The Role of Red Raspberry (Rubus Idaeus) on Inflammation, Lipid Metabolism, and Endothelial Dysfunction as Related to the Metabolic Syndrome

Natalie VandenAkker
University of Maine, natalie.marchi@maine.edu

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THE ROLE OF RED RASPBERRY (RUBUS IDAEUS) ON INFLAMMATION, LIPID METABOLISM, AND ENDOTHELIAL DYSFUNCTION AS RELATED TO THE METABOLIC SYNDROME.

By

Natalie E. VandenAkker

B.S. Worcester State University, Massachusetts, 2013

M.S. Worcester State University, Massachusetts, 2015

A THESIS

Submitted in Partial Fulfillment of the Requirements of the Degree of Doctor of Philosophy (in Food and Nutrition Sciences)

The Graduate School
The University of Maine
August 2021

Advisory Committee:

Dorothy J. Klimis-Zacas, Ph.D., FACN, Professor of Clinical Nutrition, Advisor
Cristian Del Bo’, Ph.D., Associate Professor of Food and Nutrition Sciences, University of Milan
Robert Gundersen, Ph.D., Associate Professor of Biochemistry, University of Maine
Brian Perkins, Ph.D., Research Assistant Professor of Food Science, University of Maine
Stefano Vendrame, Ph.D., Italian State Licensed Dietician, Milano, Italy
THE ROLE OF RED RASPBERRY (*RUBUS IDAEUS*) ON INFLAMMATION, LIPID METABOLISM, AND ENDOTHELIAL DYSFUNCTION AS RELATED TO THE METABOLIC SYNDROME.

By Natalie E. VandenAkker

Thesis Advisor: Dr. Dorothy Klimis-Zacas

An Abstract of the Thesis Presented
In Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy (in Food and Nutrition Sciences)
August 2021

Metabolic syndrome (MetS) is major public health concern. Diet can play a major role in the prevention and/or progression of the MetS. At 8 weeks of age, male obese Zucker rat (OZR) and their lean littermates (LZR) were placed on a control or an 8% w/w whole red raspberry (WRR)-enriched diet for 8 weeks. Circulating levels of inflammatory cytokines and their gene expression in the liver and adipose tissue were evaluated. Several lipid markers were measured in the plasma, liver and adipose tissue. The expression of eight genes related to lipid metabolism were evaluated, both in liver and adipose tissue. Phenylephrine (Phe)-induced vasoconstriction and acetylcholine (Ach)-induced vasorelaxation were measured in aortic rings in the presence or absence of L-N-monomethyl-arginine (L-NMMA) and mefenamic acid (MFA). Prostanoid levels were measured in the aortic effluent. Vascular function related gene were analyzed in the aorta. Plasma levels of interleukin-6 (IL-6) decreased in the OZR consuming a WRR diet compared to the OZR-C (*p*<0.05). Hepatic expression of pro-inflammatory markers IL-6, tumor necrosis
factor-alpha (TNF-α), and nuclear factor kappa-B (NF-κB) was also significantly down-regulated ($p<0.05$) with the WRR diet in the OZR. Following consumption of WRR, the expression of IL-6 and TNF-α in the OZR were significantly down-regulated ($p<0.05$) in the adipose tissue. A WRR enriched-diet reduced plasma cholesterol and HDL-C and increased plasma TG while it decreased hepatic TG accumulation in the OZR. The OZR assigned to a WRR exhibited up-regulation of microsomal triglyceride transfer protein and down-regulation of fatty acid synthase expression in the liver. The WRR diet partially restored aortic response in the OZR-WRR aorta ($p<0.05$) compared to OZR- C. The OZR-WRR group pre-treated with L-NMMA increased ($p<0.05$). Pre-treatment with L-NMMA, maximal relaxation response was higher in the OZR compared to the LZR ($p<0.05$). With L-NMMA, maximal relaxation response in OZR-WRR ($p<0.05$) was lower. Prostacyclin I2 concentration was attenuated in the OZR-WRR ($p<0.05$). Aortic expression of eNOS and COX-2 were downregulated in the OZR-WRR ($p<0.05$). In conclusion, WRR attenuates inflammation, plays a hepatoprotective role and restores impaired vascular tone in the OZR.
DEDICATION

This dissertation is dedicated to my mother, Ms. Joanne M. Marchi.
ACKNOWLEDGEMENTS

I would like to acknowledge my mother, Ms. Joanne M. Marchi and husband, Mr. Jacob E. VandenAkker for their unwavering constant love, support, encouragement, and serving as my backbone both near and far. My family and friends opened their arms and heart, many times several miles apart, with comfort, love and confidence in my ability.

I would like to express my gratitude to my advisor and mentor, Dr. Dorothy Klimis-Zacas Ph.D., FACN for her consistent and constant support of my PhD study both in and out of the laboratory. Her guidance, patience and motivation were a guiding light. I would also like to acknowledge my advisory committee board, Dr. Cristian Del Bo’, Ph.D., Dr. Robert Gundersen, Ph.D., Dr. Brian Perkins, Ph.D., and Dr. Stefano Vendrame, Ph.D for all their expertise. I would like to thank colleagues and students Dr. Panagiotis Tsakiroglou, Ph.D., Jasmine Waite, Marissa McGilvrey, Dr. James Weber and Brenda Kennedy-Wade for their assistance in this study.
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LIST OF ABBREVIATIONS

Abca1: ATP-binding cassette transporter
Acat1: Acyl-CoA
Ach: Acetylcholine
Acox1: Acyl-CoA oxidase 1
ApoB: Apolipoprotein B
AT: Adipose Tissue
ATP: Adenosine Triphosphate
BH₄: Tetrahydrobiopterin
Ca²⁺: Calcium
cAMP: Cyclic AMP
cGMP: Cyclic GMP
COX: Cyclooxygenase
COX1: Cyclooxygenase-1
COX2: Cyclooxygenase-2
CRP: C-Reactive Protein
CVD: Cardiovascular Disease
EDCF: Endothelial-Derived Constricting Factors
EDRF: Endothelial-Derived Relaxing Factors
eNOS: Endothelial Nitric Oxide Synthase
Fas: Fatty Acid Synthase
GTP: Guanosine Triphosphate
H&E: Hematoxylin and Eosin Stain
HDL: High-Density Lipoprotein
IL-1β: Interleukin 1-Beta
IL-4: Interleukin-4
IL-6: Interleukin-6
IL-10: Interleukin-10
IP: Prostacyclin Receptor
L-NMMA: L-N-Monomethyl-Arginine
LDL: Low-Density Lipoprotein
Lepr: Leptin Receptor Gene
Lpl: Lipoprotein Lipase
Lrp1: LDL Receptor Protein
LZR: Lean Zucker Rat
MCP-1: Monocyte Chemoattractant Protein 1
MetS: Metabolic Syndrome
MFA: Mefenamic Acid
MLCK: Myosin Light Chain Kinase
Mttp: Microsomal Triglyceride Transfer Protein
NAFLD: Non-Alcoholic Fatty Liver Disease
NF-κB: Nuclear Factor Kappa
NLRP3: NOD-, LRR- and pyrin domain-containing protein 3
NO: Nitric Oxide
O₂: Superoxidase Anion
ONOO⁻: Peroxinitrite
ORAC: Oxygen Radical Absorbance Capacity
OZR: Obese Zucker Rat
PGI2: Prostacyclin I2
Phe: Phenylephrine
Pparα: Peroxisome Proliferator-Activated Receptor Alpha
Pparγ: Peroxisome Proliferator-Activated Receptor Gamma
ROS: Reactive Oxygen Species
sGC: Soluble Guanylyl Cyclase
T2D: Type 2 Diabetes Mellitus
TG: Triglycerides
TNF-α: Tumor Necrosis Factor-Alpha
TP: Thromboxane Receptor
TXA2: Thromboxane A2
VLDL: Very Low-Density Lipoprotein
WRR: Whole Red Raspberry
CHAPTER 1
1. INTRODUCTION

The term Metabolic Syndrome (MetS) was first coined by Haller and Hanefield in 1975. The MetS is characterized by a combination of obesity, hypertension, dyslipidemia and elevated glucose increasing the risk of cardiovascular disease and type 2 diabetes (O’Neill & O’Driscoll, 2015). The major contributor of the MetS is lifestyle such as physical inactivity and excess dietary intake of fats and carbohydrates (O’Neill & O’Driscoll, 2015). There is a high prevalence of the MetS globally and it is expected to increase (O’Neill & O’Driscoll, 2015). According to the United States 2000 census it is estimated that about 47 million of the U.S. population had the MetS (O’Neill & O’Driscoll, 2015).

One condition associated with the MetS is inflammation. Inflammation is a defense response that is facilitated with persistent metabolic disturbances (Mittal, Siddiqui, Tran, Reddy, & Malik, 2014). Obesity-induced inflammation is associated with insulin resistance, hyperglycemia, dyslipidemia and hypertension (Castro, Macedo-de la Concha, & Pantoja-Meléndez, 2016). This inflammatory state increases the production of pro-inflammatory adipokines, chemokines, cytokines by adipocytes and oxidative stress (Castro et al., 2016). Unresolved inflammatory condition results into systemic inflammation causing further metabolic damages (Castro et al., 2016).

Dyslipidemia associated with the MetS, consists of three components: fasting and post-prandial triglycerides, decreased high-density lipoprotein and increased small, dense low-density lipoprotein particles (Ruotolo & Howard, 2002). Abnormal lipid ratios increases an individual’s risk of atherosclerotic lesions and fatty liver disease (S. Grundy, 1997).
Patients with the MetS or contributing elements to it, commonly have altered vascular function, such as an abnormal vascular tone from a physiological contribution such as reactive hyperemia, elevated metabolic demand or pharmacological contribution such as infusion of endothelium-dependent agonist (e.g. acetylcholine, methacholine, and insulin) (Frisbee & Delp, 2006). Vascular dysfunction is accompanied by a greater risk of cardiovascular events.

