the literature.

The results from the current study did not find significant differences in the percent of collagen in the aorta between the LZR-WB and the LZR-C groups. Compared to a group of lean Wistar control rodents, a group of lean Wistar rodents that consumed green tea extract did not display any significant differences between aortic collagen content (Babu et al., 2006). It appears that lean control rodents compared to treatment rodents may be stable and healthy enough to produce similar results regardless of treatment.

Aortic width as assessed by Sirius Red: Increased levels of intima-media thickness in the aorta has been associated with low levels of plasma antioxidants, higher levels of inflammatory factors, as well as greater levels of clotting factors, such as fibrinogen (Riccioni et al., 2009). Increases in intima-medial thickness is also positively associated with increasing levels of glycaemia in human subjects (Thomas et al., 2004). Observing structural changes in the aorta may serve to evaluate the short and long-term effects of antioxidant-rich wild blueberries as well as the deleterious effects from components of the MetS.

The current study did not detect statistically significant differences between the OZR-C and LZR-C medial thicknesses (Figure 22 and Table 8). Although non-significant, there was a trend for the average medial thickness in the OZR-C to be greater than the medial thickness in the LZR-C group. These findings are similar to the results found in Orozco et al (Orozco et al., 2012) in which the aortic thickness in OZR group was greater than those observed in the LZR groups at two weeks (baseline) and four weeks after aortic injury. Finally, in obese Zucker rats, the interior diameters of the aortas were both smaller than those of lean rats after a blood flow test (Bouvet et al., 2007). Scientists from the same study (Bouvet et al., 2007) also found that the arteries in the OZR had greater surface

areas compared to those found in the LZR; both results from the OZR group suggest the thickening of the aorta due to obesity and the MetS.

Results from the current study did not observe significant differences between the OZR-WB and OZR-C groups. Although non-significant, the OZR-C group had an overall greater increase in medial thickness compared to the OZR-WB. Male Sprague-Dawley rats consuming a chow diet supplemented with quercetin, a dark colored pigment also present in blueberries, resulted in significantly lower medial thickness and carotid systolic blood pressure compared to rodents consuming a normal chow diet after both groups were subjected to an abdominal aortic constriction (Carlstrom et al., 2007); these results indicate antioxidant activity may have played a role in the prevention of medial thickening and hypertension. A clinical study (Aviram et al., 2004) with overweight subjects who had severe carotid artery stenosis found that those who did not consume polyphenolic antioxidant-rich pomegranate juice, which contains anthocyanins, displayed significant increases in intima-media thickness suggesting both the morphological and biochemical benefits of polyphenolic consumption. Although the results of this current study did not display significant differences, there was a trend for the OZR-WB and LZR-WB groups to have less medial thickness compared to controls which appears to align with the literature presented for both rodents and human subjects. The non-significant differences may be due to a low sample size and sample processing. Additionally, since a digital measuring tool was used to assess the randomized images, the measurements still required the precision of the scientist to assess the thickness without including the tunica intima and the tunica adventitia which contains bias.

Including the morphology of the intima and the adventitia would increase the average thickness for each group yielding inaccurate results.

The current results indicate no significant differences in medial thickness in LZR-WB compared to LZR-C groups. The LZR-C group did however have a non-significant increase in medial thickness compared to the LZR-WB group. There was no difference between lean and obese Zucker rats regarding the surface area of the tunica media after exposure to aortic injury (Haudenschild et al., 1981) which continues to suggest there may be no notable differences between lean rodents.

Aortic nuclei as assessed by Hematoxylin and Eosin: The tunica medial layer of the aorta contains layers of smooth muscle cells, elastic laminae surrounded by microfibrils, as well as collagen fibers all of which enable aortic contraction and ensure proper elasticity and stiffness to buffer blood flow circulating toward peripheral tissues (Karimi et al., 2016).

The current study did not observe statistically significant differences in the number of nuclei between the OZR-C and the LZR-C groups (Figure 24 and Table 9). The OZR-C group did however display a trend for a greater average number of nuclei compared to the LZR-C group, although non-significant. One study found that in spontaneously hypertensive (SH) rats of the Okamoto strain, the volume of nuclei significantly decreased with age more so than compared to the control Wistar-Kyoto (WK) rats while medial thickness increased by 150% in the SH rats and by 87% in WK rats (Olivetti et al., 1982). This indicated there was a decrease in nuclei and subsequent increase in medial thickening as hypertension progressed over time suggesting that if the current experiment was studied for a longer duration of time, similar findings may be produced.