Over the past several years pharmacotherapies have been used to reduce the risks of MetS. Current therapies have side effects which have been identified as the major reason patients discontinue therapy, increasing risks associated with the MetS (Ahuja & Chou, 2016). An alternative to the use of pharmacotherapies is a diet rich in plant foods as a preventative and/or reversible approach for many conditions associated with the MetS (Pandey & Rizvi, 2009). There is a growing interest in nutraceutical agent interventions due to their various medicinal properties with little to no side effects (Probst, Guan, & Kent, 2017).

In particular, red raspberries (*Rubus idaeus*) are unique compared to other berries due to their distinct phenolic profile and are good dietary sources of antioxidants (Cevik, Turker, & Kizilkaya, 2013). Their main components are ellagitannins and anthocyanins as well as vitamin C, manganese, fiber and other nutrients (Venketeshwer Rao & Snyder, 2010). Whole red raspberries and their extracts have been shown to have various potential health-promoting benefits including attenuating inflammation, altering lipid metabolism and restoring vascular-related abnormalities (Burton-Freeman, Sandhu, & Edirisinghe, 2016) (Teng et al., 2017). However, scarce research investigates the benefits of whole red raspberries (WRR) related to inflammation, dyslipidemia and vascular dysfunction in a valid *in vivo* model of the MetS.

The obese Zucker rat (OZR) is an ideal model for the MetS due to its similar multifactorial conditions to that of human diagnosed with MetS. The OZR has a mutation in the
leptin receptor gene (*Lepr*) (*fa/fa*). This mutation causes early-onset morbid obesity, hyperphagia, reduced energy expenditure, alteration of glucose homeostasis, dyslipidemia, and inflammation (Clément et al., 1998). At 8 weeks all the abnormalities associated with MetS are present in the OZR (Aleixandre de Artiñano & Miguel Castro, 2009). Thus, in this study we examined the role of WRR as a potential candidate to improve conditions associated with the MetS, in the OZR, a genetic model of the MetS.

Thus, the focus of this study is to explore the possible relationship between WRR consumption and conditions of the MetS, in particular inflammation, dyslipidemia and endothelial dysfunction in the obese Zucker rat, a model of the MetS.

The goal of this research was to examine the role of WRR as a potential candidate to attenuate inflammation, normalize lipid metabolism, and improve endothelial dysfunction associated with MetS, in the OZR. The objectives were to determine whether WRR have the potential to:

1. Attenuate inflammation by measuring concentrations and gene expression of pro-and anti-inflammatory markers in the plasma, liver and abdominal adipose tissue;

2. Improve blood lipid levels, gene expression involved in lipid metabolism in the liver and abdominal adipose tissue and hepatic structure and;

3. Improve endothelial dysfunction associated with MetS, by studying its aortic response to vasoconstriction, vasodilation, determine the metabolic pathways red raspberries modulate related to vascular tone and aortic gene expression associated with the MetS.
CHAPTER 2

2. LITERATURE REVIEW

2.1 Metabolic Syndrome

Metabolic syndrome (MetS) started as a concept rather than a diagnosis (Kaur, 2014). Since the 1920’s, literature demonstrates the association between obesity, atherogenic dyslipidemia, elevated blood pressure, elevated glucose and a pro-thrombotic and pro-inflammatory state (Table 1). Moreover, MetS increases an individual susceptibility to additional conditions such as fatty liver, sleep disturbances, cholesterol gallstones, polycystic ovary syndrome, asthma and cancer (S. M. Grundy, Brewer, Cleeman, Smith, & Lenfant, 2004). For an individual to be diagnose with MetS, they must present at least three of the above risk factors. MetS has been come to known as the constellation of interconnected physiological, metabolic, biochemical and clinical abnormalities that is directly linked to cardiovascular disease (CVD) and type 2-diabetes mellitus (T2D) (Kaur, 2014) (Figure 1). MetS is a major public health problem and is escalating quickly, both in developed and underdeveloped countries. MetS does not only affect the individual, but also increase risks for their offspring. Over 50% of women who enter pregnancy are classified as obese, resulting in short and long-term child abnormalities (Robson & Norman, 2017).
Table 1: Criteria for clinical diagnosis of the Metabolic Syndrome.\(^a\)

<table>
<thead>
<tr>
<th>Measure</th>
<th>Reference Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Elevated Waist Circumference</td>
<td>Male: (\geq 40) in. ((\geq 102)cm)</td>
</tr>
<tr>
<td></td>
<td>Female: (\geq 35) in. ((\geq 88)cm)</td>
</tr>
<tr>
<td>Elevated Triglycerides</td>
<td>(\geq 150) mg/dL</td>
</tr>
<tr>
<td>Reduced HDL-C</td>
<td>Male: &lt;40 mg/dL</td>
</tr>
<tr>
<td></td>
<td>Female: &lt;50 mg/dL</td>
</tr>
<tr>
<td>Elevated Blood Pressure</td>
<td>Systolic: (\geq 130) mmHg</td>
</tr>
<tr>
<td></td>
<td>Diastolic: (\geq 85) mmHg</td>
</tr>
<tr>
<td>Elevated Fasting Glucose</td>
<td>(\geq 100) mg/dL</td>
</tr>
</tbody>
</table>

Key:
- HDL-C: High-Density Lipoprotein Cholesterol
- mg/dL: Milligrams per Deciliter
- mmHg: Millimeter of Mercury

\(^a\)Diagnosis based on the of three of five factors (S. M. Grundy, 2016)

Obesity is excess body fat and is typically measured using body mass index or weight circumference (O’Neill & O’Driscoll, 2015). The American Heart Association reported that 154-157 million adults in the United States are overweight or obese (O’Neill & O’Driscoll, 2015).

Subcutaneous-abdominal obesity is metabolically more damaging due to the increase size, number of fat deposits and dysfunction adipocytes within the abdominal cavity which surrounds the major organs creating a greater risk for MetS (Nugent, 2004). Excess adipose contributes and influences of mediators including pro-inflammatory factors such as interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-\(\alpha\)), resistin, leptin, angiotensinogen, and plasminogen activator inhibitor -1 (S. M. Grundy, 2016). Additionally, dysfunctional adipocytes associated with obesity induces oxidative stress (Soares & Costa, 2009). Chronic imbalance of adipocyte function result in several risk factors such as: insulin resistance, hyperglycemia, elevated lipid profile (increase cholesterol, triglycerides and small, dense-LDL particles, decrease HDL-particles), hypertension, and atherosclerosis (Soares & Costa, 2009).
Obesity-induced inflammation have an unfavorable effect on lipid metabolism. Dyslipidemia is characterized as an abnormal amount of lipids, increased fatty acids, elevated triglycerides, decreased high-density lipoprotein (HDL)-cholesterol, and heightened levels of small, dense low-density lipoprotein (LDL) (O’Neill & O’Driscoll, 2015). Lipid abnormalities are associated with increased risk of cardiovascular events and fatty liver disease (Tóth, Potter, & Ming, 2012). According to the Nation Health and Nutrition Examination Survey, the prevalence of dyslipidemia is approximately 21% of adults in the United States (Tóth et al., 2012).

The combination of obesity-induced inflammation and dyslipidemia negatively influences the function of blood vessels resulting in vascular dysfunction. Vascular dysfunction contributes to atherosclerotic plaques leading to severe cardiovascular complications such as hypertension, stroke, ischemic heart disease, myocardial infarction or coronary syndromes (Figure 1) (Sitia et al., 2010). Each year approximately 1.5 million U.S. adults suffer from a heart attack or stroke (Ritchey, Wall, Gillespie, George, & Jamal, 2014).
2.2 Metabolic Syndrome and Inflammation

Inflammation is a major hallmark in obesity, atherosclerosis, dyslipidemia, T2D, and hypertension (Ouchi, Kihara, Funahashi, Matsuzawa, & Walsh, 2003). In most cases the underlining cause of the MetS is obesity (Gade, Schmit, Collins, & Gade, 2010). Obesity is associated with other medical conditions such as several forms of cancer (J. Park, Morley, Kim,

Increased pro-inflammatory markers, such as C-reactive protein (CRP), tumor necrosis factor (TNF)-alpha, interleukin-6 (IL-6), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and decreased anti-inflammatory markers such as adiponectin correlate with increased risks associated with MetS (Esser et al., 2014). Additionally, production of reactive oxygen species (ROS) is central to the progression of the inflammatory state (Belemets et al., 2017). These ROS act as a signaling molecule and a mediator of inflammation (Belemets et al., 2017). ROS that contribute to prolonged inflammation include superoxide anion, hydroxyl radical hydrogen peroxide and hypochlorous acid (Belemets et al., 2017).

There are three major sites that are implicated by inflammation: the liver, intestine and adipose depots (Mendrick et al., 2018). The release of inflammatory factors at one of these sites promotes an inflammation in neighboring tissues, thereby amplifying the inflammatory response and tissue dysfunction/damage (Mendrick et al., 2018). At sites of inflammation cytokines are recruited and macrophages differentiate (Baker, Hayden, & Ghosh, 2011). Two types of macrophages M1 and M2 are responsible for the inflammatory response based on function and cytokine expression (Figure 2) (Baker et al., 2011). The M1 macrophages produces pro-inflammatory cytokines such as IL-1, IL-6 and TNF-α (Baker et al., 2011). These M1 macrophages are responsible for chronic inflammation in metabolic diseases (Baker et al., 2011). For example, accumulation of M1 macrophages in the adipose tissue in the obese state of mice
and humans (Cinti et al., 2005). The second type of macrophage, M2, is responsible for wound healing responses, and secretes anti-inflammatory cytokines such as IL-4 and IL-10 (Baker et al., 2011). It has been previously reported that lower IL-10 levels are associated with obesity (Manigrasso et al., 2005), insulin sensitivity (Han, Patterson, Speck, Ehses, & Levings, 2014) and T2D (Mittal et al., 2014).

**Figure 2: Pro-inflammatory response to stimuli.**

Key: NF-κB (Nuclear Factor Kappa); TNF-α (Tumor Necrosis Factor-Alpha); IL-6 (Interleukin-6); CRP (C-Reactive Protein)

Figure modified from (Jung & Choi, 2014)
Nuclear Factor-κB - The central regulatory of the immune system and inflammation is the transcription factor, nuclear factor κB (NF-κB) (Baker et al., 2011). There are five structurally related members of NF-κB: NF-κB1 (p50), NF-κB1 (p52), RelA (p65), RelB, and c-Rel (Liu, Zhang, Joo, & Sun, 2017). The NF-κB protein is normally sequestered in the cytoplasm with a family of inhibitory proteins, I-Kappa B Kinase and activated by one of two major pathways, the canonical that leads to p50/RelA and P50/c-Rel dimers and noncanonical that leads to p52/RelB dimer (Liu et al., 2017)(M. Park & Hong, 2016). The canonical pathway responds to cytokine receptors, pattern-recognition receptors, tumor necrosis factor (TNF) receptors, T-cell and B-cell receptors (Liu et al., 2017). Expression of inflammatory cytokines, such as IL-1, IL-6, IL-12, TNF-α, cyclooxygenase-2 chemokines and additional inflammatory mediators are mediated by the recruitment of either M1 or M2 macrophages by NF-κB (Baker et al., 2011). This pathway activates an auto feedback loop, which results in increased levels of NF-κB activity (M. Park & Hong, 2016). The NF-κB signaling pathway plays a key role in a number of inflammatory diseases such as rheumatoid arthritis, inflammatory bowel disease, multiple sclerosis and MetS (M. Park & Hong, 2016). Thus, suppressing NF-κB activation is the focus of combating chronic inflammatory diseases.

Tumor Necrosis Factor-Alpha - Tumor necrosis factor – alpha (TNF-α) is typically not detectable in healthy individuals (Bradley, 2008). However, elevated levels of TNF-α are found in acute and chronic inflammatory conditions including trauma, rheumatoid arthritis, and MetS (Bradley, 2008). NF-κB activity regulates TNF-α transduction, also referred to as the TNF-NF-κB pathway (Bradley, 2008). The expression of TNF-α is triggered by binding to one of two receptors TNFR1 and TNFR2 (Popa, Netea, van Riel, van der Meer, & Stalenhoef, 2007). The
expression of TNF receptors are regulated by a number of stimuli including TNF, IL-1, IL-10 (Bradley, 2008). The signaling cascade leads to cell death, survival, differentiation, proliferation, and migration (Bradley, 2008). Circulating TNF-α is a major contributor in lipid modifications resulting in the development of atherosclerotic plaque through increase in leukocyte adhesion, transendothelial migration, and lipid modifications (Bradley, 2008)(Popa et al., 2007). Additionally, pro-inflammatory cytokine TNF-α contributes to impaired insulin sensitivity and increase production of leptin (Popa et al., 2007).

*Interleukin-6*- The cytokine IL-6 is elevated in an inflammatory state (Scheller, Chalaris, Schmidt-Arras, & Rose-John, 2011). IL-6 binds to its complex receptor consisting of the IL-6 binding type 1 transmembrane glycoprotein (IL-6R) and type 1 transmembrane signal transducer protein gp130 (CD130) (Scheller et al., 2011). Binding of IL-6 to its receptors leads to cell division, cell death, tumor formation, proliferation and migration (Scheller et al., 2011). IL-6 is responsible for the transition from acute to chronic inflammation by monocyte recruitment, angioproliferation and anti-apoptotic functions on T-cells (Gabay, 2006). Elevated IL-6 are elevated in several diseases including rheumatoid arthritis, Crohn’s disease, and the MetS (Tanaka, Narazaki, & Kishimoto, 2014).

*C- Reactive Protein*- Elevated levels of CRP are expressed during several inflammatory diseases such as rheumatoid arthritis, Crohn’s disease, and the MetS (Sproston & Ashworth, 2018). Human CRP has high affinity to phosphocholine residues, but it can also bind to autologous ligands include native and modified plasma lipoproteins, damaged cell membranes, phospholipids, and apoptotic cells (Pepys, Hirschfield, Pepys, & Hirschfield, 2003). Plasma CRP
is synthesized in the liver by hepatocytes (Sproston & Ashworth, 2018). The main inducer of the acute-phase response of CRP is IL-6 and to a lesser extent IL-1 and TNF-α, however, it is not sufficient to achieve this alone (Sproston & Ashworth, 2018).

Adiponectin- Adiponectin is an adipocyte-derived anti-inflammatory molecule that is abundantly present in human plasma (López-Jaramillo et al., 2014). Levels of adiponectin are negatively regulated by the accumulation of adipose tissue and positively associated with insulin sensitivity (López-Jaramillo et al., 2014)(Achari & Jain, 2017). The anti-inflammatory effect of adiponectin is activated when it binds to one of three receptors (AdipoR1, AdipoR2, and T-cadherin) (López-Jaramillo et al., 2014). Binding to receptors AdipoR1, abundant in skeletal muscle and AdipoR2, abundant in the liver result in increased hepatic and skeletal muscle fatty acid oxidation, increased skeletal muscle lactate production, reduced haptic gluconeogenesis, increased cellular glucose uptake, and inhibition of inflammation and oxidative stress (López-Jaramillo et al., 2014). Binding to T-cadherin in vascular endothelial cells inhibits oxidative stress-induced apoptosis (López-Jaramillo et al., 2014).

2.3 Metabolic Syndrome and Lipid Metabolism

The MetS is associated with atherogenic lipid profile consisting of reduced HDL-C, increased plasma triglyceride, apolipoprotein B, intermediate-density lipoprotein levels, and smaller, dense LDL particles (Nugent, 2004). Additionally, the MetS contributes to hepatic imbalance from excess lipid droplets due to impaired export of triglycerides in the form of VLDL, rise in fatty acid delivery, increase de novo lipogenesis, and decrease in oxidation of fatty acids in hepatocytes results in fatty liver (Arvind, Osganian, Cohen, & Corey, 2019). Under
normal physiological conditions LDL-C levels are normal or marginally elevated, however, individuals with the MetS have raised levels of small, dense LDL particles and oxidized LDL concentrations which are associated with increased risk of atherosclerosis (Nugent, 2004).

The liver plays a major role in lipid metabolism serving as the center for lipoprotein uptake, formation, and export into circulation, glucose and fatty acid metabolism (Figure 3) (Reddy & Rao, 2006). The uptake, transport and storage of lipids require numerous metabolic processes (Klop, Elte, & Cabezas, 2013). Following oral intake of dietary fats, triglycerides are lipolyzed in the intestinal lumen into free fatty acids and 2-monoacylglycerols and enter the enterocytes through passive diffusion or CD36 transporters (Klop et al., 2013). Dietary cholesterol enters the enterocytes through the Niemann-Pick C1 Like 1 Protein (Klop et al., 2013). Once in the enterocyte, triglycerides are reassembled and cholesterol is transformed into cholesterol-esters, both packed together into chylomicrons (Klop et al., 2013). Chylomicrons are released into the lymphatic system for circulation (Klop et al., 2013). The liver synthesizes very low-density lipoprotein (VLDL) containing the apoB100 protein, when dietary triglycerides and free fatty acid reach the liver (Klop et al., 2013). Chylomicrons and VLDL deliver free fatty acids to heart, skeletal muscle, and adipose tissue for energy and storage (Klop et al., 2013).

Several enzymes and proteins regulate these particles, such as lipoprotein lipase (LPL) which is stimulated by insulin and serves as a docking station and is the primary enzyme for triglyceride lipolysis in circulation (Klop et al., 2013). Eventually, chylomicron and VLDL shrink into chylomicron remnants and LDL, respectively both being taken up by the liver via multiple pathways (Klop et al., 2013). Alternatively, HDL particles are responsible for the uptake of excess free cholesterol from peripheral tissue and turning it back to the liver, this is referred to as the reverse cholesterol transport (Klop et al., 2013). During transport free cholesterol is esterified
into cholesterol-ester by lecithin-cholesterol acyltransferase within the HDL particle (Klop et al., 2013). Within circulation HDL particle exchanges cholesterol-esters for triglyceride from triglyceride-rich lipoprotein via cholesteryl ester-transfer-protein (Klop et al., 2013).

**Figure 3: Major pathways of lipoprotein metabolism.**

**Key:** CM (Chylomicron); HDL (High Density Lipoprotein); LDL (Low-Density Lipoprotein); IDL (Intermediate Density Lipoprotein); VLDL (Very Low-Density Lipoprotein).

Figure modified from (Helkin et al., 2016)

*Triglycerides*- Dietary fat, primarily in the form of triglycerides (TGs), are absorbed into the small intestine, incorporated into chylomicrons and released into the lymphatic system (Kersten, 2017). Lipoprotein lipase breakdowns TGs into fatty acids and are stored or used as fuel by fat and muscle cells (Kersten, 2017). Under physiological conditions, all chylomicrons are removed by the liver by binding to the LDL receptor, the LDL receptor-related protein, hepatic triglyceride lipase, and cell-surface proteoglycans (Miller et al., 2011). However, several factors
can reduce the fate of chylomicrons resulting in hypertriglyceridemia (Goldberg, Eckel, & McPherson, 2011). Hypertriglyceridemia has been associated with vascular disease such as atherosclerosis and increased cardiovascular events (Goldberg et al., 2011).