One study (Owens et al., 1981) found that SH rats contained a greater smooth muscle cell DNA/aortic length compared to normotensive Wistar-Kyoto and Sprague-Dawley rats, although the number of smooth muscle cells per length did not display differences between the rodents suggesting that hypertension, as seen in the MetS, may cause hypertrophy and hyperplasia of smooth muscle cells. The process of counting nuclei may have been hindered due to adequate visualization of the nuclei in the aortic tissue. Factors which may have contributed to the current results include how the tissue was sliced, the final quality of the stain, the process of capturing images, and evaluating the number of nuclei. Some of the aortic tissue contained nuclei that were well visible and sufficient enough to include in the count. On the other hand, some of the aortic tissues contained artifacts and displayed nuclei that appeared to be sliced at a different angle resulting in small, dot-like nuclei rather than longitudinal or oval-shaped nuclei. Also the visibility of the nuclei was sometimes diminished due to the smooth muscle tissue covering the nuclei resulting in translucent cells which may have not been included in the final average. Although randomized, the location in which the nuclei cells were located and sampled from in the aorta may have also contributed to the current results.

The current study did not observe significant differences between the number of nuclei between the OZR-WB and the OZR-C groups. Studies using cell cultures of rat aorta (Chang et al., 2008) found that rat aortic smooth muscle cells incubated with oxidized LDL-C displayed an increase in smooth muscle cell proliferation (S-phase) and was reduced when ellagic acid, a phenolic compound present in blueberries, was added to the medium. Another study (Park et al., 2010) found that proliferation of rat aortic smooth muscle cells stimulated by platelet-derived growth factor-BB was reduced after cells

were subjected to pterostilbene, a (natural) dimethylated analog of resveratrol. The results appear to suggest that, under atherogenic conditions commonly associated with the MetS, antioxidant rich compounds have the potential to attenuate smooth muscle cell proliferation, and therefore, number of smooth cell nuclei. Due to the small sample size the results from the study may not display the true average of nuclei in the tissue. Since the number of nuclei was determined by counting the cells, bias from the researcher may have contributed to the current results.

The current study did not observe statistically significant differences in the number of nuclei between the LZR-WB and the LZR-C groups. One explanation for the results of the current study are that, since both groups of rodents were lean, assumingly healthy, and consuming a similar isocaloric diet, both rodent groups may have been physiologically stable enough to maintain normalized smooth muscle cells and antioxidant status therefore resulting in non-proliferative smooth muscle cells.

Aortic glycosaminoglycans as assessed by Alcian Blue: The three main components of atherosclerosis are proliferation of smooth muscle cells, synthesis of connective tissue, such as GAG and collagen, and deposition of extracellular and intracellular lipids (Campbell et al., 1985). Glycosaminoglycans, which are linear polysaccharides, play an important role in vascular function through cellular proliferation, adhesion and migration (Kalea et al., 2006) and also play a role in arterial wall dysregulation, especially through the binding of LDL-C resulting in aortic lesions and atherosclerosis (A. M. Tovar et al., 1998). There appears to be a positive association between the increasing length and GAG sulfation to the binding of LDL-C to proteoglycans resulting in the enhancement of intimal thickening and atherosclerosis development due to prolonged binding (Tannock et

al., 2008). Proliferating vascular smooth muscle cells appear to increase GAG size and sulfation resulting in increased binding of LDL-C (Camejo et al., 1993).

To the author's knowledge, there are currently no studies which have investigated the concentration of GAG in the aorta of the Zucker rat utilizing the Alcian Blue stain and image analysis.

Results from the current study indicate no statistically significant differences of the percentage of GAG between the OZR-C and LZR-C groups (Figure 26 and Table 10).

However, the average percentage of aortic GAG in the OZR-C group was (non-significantly) greater than in the LZR-C group. To the author's knowledge there are currently no direct studies exploring aortic glycosaminoglycans in the OZR. However, in one study (Ihara et al., 2005), the aortas of obese Zucker rats displayed significant increases in both C-mannosylation, a c-glycosylation molecule, as well as thrombospondin-1 (TSP-1), a multimeric glycoprotein, compared to LZR indicating an increase in glycosylation in response to a hyperglycemic environment. TSP-1 is associated with glycosaminoglycan binding and has been shown to be up-regulated during inflammation, in endothelial cells during the response to healing, and in atherosclerotic lesions (Adams, 2001). C-glycosylation (and O-glycosylation) have been linked to the interaction between TSP-1 and GAG (Hofsteenge et al., 2001). Another study (Ichida et al., 1968) found that lean Sprague-Dawley rats (SDR) displayed similar levels of sulfated GAG as in diabetes-induced SDR, although diabetic rats tended to have greater levels of non-sulfated hyaluronic acid (HA) in the aorta as they aged.

Results from the current study indicate no significant differences between the OZR-WB and the OZR-C groups nor were there differences between the LZR-WB and the LZR-C