**Total Plasma Cholesterol-** Cholesterol in the body is either synthesized de novo within our cells or consumed from certain foods (Cerqueira et al., 2016). Cholesterol is a major sterol and plays a vital role in the functioning of cells as it is a major component of cell membranes and a precursor to several steroid hormones (Cerqueira et al., 2016). Cholesterol concentrations contribute to membrane fluidity where higher cholesterol content in cell membranes will be more rigid and packed whereas, less cholesterol will be more fluid (Cerqueira et al., 2016). Additionally, cholesterol is important for cell membrane endocytosis (Cerqueira et al., 2016). Cholesterol also serves as a precursor for bile acid, vitamin D, and steroids including androgens, estrogens, and progestogens (Cerqueira et al., 2016). Cholesterol itself cannot be dissolved in the bloodstream, therefore it is packaged into lipoproteins with other fats for transport (Cerqueira et al., 2016). When the ratio of cholesterol to lipids (other fats) in the lipoprotein is high, it is referred to as high-density lipoprotein (HDL), whereas when the ratio of cholesterol to lipids in the lipoprotein is small, it is referred to as low-density lipoprotein (LDL) (Cerqueira et al., 2016). High levels of cholesterol increases an individual’s risk of atherosclerosis and cardiovascular events when concentration of LDL is higher than HDL (Cerqueira et al., 2016).

**High-Density Lipoprotein Cholesterol-** High-Density Lipoprotein (HDL) is a carrier of free cholesterol from peripheral cells back to the liver referred to as reverse cholesterol transport (Lingyan Zhou, Li, Gao, & Wang, 2015). Apolipoprotein (Apo) A1 is the primary structural
protein of HDL (Lingyan Zhou et al., 2015). HDL particles are dynamic and can be divided into several sub-types based on density and size (Lingyan Zhou et al., 2015). HDL is secreted by the liver (~70-80% of total plasma HDL) and small intestines (Lingyan Zhou et al., 2015). When HDL reaches the liver, HDL receptors (SR-BI) located on hepatocytes, transport cholesterol from the particle into the liver (Lingyan Zhou et al., 2015). The cholesterol is then metabolized into bile acid which can be excreted as bile or in the feces (Lingyan Zhou et al., 2015). This process explains the anti-atherosclerotic effect of HDL (Lingyan Zhou et al., 2015).

**Very Low-Density Lipoprotein-** Lipid storage pool in the liver, de novo synthesis of fatty acids and phospholipids contribute to VLDL synthesis (Ramasamy, 2014). ApoB100 is the main protein of VLDL, once in the plasma VLDL is hydrolyzed by LPL that form intermediate (IDL) (Ramasamy, 2014). With further catabolism IDL becomes LDL which binds to LDL receptors of cells membranes in the clathrin-coated pits (Ramasamy, 2014). Receptor bound LDL is internalized by endocytosis and cholesterol is released into the cell (Ramasamy, 2014). The expression of the LDL receptor is regulated by both transcriptional and post-transcriptional levels of cholesterol (Ramasamy, 2014). Extensive clinical and epidemiological studies have demonstrated that increase levels of LDL is associated with development and progression of atherosclerosis and cardiovascular events (Crismaru et al., 2020).

**Fatty Acid Synthase-** The fatty acid synthase (FAS) enzyme is responsible for de novo synthesis of fatty acids used for energy storage, membrane assembly and repair, and secretion in the form of lipoprotein triglycerides (Jensen-urstad & Semenkovich, 2012). Hepatic fatty acid synthase is regulated by insulin, glucagon, cyclic AMP, fructose, glucose, and dietary fat (Jensen-urstad &
Semenkovich, 2012). In a fed state, hepatic FAS synthesizes lipids from stored lipid droplets or secreted VLDL. Alternatively, FAS promotes $\beta$-oxidation of fatty acids through PPAR$\alpha$ activation under nutrient-deficient conditions (Jensen-urstad & Semenkovich, 2012).

Microsomal Triglyceride Transfer Protein—Microsomal Triglyceride Transfer Protein (MTTP) is an intracellular protein that transfers lipids, triglycerides and cholesterol esters between phospholipid particles (Hussain, Rava, Walsh, Rana, & Iqbal, 2012). MTTP activity is primarily present in epithelial cells, small intestine and the liver (Hussain et al., 2012). MTTP is essential for the assembly of triglycerides in nascent ApoB-lipoproteins, ApoB binding, lipid transfer activity, and membrane association (Hussain et al., 2008).

ATP Binding Cassette Transporter A1—ATP Binding Cassette Transporter A1 (ABCA1) is a ubiquitous membrane protein that plays an important role in the formation of HDL particles, HDL metabolism and lipid clearance from foam cells (Lingyan Zhou et al., 2015). ABCA1 mediates the transport of cholesterol across cellular membranes to lipid-poor HDL particles (Yin, Liao, & Tang, 2013). Expression level of ABCA1 in cells is regulated by transcription or post-transcription levels (Yin et al., 2013).

Low Density Lipoprotein Receptor-Related Protein 1—The clearance of circulating atherogenic lipoprotein particles via the liver is regulated by low density lipoprotein receptor-related protein 1 (LRP1) (Van De Sluis, Wijers, Herz, Opin, & Author, 2017). Hepatic LRP1 binds ApoE rich particles such as TG rich chylomicron remnants and VLDL (Van De Sluis et al., 2017). LRP1 is
the only member of the LDL receptor gene family that is expressed at high levels in hepatocytes thus increasing the transport of ApoE rich lipoproteins (Van De Sluis et al., 2017).

*Peroxisome Proliferator-Activated Receptor Alpha*—Peroxisome Proliferator-Activated Receptor Alpha (PPARα) is ligand-activated transcription factor important for metabolism and inflammation (Kersten & Stienstra, 2017). PPARα is expressed at the highest levels in the liver followed by brown adipose tissue, kidney, heart, skeletal muscle and small intestines (Green & Wahli, 1994). PPARα promotes and initiates lipid uptake and catabolism of fatty acids via β-oxidation to produce ketone bodies (Jensen-urstad & Semenkovich, 2012).

*Peroxisome Proliferator-Activated Receptor Gamma*—Peroxisome Proliferator-Activated Receptor Gamma (PPARγ) exists in two isoforms PPARγ1 and PPARγ2 (L. Wang et al., 2014). PPARγ1 is expressed in adipose tissue, large intestines, hematopoietic cells, kidney, liver, muscles, pancreas and small intestine. PPARγ2 is primarily expressed in white and brown tissue (L. Wang et al., 2014). Overall, PPARγ regulates adipocytes differentiation, fatty acid and glucose metabolism, insulin sensitivity and inflammation (Vamecq & Latruffe, 1999).

*Acyl-CoA Oxidase 1*—The expression of Acyl-CoA Oxidase 1 (ACOX1) is a rate-limiting enzyme that is regulated by peroxisomes (Keller et al., 1993). ACOX1 is the first enzyme of β-oxidation, the pathway responsible for lipid catabolism (Nguyen et al., 2008). In peroxisomes, β-oxidation is responsible for the metabolism of very long chain fatty acids (Nguyen et al., 2008).
Acetyl-CoA acyltransferase- Acetyl-CoA acyltransferase (ACAT), also known as acetoacetyl-CoA thiolase is an enzyme that transforms cholesterol to cholesteryl esters (Chistiakov, Melnichenko, Myasoedova, Grechko, & Orekhov, 2017). The ACAT enzyme can be found in the mitochondria (ACAT1) or cytoplasm (ACAT2) (Chistiakov et al., 2017). Acetyl-CoA is derived from glucose, fatty acid, and amino acid catabolism (Shi & Tu, 2015).

Lipoprotein Lipase- Lipoprotein lipase is an enzyme responsible for the metabolism and transport of lipids (H. Wang & Eckel, 2009). LPL produced by many tissues including adipose tissue, cardiac and skeletal muscle, islets, and macrophages (H. Wang & Eckel, 2009). The LPL enzyme produces chylomicron remnants and intermediate LDL by the hydrolyzing TGs of chylomicrons and VLDL particles (H. Wang & Eckel, 2009). Additionally, LPL anchors lipoproteins to vessel walls for the uptake of lipoprotein particles, promotes lipid exchange between lipoproteins, acts as a ligand lipoprotein receptors for lipoprotein uptake and selectively facilitates the uptake of lipoprotein-associated lipids and lipophilic vitamins (H. Wang & Eckel, 2009). Thus, LPL regulates supply of fatty acids to various tissues for either storage or oxidation (H. Wang & Eckel, 2009).

2.4 Endothelial Dysfunction

The blood vessel is composed of three layers: intimal, medial and adventitia (Y. Zhao, Vanhoutte, & Leung, 2015). The intimal layer is made-up of a single layer of endothelial cells (Y. Zhao et al., 2015). This endothelial layer regulates blood vessel diameter by releasing endothelium-relaxant factor nitric oxide and endothelium-contracting factors proteinoids and peptides (Y. Zhao et al., 2015). The medial layer is made-up of vascular smooth muscle cells and
mediates constriction and dilation of blood vessels (Y. Zhao et al., 2015). Mechanical and/or pharmacological activation initiates the contraction of smooth muscle cells by increasing intracellular calcium either by the release of internal stores or influx into the cell via calcium channels (Y. Zhao et al., 2015). Intracellular calcium binds to calmodulin and activates myosin light chain kinase (MLCK) (Y. Zhao et al., 2015). The activated MLCK phosphorylates the myosin light chain and leads to a cross-chain between myosin head and actin filaments, resulting in contraction (Y. Zhao et al., 2015). The adventitia layer contains nerve endings, perivascular adipose tissue and connective elements (Y. Zhao et al., 2015).

The vascular endothelium is dynamic and has numerous functions including regulation of vascular tone, platelet aggregation, coagulation and fibrinolysis (Nugent, 2004). Thus, dysregulation of one or more of these functions can occur in the MetS resulting in endothelial dysfunction (Nugent, 2004). Hyperinsulinemia and elevated levels of circulating free fatty acids disturb the endothelium by impairing the loss of NO bioavailability in the vessel wall (Nugent, 2004). Damage to the wall initiates coagulation and inflammation further damaging the endothelial wall (Nugent, 2004). Thus, altered vascular tone and endothelial dysfunction increases clotting factors: fibrinogen, clotting factors VII and VIII, von Willebrand factor which result in a pro-thrombotic state and recruitment of inflammatory factors: IL-1, IL-6, TNF-α and CRP which result in mild chronic inflammation in individuals with the MetS, which can lead to hypertension (Nugent, 2004).

Vascular function is a major component of vascular health and disease. It is regulated by two major pathways: nitric oxide synthase (NOS) and cyclooxygenase (COX) (Figure 4) (Steinberg & Baron, 2002) (Kristo, Kalea, Schuschke, & Klimis-Zacas, 2013). In healthy blood vessels these pathways produce a balance of vasoactive mediators such as nitric oxide (NO),
thromboxane A2 (TXA\textsubscript{2}), and prostacyclin (PGI\textsubscript{2}) (Suganya, Bhakkiyalakshmi, Sarada, & Ramkumar, 2016). Nitric oxide, a product of NOS, is a vasodilator that suppresses vascular smooth muscle cell proliferation, blood cell adhesion, and lipid peroxidation (Kleinbongard et al., 2003)(Napoli et al., 2006). The COX pathway is responsible for prostanoid formation including TXA\textsubscript{2} and PGI\textsubscript{2}. Prostacyclin, is a vasorelaxant agent that acts synergistically with NO to inhibit platelet aggregation (Vanhoutte, 2003), whereas TXA\textsubscript{2} is an endothelium-dependent vasoconstrictor and a potent inducer of platelet aggregation (Paul, Jin, & Kunapuli, 1999). Alterations in the production of these endothelially-derived factors leads to vascular dysfunction which has been observed in individuals with MetS. Thus, initiating and promoting the pathogenesis of early vascular events such as atherosclerotic lesions (Tziomalos, Athyros, Karagiannis, & Mikhailidis, 2010)(Oak et al., 2018).
Nitric Oxide - In 1980, Furchgott & Zawadzki, first described endothelium-dependent relaxation of the blood vessels, later proposed to be nitric oxide (NO) (Furchgott & Zawadski, 1980). Nitric oxide is a soluble gas with a short half-life, that is synthesized by L-arginine in endothelial cells by the enzyme nitric oxide synthase (NOS) (Cannon III, 1998). Nitric oxide is a regulator of blood vessel tone, neurotransmission and modulation of the immune system and inflammation (Robbins & Grisham, 2003). There are three isoforms of nitric oxide: neuronal NOS (nNOS), cytokine-inducible NOS (iNOS) and endothelial NOS (eNOS) (Robbins & Grisham, 2003). An important aspect of the production of NO is the presence of tetrahydrobiopterin (BH₄) (Silva,
Paula, Paulo, & Bendhack, 2016). The cofactor BH₄ plays an important part in the oxidation of L-arginine for the production of NO (Silva et al., 2016). Depletion and/or oxidation of BH₄ by various diseases and environmental factors alters the function of NOS by acting as an oxidase rather than generating NO (Silva et al., 2016). The uncoupling of NOS generates superoxidase anion (O₂⁻) which reacts with NO to form highly reactive and cytotoxic reactive oxygen species (ROS) such as peroxinitrite (ONOO⁻) (Silva et al., 2016).

**Inducible Nitric Oxide Synthase-** Under normal physiological conditions the expression of iNOS is minimal but is increased during infection, chronic inflammation and in tumors (Y. Zhao et al., 2015). Activation of iNOS requires interferon regulatory factor 1 and NF-κB (Y. Zhao et al., 2015). Production of iNOS may contribute to vascular dysfunction by limiting the availability of BH₄, a critical cofactor for eNOS (Y. Zhao et al., 2015).

**Endothelial Nitric Oxide Synthase-** The eNOS isoform is responsible for the regulation of vascular function, in particular the endothelium-derived relaxing factor (Y. Zhao et al., 2015). The main source of NO in the vascular endothelium is via eNOS (Mónica, Bian, & Murad, 2016). The production of NO from eNOS is initiated by several stimuli such as acetylcholine, bradykinin, and histamine (Y. Zhao et al., 2015). Activation of eNOS results in an increase of intracellular concentration of calcium (Ca²⁺), the binding of Ca²⁺ to Ca²⁺/calmodulin complex which initiates the production of NO (Silva et al., 2016). Alternatively, shear stress generated by blood flow across endothelial cells activate eNOS without the direct increase of Ca²⁺ but rather by activating phosphatidylinositol 3-kinase and the subsequent Akt and protein kinase A pathway (Silva et al., 2016). Additionally, eNOS regulates suppression of vascular smooth
muscle cell proliferation, modulation of leukocyte-endothelial interactions and modulation of thrombosis (Huang, 2009).

\textit{Cyclooxygenase Pathway}- The cyclooxygenase (COX) pathway, releases long-chain polyunsaturated fatty acids, mainly arachidonic acid (20:4 n-6) from lipids in the cell membrane to produce tissue hormones (prostanoids) (Korbecki, Baranowska-Bosiacka, Gutowska, & Chlubek, 2014). Four main prostanoids \textit{in vivo} include: prostaglandin E$_2$, prostacyclin I$_2$, prostaglandin D$_2$, and prostaglandin F$_2$ (Ricciotti & Fitzgerald, 2011). The production of prostanoids changes due to the inflammation response. Low/uninflamed tissues produce generally low levels of prostanoids, whereas there is an increase in prostanoid production in acute inflammation (Ricciotti & Fitzgerald, 2011). The production of prostanoids is dependent on two main isoforms of COX are cyclooxygenase-1 (COX-1) and cylooxygenease-2 (COX-2) (Korbecki et al., 2014). COX-1 is constitutively expressed within tissues whereas COX-2 is induced when inflammation is present (Mitchell & Kirkby, 2019). However, both COX’s contribute to autoregulation and homeostatic production of prostanoids, thus both can contribute during inflammation (Ricciotti & Fitzgerald, 2011).

\textit{Cyclooxygenase-2}- Cyclooxygenase-2 (COX-2) expression is induced in monocytes/macrophages, vascular endothelial cells and colorectal cancer cells in response to inflammatory cytokines, laminar shear stress and growth factors (Patrono, 2016). COX-2 is the predominante source of prostaglandin formation in inflammation (Ricciotti & Fitzgerald, 2011). COX-2 is regulated by post-transcriptional and enzymatic level such as nitric oxide (Tsatsanis, Androulidaki, Venihaki, & Margioris, 2006).
Prostacyclin I2- Prostacyclin plays a major role in the cardiovascular system as a potent inhibitor of platelet aggregation, leukocyte adhesion, vascular smooth muscle cell proliferation, vasodilator, anti-mitogenic and inhibits DNA synthesis (Mitchell & Kirkby, 2019). These actions are mediated by the binding of Prostacyclin I2 to a rhodopsin-like seven transmembrane spanning G-protein receptor, PGI receptor (IP) (Ricciotti & Fitzgerald, 2011). The IP receptor is expressed in kidney, liver, lung, platelets, heart and aorta (Ricciotti & Fitzgerald, 2011). Major sources of PGI2 are endothelial cells, vascular smooth muscle cells and endothelial progenitor cells (Ricciotti & Fitzgerald, 2011). COX2 is the dominant source of PGI2 (Ricciotti & Fitzgerald, 2011). Once generated, PGI2 acts on neighboring vascular smooth muscle cells and is quickly converted to 6-keto-PGF₃α (Ricciotti & Fitzgerald, 2011).

Thromboxane A2- Thromboxane A2 is predominately derived from platelet COX1, and binds to the TP receptor (Ricciotti & Fitzgerald, 2011). The TP receptor couples with Gq/G12/13 and small G-proteins that regulate phospholipase C, Rho and adenylyl cyclase (Ricciotti & Fitzgerald, 2011). The TP receptor is expressed in platelets, smooth muscle and endothelial cells, lungs, kidneys, heart, thymus and spleen (Rucker & Dhamoon, 2019). Activation of the TP receptor results in platelet adhesion and aggregation, smooth muscle contraction and proliferation, and activation of endothelial inflammatory responses (Ricciotti & Fitzgerald, 2011). Thromboxane A2 has a 30-second half-life and is quickly converted to TXB₂ (Ricciotti & Fitzgerald, 2011). Due to the short half-life, TXA₂ acts in an autocrine or paracrine manner to activate adjacent platelets to amplify the response and produce more TXA₂ (Rucker & Dhamoon, 2019).
2.5 Red Raspberries (*Rubus idaeus*)

Red raspberry (*Rubus idaeus*), known as the “golden fruit” is a species of the *Rosaceae* family, originated in Europe, northwest Africa and western Asia (Teng et al., 2017)(Matkowski et al., 2014). Today, these cultivated berries are produced in United States Washington, Oregon, and California regions (Burton-Freeman et al., 2016). Many ancient references of red raspberry did not consider the berry as food, but instead plant parts used in folk medicine (Hummer, 2010). Raspberries were used as a medicinal plant by the native people soon after the Ice Age and harvested by the ancient Greeks as early as 370 BC (Hummer, 2010). Traditionally, the flesh has been used as anti-inflammatory and antimicrobial remedies, the shoots have been used to treat common cold, fever and flu-like infections and the leaves have been used to relieve menstrual cramps, diarrhea, chronic skin conditions and conjunctivitis (Matkowski et al., 2014).