groups. Although non-significant, the OZR-WB group appeared to have less total percentage of GAG compared to the OZR-C group while both the LZR-WB and the LZR-C groups remained the same. A study from our lab (Kristo et al., 2012) found that spontaneous hypertensive rats (SHR) consuming a WB diet displayed a reduction in atherogenic galactosaminoglycans disaccharides (GalAG) along with an increase in anti-atherogenic hyaluronic acid (HA) and heparan sulfate (HS); both HS and HA appear to promote nitric oxide synthesis and inhibit SMC proliferation. The SHR has been known to contain greater levels of chondroitin sulfate contributing to elevated glycosaminoglycan levels. In the current study, it cannot be determined if the slight, non-significant decrease in levels of GAG are of benefit. Since this study did not use a specific biochemical or morphological assay to identify the potential state of flux for GAG subcategories, such as evaluating for HS and HA, this factor creates great difficulty in determining the benefits of blueberries based upon GAG imagery alone. Another study from our lab (Kalea et al., 2006) found that Sprague-Dawley rats who reached an obese state and fed an 8% w/w WB enriched diet displayed a significant decrease in overly-sulfated GAG as well as a significant increase in non-sulfated disaccharide chains of galactosaminoglycans (GalAG) concentrations in the aorta compared to the obese control group indicating anti-atherogenic effects. The current alcian blue stain may only be useful to determine the total presence of GAG in the aortic tissue rather than the quality of the GAG. As the literature reported, the degree of sulfation of GAG appears to play an imperative role in determining the atherogenic or antiatherogenic properties of the aorta at any given moment. The current study cannot conclude if the WB diet provided

beneficial effects due to the indiscriminant nature of the stain for all GAG and its inability to determine sulfated from non-sulfated GAG.

5.4. Recommendations for future research

The current study evaluated the morphological and biochemical effects of an 8-week, 8% w/w wild blueberry diet in obese Zucker rats, a model of the metabolic syndrome. The obese rodents begin to develop symptoms of the MetS at an early age and quickly progress toward an obese state while exhibiting adverse effects both morphologically and biochemically. Studying the effects of a WB diet in the rodents has provided greater insight for human health, specifically in the aorta and liver, in relation to disease pathogenesis of the MetS.

To achieve greater insight and deeper understanding of morphological and biochemical changes that occur after consuming a wild blueberry diet in the MetS model, it is important to study the OZR using different techniques to assess the disease progression. This study found subtle morphological changes between the OZR-WB and OZR-C groups resulting in non-significant differences. A future study could include different increments in time to assess the baseline characteristics, after 8-weeks, 16-weeks, and additional time periods if desired to assess the changes over a duration of time. This approach would further characterize when morphological changes occur in the Zucker rat and map the lifespan of the rodent from a young to older age. Studying this area is especially important as society is now seeing a greater proportion of younger and older people with the MetS which may impact both developmental and ill health-related decline.

Since morphological characteristics do not always agree with biochemical assays, such as comparing the ORO stain to the hepatic TG assay, each image analysis test should be paired with a biochemical evaluation. Including a glycogen assay to compliment image analysis would further prove or disprove the visual findings. Testing for specific glycosaminoglycans from an assay kit in combination with the Alcian Blue stain with image analysis would provide both a visual of the physical morphology as well as a biochemical representation for the ratio pro- and anti-atherogenic GAG molecules. Future studies on the morphology of liver and aorta tissues may benefit from using whole-slide imaging techniques. The whole-slide imaging has the capability to scan entire images providing a larger, more visually uniform, surface area to evaluate. This method may decrease the time and energy required compared to traditional methods of capturing pictures from single points on the tissue as well as decrease the chance of capturing images that overlap. ImageJ is an excellent tool used by thousands of scientists to evaluate tissue and cells. For more advanced research projects, utilizing an image analysis program with greater processing capabilities in conjunction with whole-slide scanning technology will enable the user to customize and seek unique traits of the tissue which are otherwise more difficult to do with simpler programs.

As for the histopathological assessment of the tissue, utilizing a scaling system which incorporates greater detail, such as defining lipid droplets as micro or macrovesicular steatosis or assessing for abnormalities in tissue development, may also further characterize the pathogenesis of the MetS in the OZR. A larger panel of pathologists will also be required to diminish any bias and increase the confidence and strength of the results.

5.5. Significance

Approximately 20-25% of Americans have the metabolic syndrome (Association, 2012), a serious health concern to the U.S. population. The MetS is a cluster of symptoms, including as obesity, hyperglycemia & insulin resistance, hypertension, dyslipidemia with low HDL-C, high sdLDL-C, and is often manifested over years of poor nutritional care. Antioxidant rich foods, such as wild blueberries, may decrease the risk of acquiring the MetS, prevent cardiovascular disease, and provide a natural treatment for patients who currently have the MetS. Studies have revealed how healthcare expenses in patients with the MetS are higher than healthy patients and are expected to increase in the future (Boudreau et al., 2009; Scott M. Grundy, 2011). Consuming wild blueberries may be used as an alternative to expensive pharmaceutical treatment and offer an inexpensive alternative to disease prevention.

For the first time, this project has explored the effects of wild Maine blueberries on morphological and biochemical properties in hepatic and aortic tissues of the Zucker rat. In addition, due to the robust nutraceutical value of wild blueberries, such as the phenolic content, antioxidant capability, as well as vitamin and mineral composition, future studies with a larger sample size and longer duration may influence the morphology of hepatic and aortic tissue. These results can be used to further characterize the morphology of obese Zucker rat as well as contribute to the current understanding of beneficial health effects from wild blueberries, such as advancing the field of complementary alternative medicine (CAM) and treatment for chronically ill patients with MetS.

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