Red raspberry have many cultivars mainly due to selection for good yield and pest resistance (Beekwilder, Hall, & De Vos, 2005). Raspberries contain essential minerals, vitamins, fatty acids, fiber and a range of phytochemicals that have been shown to have healthful properties (Venketeshwer Rao & Snyder, 2010). Red raspberries are low in calories providing 52 kcal per 100 grams of fresh fruit and high in dietary fiber providing 6.5 grams/ 100 grams of fruit (Table 2) (Venketeshwer Rao & Snyder, 2010). The seeds of raspberries contain healthy source of essential fats with 97.8% unsaturated fatty acids and low ratio (1.64) of omega-6 to omega-3 fatty acids, and fat-soluble vitamins, including carotenoids and tocopherols (Venketeshwer Rao & Snyder, 2010).
Red raspberries have a distinct phytochemical profile characterized primarily by ellagitannins and anthocyanins (Figure 5) (Beekwilder et al., 2005). Ellagitannins contribute to more than 50% of total antioxidant capacity (Beekwilder et al., 2005). Ellagitannins are hydrolyzable tannins making them more stable such as ellagic acid or gallic acid (Venketeshwer...
Rao & Snyder, 2010). Ellagitannins can be defined as monomers, oligomers, and complex polymers and have hexahydroxydiphenoyl (HHDP) esters of carbohydrates and cyclitols backbone that can include additional oxidative transformations (Figure 5) (Venketeshwer Rao & Snyder, 2010). The main ellagitannins in red raspberries are oligomers, lambertianin C and sanguin H6 which are trimers and tetramers, respectively that contain an ether link between a galloyl hydroxyl oxygen and an HHDP group (Beekwilder et al., 2005) (Venketeshwer Rao & Snyder, 2010). Ellagitannins are large molecules (Mw > 1000 Da) and contain between 1 and 3 gram per 100 gram of dry weight of fruit (Beekwilder et al., 2005). Ellagitannins are found in few berries such as cloudberry and raspberry with a lesser extent in strawberries (Beekwilder et al., 2005). Anthocyanins contribute about 25% are involved in the pigmentation of the fruit (Beekwilder et al., 2005). Anthocyanins are glycosylated polyhydroxy or polymethoxy derivative of 2-phenylbenzopyrylium that contain two benzoyl rings separated by a heterocyclic ring (Figure 6) (Venketeshwer Rao & Snyder, 2010). The main anthocyanins in red raspberries are cyanidin-3-O-sophoroside, cyanidin-3-O-(2⁵-glucosylrutinoside) and pelargonidin glycosides (Beekwilder et al., 2005). The average content of anthocyanins in red raspberries are 200 to 300 milligram per 100 gram dry weight of fruit (Beekwilder et al., 2005).
Red raspberries have an oxygen radical absorbance capacity (ORAC) value of 16 to 20 μmol (Trolox equivalents) per gram of fruit (Beekwilder et al., 2005). The ORAC value varies depending on stage of ripeness at harvest and storage conditions (Beekwilder et al., 2005). When the fruit transitions from pink to red anthocyanins accumulate, while ellagitannins diminish by 30% (Beekwilder et al., 2005). When raspberries are snap-frozen in liquid nitrogen the quality and quantity of antioxidants are not affected (Beekwilder et al., 2005). However, the positive health benefits of dietary antioxidants are only beneficial to the individual depending on bioavailability. Antioxidants are absorbed by the gut before circulating in the blood (Beekwilder et al., 2005). Due to the size of ellagitannins direct absorption in the gut may be detrimental, thus ellagitannins are hydrolyzed into ellagic acid which is quickly converted into urolithin B by colon microbes (Beekwilder et al., 2005). Anthocyanin are typically unchanged from their
dietary form, thus indicating that anthocyanins are metabolized in the body, and are not effectively absorbed by the digestive system (Beekwilder et al., 2005).

![Figure 6: Chemical structures of major polyphenols found in red raspberry.](image)

<table>
<thead>
<tr>
<th>Ellagitannins</th>
<th>Anthocyanins</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lambertianin C</td>
<td>Cyanidin-3-O-Sophoroside</td>
</tr>
<tr>
<td>Sanguin H-6</td>
<td>Cyanidin-3-O-(2β-glucosylrutinoside)</td>
</tr>
</tbody>
</table>

2.6 Red raspberries and phytochemical compounds

Red raspberry health benefits have been documented in several studies. Previous research used whole red raspberry and red raspberry fractions in different *in vitro* and *in vivo* models for various health conditions (Table 3), sorted by publication date. However, limited studies have
demonstrated the effect of whole red raspberries on the MetS, inflammation, lipid metabolism and endothelial dysfunction in a model of the MetS.

### Table 3: Review of red raspberry literature sorted by publication date.

<table>
<thead>
<tr>
<th>Author</th>
<th>Amount</th>
<th>Red Raspberry</th>
<th>Duration</th>
<th>Model</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Bartosz Fotschki et al., 2019)</td>
<td>7%</td>
<td>Raspberry Pomace</td>
<td>8 Weeks</td>
<td>Wistar Rats</td>
<td>Increased plasma HDL-C; decreased atherogenic index and plasma glucose level</td>
</tr>
<tr>
<td>(Xue, Du, &amp; Zhu, 2019)</td>
<td>10 or 15 μg/mL</td>
<td>Raspberry Extract</td>
<td>4 Hours</td>
<td>Caco-2 cells infected with <em>E. coli</em> induced NLRP3 inflammasome</td>
<td>Reduced inflammation and oxidative stress</td>
</tr>
<tr>
<td>(D. Xiao et al., 2019)</td>
<td>125g (~1 cup) or 250g (~2 cups)</td>
<td>Frozen Red Raspberry</td>
<td>Single Dose (Breakfast)</td>
<td>Adults (overweight or obese with prediabetes and insulin resistance)</td>
<td>Reduced 2-hour insulin, peak insulin, peak glucose, 2-hour glucose, and postprandial TG</td>
</tr>
<tr>
<td>(V. Khan et al., 2019)</td>
<td>100 or 200 mg/kg</td>
<td>Raspberry Ketones</td>
<td>28 Days</td>
<td>Wistar Rats induced with isoproterenol (ISO)-induced cardiotoxicity</td>
<td>Protected against oxidative stress, inflammation and dyslipidemia. RK had binding affinity with PPARα (docking analysis); increase expression and concentration of PPARα</td>
</tr>
</tbody>
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</tr>
</thead>
<tbody>
<tr>
<td>(A. Al-Salmi, 2019)</td>
<td>300 mg/kg$^{-1}$</td>
<td>Red Raspberry</td>
<td>30 Days</td>
<td>Mice-Acrylamide (ACR)</td>
<td>Prohibited against ACR-induced liver damage</td>
</tr>
<tr>
<td>(P. W. Wang et al., 2019)</td>
<td>750 $\mu$g/mL</td>
<td>Red Raspberry Extract</td>
<td>5 Days</td>
<td>Nude Mice: UVB Exposure</td>
<td>Alleviated photodamage to the skin by UVB exposure through the ROS scavenger and protection against inflammatory response by abolishing cells apoptotic signaling pathways (caspase-3, c-jun) and activation of NF-kB and COX2</td>
</tr>
<tr>
<td>(X. Zhang, Sandhu, Edirisinghe, &amp; Burton-Freeman, 2018)</td>
<td>25 or 50 mg/mL</td>
<td>Red Raspberry Fruit</td>
<td>24, 48, 72 or 96 Hours</td>
<td>Hepatocellular carcinoma cells</td>
<td>Inhibited the proliferation of HCC cells by regulating the PTEN/AKT signaling pathway (cellular processes involved in growth, proliferation, and metastasis)</td>
</tr>
<tr>
<td>(Le Zhou, Yao, Song, et al., 2018)</td>
<td>50 $\mu$M</td>
<td>Neolignans from Red Raspberry</td>
<td>1 Hour</td>
<td>SH-SY5Y Cells</td>
<td>Protective effects against H2O2 induced neurotoxicity, in addition to</td>
</tr>
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</table>
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</tr>
</thead>
<tbody>
<tr>
<td>(Bartosz Fotschki, Laparra, &amp; Sójka, 2018)</td>
<td>5%</td>
<td>Raspberry Polyphenol Extract</td>
<td>3 Hours</td>
<td>Wister Rats (hepatocytes)</td>
<td>attenuating H2O2 apoptosis, ROS generation, and mitochondrial dysfunction</td>
</tr>
<tr>
<td>(Gao et al., 2018)</td>
<td>1, 10 or 100 (\mu)g/ml</td>
<td>Red Raspberry</td>
<td>72 Hours</td>
<td>Human Dermal Fibroblasts (UVB treatment)</td>
<td>Regulated obesity-related signals by modulating immune-metabolic mechanisms</td>
</tr>
<tr>
<td>(Istas et al., 2018)</td>
<td>200 or 400 g</td>
<td>Red Raspberry</td>
<td>Single Dose</td>
<td>Healthy Males</td>
<td>Prevented UVB induced skin photoaging</td>
</tr>
<tr>
<td>(H. Zhang et al., 2018)</td>
<td>5%</td>
<td>Red Raspberry</td>
<td>10 Weeks</td>
<td>WT Mice and Mice Deficient in the catalytic subunit (a1) of AMPK</td>
<td>Improved endothelial function. Flow mediated dilation was significantly improved up to 24h after consumption. Ellagitannin metabolites were detected in plasma at 2 and 24 hours.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Reduced ectopic lipid storage, alleviated inflammatory response, improved insulin sensitivity and promoted</td>
</tr>
</tbody>
</table>
Table 3: Review of red raspberry literature sorted by publication date.

<table>
<thead>
<tr>
<th>Author</th>
<th>Amount</th>
<th>Red Raspberry</th>
<th>Duration</th>
<th>Model</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Bibi, Du, &amp; Zhu, 2018)</td>
<td>5%</td>
<td>Red Raspberry</td>
<td>10 Weeks</td>
<td>C57 Mice induced with DSS (induced colitis)</td>
<td>Reduced disease activity index score, expression of inflammatory mediators (IL-6 and COX2), infiltration of CD4 T Cells and adhesion molecules and repaired epithelium repair.</td>
</tr>
<tr>
<td>(L. Zhao et al., 2018)</td>
<td>5%</td>
<td>Red Raspberry</td>
<td>10 Weeks</td>
<td>WT Mice and Mice Deficient in the catalytic subunit (α1) of AMPK</td>
<td>Reduced ectopic lipid storage, inflammation response and improved insulin sensitivity</td>
</tr>
<tr>
<td>(S. Khan et al., 2018)</td>
<td>5%</td>
<td>Red Raspberry</td>
<td>12 Weeks</td>
<td>C57BL/6J Mice induced high-fat diet</td>
<td>Decreased hypertrophy, pro-inflammatory cytokine expression and macrophage infiltration in white adipose tissue and</td>
</tr>
</tbody>
</table>
Table 3: Review of red raspberry literature sorted by publication date.

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<thead>
<tr>
<th>Author</th>
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<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Wu et al., 2018)</td>
<td>200 mg/kg</td>
<td>Raspberry Anthocyanins</td>
<td>12 Weeks</td>
<td>C57BL/6J Mice induced high-fat diet</td>
<td>Improved insulin sensitivity, Elevated superoxide dismutase and glutathione peroxidase, down-regulated inflammatory status in serum, improved hepatic lipid profile, and promoted recovery of metabolites involved in glycerophospholipid metabolism</td>
</tr>
<tr>
<td>(Zhu et al., 2018)</td>
<td>5%</td>
<td>Red Raspberry</td>
<td>12 Weeks</td>
<td>Wild Type Mice (High-fat induced)</td>
<td>Improved insulin resistance, reduced hepatic IL-1B and IL-18, reduced hepatic lipid accumulation, and suppressed NLRP3 inflammasomes</td>
</tr>
<tr>
<td>(Kirakosyan et al., 2018)</td>
<td>2%</td>
<td>Red Raspberry</td>
<td>12 Weeks</td>
<td>Zucker Fatty Rats</td>
<td>Reduced fasting TG, glucose, heart rate, left ventricular enlargement and wall thickening, up-regulated myocardial adiponectin receptor</td>
</tr>
<tr>
<td>Author</td>
<td>Amount</td>
<td>Red Raspberry</td>
<td>Duration</td>
<td>Model</td>
<td>Results</td>
</tr>
<tr>
<td>---------------------------------------------</td>
<td>-----------------</td>
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<td>-------------------------------------------------------------------------</td>
</tr>
<tr>
<td>(Bibi, Kang, Du, &amp; Zhu, 2018)</td>
<td>5%</td>
<td>Red Raspberry</td>
<td>6 Weeks</td>
<td>DSS-induced mice</td>
<td>Protected against inflammation and colitis systems induced by DSS</td>
</tr>
<tr>
<td>(V. Khan, Sharma, Bhandari, Ali, &amp; Haque, 2017)</td>
<td>100 or 200 mg/kg</td>
<td>Raspberry Ketones</td>
<td>28 Days</td>
<td>Wistar Rats induced with isoproterenol-induced cardiotoxicity</td>
<td>Reduced cardiac marker enzymes and pro-inflammatory markers, altered lipid profile, improved antioxidant status</td>
</tr>
<tr>
<td>(Luo, Miranda-Garcia, Sasaki, &amp; Shay, 2017)</td>
<td>2.5% kcal</td>
<td>Red Raspberry Juice concentrate</td>
<td>10 Weeks</td>
<td>C57 Mice (High-Fat diet induced)</td>
<td>Altered heme oxygenase 1 (HMOX1) and hormone sensitive lipase</td>
</tr>
<tr>
<td>(Tsai et al., 2017)</td>
<td>50, 150, 300 or 400 μM</td>
<td>Raspberry Ketone</td>
<td>2 Days</td>
<td>3T3-Preadipocytes</td>
<td>RK may exert anti-adipogenic effects through modulation of the HO-1/Wnt/beta-catenin signaling pathways</td>
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<tr>
<td>Author</td>
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<td>Duration</td>
<td>Model</td>
<td>Results</td>
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<tr>
<td>(G. D. Noratto, Chew, &amp; Atienza, 2017)</td>
<td>5.3%</td>
<td>Red Raspberry</td>
<td>8 Weeks</td>
<td>Obese diabetic mice</td>
<td>RR improved antioxidant status and decreased plasma IL-6, enhanced glutathione peroxidase in liver and blood.</td>
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<tr>
<td>(Sousa et al., 2016)</td>
<td>10, 25 or 50 μg GAE/mL</td>
<td>Red Raspberry</td>
<td>16 Hours</td>
<td>Human microvascular endothelial cells</td>
<td>Decrease in Phos-VEGFR2 expression, rearrangement of filamentous actin cytoskeleton</td>
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<tr>
<td>(Luo, Miranda-Garcia, Adamson, Sasaki, &amp; Shay, 2016)</td>
<td>10% kcal (juice and puree concentration) 0.2% w/w (Purified phytochemicals)</td>
<td>Red Raspberry Juice Concentrate Red Raspberry Puree Ellagic Acid Raspberry Ketones</td>
<td>10 Weeks</td>
<td>C57 Mice</td>
<td>Decreased body weight, resistin levels were decreased and histological differences</td>
</tr>
<tr>
<td>(G. Noratto, Chew, &amp; Ivanov, 2016)</td>
<td>5.3%</td>
<td>Red Raspberry</td>
<td>8 Weeks</td>
<td>Obese diabetic mice</td>
<td>Decrease oxidative stress and inflammatory stress</td>
</tr>
<tr>
<td>(B Fotschki, Jurgoński, Juśkiewicz, &amp;</td>
<td>7%</td>
<td>Raspberry Seed Oil</td>
<td>8 Weeks</td>
<td>Wistar Rats</td>
<td>Decreased plasma alanine and aspartate transaminase</td>
</tr>
<tr>
<td>Author</td>
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<td>Red Raspberry</td>
<td>Duration</td>
<td>Model</td>
<td>Results</td>
</tr>
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<td>------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Zduńczyk, 2015)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>activities, plasma levels of TNF-α and TG concentrations. Fat concentration decreased in liver.</td>
</tr>
<tr>
<td>(K. S. Park, 2015)</td>
<td>10 or 20 μM</td>
<td>Red Raspberry Ketones</td>
<td>4 Days (Maturing pre-adipocytes)</td>
<td>3T3-L1 pre-adipocytes</td>
<td>Suppressed adipocyte differentiation, fat accumulation, adipogenesis pathway genes</td>
</tr>
<tr>
<td>(Li et al., 2014)</td>
<td>100, 150 or 200 μg/mL</td>
<td>Red Raspberries (crude extracts, anthocyanin)</td>
<td>24 Hours (Mature Adipocytes)</td>
<td>RAW264.7 macrophages (LPS stimulated)</td>
<td>Reduced expression of iNOS, COX2, IL-1B, IL-6. Pathways: NF-κB by decreasing phosphorylation of IKK</td>
</tr>
<tr>
<td>(Pieszka, Tombarkiewicz, &amp; Roman, 2013)</td>
<td>0.8 ml</td>
<td>Red Raspberry Seed Oil</td>
<td>5 Weeks</td>
<td>Wister Rats</td>
<td>No change in lipid profile, reduced SOD and GPx</td>
</tr>
<tr>
<td>(Puupponen-Pimiä et al., 2013)</td>
<td>100 g</td>
<td>Red Raspberries</td>
<td>16 Weeks</td>
<td>MetS Humans</td>
<td>No change in lipid profile, blood pressure</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Berry bioavailability dependent to composition of gut microbiota</td>
</tr>
<tr>
<td>Author</td>
<td>Amount</td>
<td>Red Raspberry</td>
<td>Duration</td>
<td>Model</td>
<td>Results</td>
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<tr>
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<td>-------------------------------------------------------------------------</td>
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<tr>
<td>(Jean-Gilles et al., 2012)</td>
<td>30 or 120 mg/kg</td>
<td>Red Raspberry Polyphenolic Extract</td>
<td>30 Days</td>
<td>Antigen-induced arthritis rat model</td>
<td>Cartilage protection</td>
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<tr>
<td>Chen et al (2011)</td>
<td>0.75, 1.5 or 3.0 g/kg</td>
<td>Red Raspberry Extract</td>
<td>20 Weeks</td>
<td>Hepatic tissues induced with DEN</td>
<td>Extract inhibited cell proliferation, VEGF expression, and induced apoptosis in hepatic lesion tissues</td>
</tr>
<tr>
<td>(Torres-Urrutia et al., 2011)</td>
<td>1 mg/mL</td>
<td>Red Raspberry extract</td>
<td>Single Dose</td>
<td>Blood samples of healthy donors</td>
<td>Anti-thrombotic</td>
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<tr>
<td>(Suh et al., 2011)</td>
<td>275 mL/by a 70kg man</td>
<td>Red Raspberry Juice</td>
<td>12 Weeks</td>
<td>Hamsters induced with an atherogenic diet</td>
<td>Decreased lipid profile and oxidative stress by increasing liver GPx and SOD</td>
</tr>
<tr>
<td>(Jia et al., 2011)</td>
<td>100 or 200 mg/kg</td>
<td>Red Raspberry Extract</td>
<td>5 Weeks</td>
<td>Spontaneously hypertensive rat</td>
<td>Decreased blood pressure, increased NO, antioxidation</td>
</tr>
<tr>
<td>(Aiyer et al., 2008)</td>
<td>5%</td>
<td>Red Raspberry and Ellagic Acid</td>
<td>3-weeks</td>
<td>CD-1 mice</td>
<td>Reduced endogenous oxidative DNA damage by increasing DNA repair mechanism</td>
</tr>
<tr>
<td>(McDougall et al., 2005)</td>
<td>18, 25.5, 42, 87 or 145 µg</td>
<td>Red Raspberry Extracts</td>
<td>Single Dose</td>
<td>Starch Assays</td>
<td>Inhibit α-amylase and α-glucosidase</td>
</tr>
</tbody>
</table>
Table 3: Review of red raspberry literature sorted by publication date.

| Author                        | Amount          | Red Raspberry       | Duration | Model                                         | Results                                                        |
|-------------------------------|-----------------|---------------------|----------|-----------------------------------------------|                                                               |
| (Morimoto et al., 2005)       | 0.5, 1, 2 %     | Raspberry Ketone    | 6 Weeks  | Mice induced by high fat diet                 | Decreased and prevented elevation of body weight, liver and adipose tissue |
| (Mullen et al., 2002)         | 24 μL           | Raspberry Extract   | Single Dose | Aorta rings                                    | Increased vasodilation                                          |

2.6.1 Red raspberry effect on inflammation

In Table 3, numerous studies have demonstrated that red raspberries can attenuate inflammation. Inflammatory response and oxidative stress were hindered in NOD-, LRR- and pyrin domain-containing protein 3 (NLRP3) inflammasome induced Caco-2 cells exposed to raspberry extract (Xue et al., 2019). Raspberry ketones (100 and 200 mg/kg) for 28 days protected Wistar rats induced with isoproterenol-cardiotoxicity against oxidative stress and inflammation (V. Khan et al., 2019). Red raspberry extract abolished oxidative damage in DNA and altered the inflammatory cascade of UVB-induced human epidermal keratinocytes and a nude mouse model (P. W. Wang et al., 2019). Similarly, red raspberry extract (1, 10, 100 μg/ml) reduced pro-inflammatory markers IL-6, NF-κB and MMP-1 production of UVB-induced photoaging in normal human dermal fibroblasts (Gao et al., 2018). Neolignans from red raspberry (50 μm) protected SH-SY5Y cells against reactive oxygen species generation and mitochondrial dysfunction (Le Zhou, Yao, Lu, et al., 2018b). Supplementation of 5% red raspberry diet reduced inflammatory mediators IL-1β, IL-6, IL-7, COX2, TNF-α and suppressed
NF-κB signaling in C57 mice induced with chronic colitis with dextran sulfate sodium (Bibi, Du, et al., 2018) (Bibi, Kang, Du, & Zhi, 2018). A 5% red raspberry diet fed to obesity induced wild-type mice and mice deficient in the catalytic subunit (α1) of AMP-activated protein kinase for 10-weeks alleviated the inflammatory response (L. Zhao et al., 2018). Raspberry anthocyanin (200 mg/kg) for 12-weeks elevated superoxide dismutase and glutathione peroxidase and down regulated hepatic expression of pro-inflammatory factors TNF-α, IL-6, NF-κB in obesity-induced C57BL/6J mice (Wu et al., 2018). Supplementation of 5% freeze dried raspberry for 12-weeks attenuated pro-inflammatory cytokine expression and macrophage infiltration in white adipose tissue and attenuated adipocyte hypertrophy in C57BL/6J mice on a high-fat diet (S. Khan et al., 2018) Obese diabetic mice fed a 5.3% red raspberry diet for 8-weeks improved antioxidant status and decreased circulating levels IL-6 and enhanced glutathione peroxidase in the liver and blood (G. D. Noratto et al., 2017) (G. Noratto et al., 2016). Raspberry ketone for 10-weeks decreased body weight and food efficiency obesity in C57BL/6J mice fed a high-fat diet (Luo et al., 2016). Obesity induced Wistar rats assigned to raspberry seed oil (7%) for 8-weeks decreased plasma TNF-α levels compared to the control group (B Fotschki et al., 2015).

Similarly, raspberry ketone suppressed adipocyte differentiation, fat accumulation and adipogenesis pathway genes in 3T3-L1 pre-adipocyte (K. S. Park, 2015). RAW264.7 macrophages stimulated with lipopolysaccharide/IFN-γ exposed to red raspberry crude extracts reduced expression levels of IL-1β, IL-6 and inhibited NF-κB activation (Li et al., 2014). Male Wistar rats administered a daily dose of 0.8 ml red raspberry seed oil for 5-weeks reduced oxidative stress (Pieszka et al., 2013). Superoxide dismutase and paraoxonase activity was increased in Syrian hamsters receiving an atherogenic diet with supplementation of red raspberry juice (equivalent to 218-305 μg/mL-1 and 45-72 μg/mL-1 anthocyanins and ellagitannins,
respectively) for 12-weeks (Suh et al., 2011). Red raspberry (3%) for 3-weeks reduced endogenous oxidative DNA damage in CD1-Mice (Aiyer et al., 2008). Raspberry ketones (0.5, 1 and 2%) for 6-weeks decreased body weight and visceral adipose tissues (epididymal, retroperitoneal, and mesenteric) in male ICR mice fed a high-fat diet (Morimoto et al., 2005).

2.6.2 Red raspberry effect on lipid metabolism

In Table 3, several studies have demonstrated that red raspberries can alter lipid metabolism. Raspberry pomace fed to Wistar rats for 8-weeks affected intestinal microbial activity, reduced short-chain fatty acid production in the caecum, increased plasma HDL-C levels, decreased atherogenic index, and decreased glucose levels (Bartosz Fotschki et al., 2019). Frozen red raspberries (125 g or 250 g) reduced 2-hour insulin and glucose, peak insulin and glucose in overweight or obese adults with prediabetes and insulin resistance (D. Xiao et al., 2019). Orally administered raspberry ketones (100 and 200 mg/kg) for 28 days protected rats against dyslipidemia, increased expression and concentration of PPAR-α and raspberry ketones demonstrated to have binding affinity with PPAR-α (V. Khan et al., 2019). Acrylamide induced-Wistar rats treated with intraperitoneal injection of red raspberry (300mg/kg) inhibited liver damage by restoring lipid profile and prevented liver toxicity (A.Al-Salmi, 2019). Hepatocellular carcinoma cells induced with red raspberry fruit (25 and 50 mg/ml) and raspberry ketone (200 μg/ml) inhibited hepatic cell proliferation by regulating PTEN/AKT signaling pathway (H. Zhang et al., 2018). Hepatocytes treated with plasma collected from Wistar rats that were fed a combination of a high-fat diet and raspberry polyphenolic extract regulated obesity-related signals in hepatocytes (Bartosz Fotschki et al., 2018). C57BL/6J mice assigned to a single daily dose of raspberry anthocyanin (200 mg/kg⁻¹) for 12-weeks reduced serum and hepatic lipid
profile (Wu et al., 2018). A 2% red raspberry diet for 12-weeks reduced fasting triglycerides and glucose, up-regulation of ApoE and down-regulation of lipoprotein lipase in Zucker Fatty rats (Kirakosyan et al., 2018). Raspberry ketone (300-400 μM) reduced PPARγ, fatty acid synthase, fatty acid-binding protein 4 expression in 3t3-L1 adipocytes (Tsai et al., 2017). Consumption of four servings of 10% kcal equivalent of raspberry juice concentrate and raspberry puree concentrate per day for 10-weeks decreased hepatic lipid accumulation and altered heme oxygenase 1 and hormone sensitive lipase in C57BL/6J mice fed a high-fat diet (Luo et al., 2017). Wistar rats induced with isoproterenol restored lipid profile by decreasing total cholesterol, triglyceride, LDL, VLDL and increasing HDL with 100 and 200 mg/kg of raspberry ketones for 28 days (V. Khan et al., 2017). Red Raspberry seed oil (7%) assigned to Wistar rats on a high-fat diet for 8-weeks attenuated plasma alanine and aspartate transaminase activities, triglyceride concentrations and decreased hepatic fat accumulation (B Fotschki et al., 2015). Atherogenic diet-induced gold Syrian hamsters consuming raspberry juice (equivalent to 218-305 μg/mL⁻¹ and 45-72 μg/mL⁻¹ anthocyanins and ellagitannins, respectively) for 12-weeks lowered plasma triglyceride, total cholesterol, and LDL-C levels and increased HDL-C (Suh et al., 2011). Using a starch assay, red raspberry extract inhibited α-amylase and α-glucosidase (McDougall et al., 2005). Hepatic triacylglycerol content was attenuated and norepinephrine-induced lipolysis was increased with raspberry ketones (0.5, 1 and %) for 6-weeks of obesity-induced ICR mice (Morimoto et al., 2005).

2.6.3 Red raspberry effect on endothelial dysfunction

In Table 3, many studies have demonstrated that red raspberries can restore vascular function. Raspberry ketones (100 and 200 mg/kg) for 28-days altered electrocardiogram patterns,
infarct size, heart weight to body weight ratio and immunohistochemical assessments of isoproterenol-induced Wistar rats (V. Khan et al., 2019). Raspberry consumption (200 and 400 g containing 204 or 403 mg of total (poly)phenols, respectively) improved endothelial function healthy adult males (Istas et al., 2018). Zucker fatty rats fed a 2% red raspberry for 12-weeks resulted in a reduced left ventricular enlargement and wall thickening, altered nicotinamide phosphoribosyl transferase expression and upregulation in myocardial adiponectin receptor 1 (Kirakosyan et al., 2018). Red raspberry phenolic compounds rearranged filamentous actin cytoskeleton and decreased phosphorylated-VEGF2 expression of human microvascular endothelial cells (Sousa et al., 2016). Lipopolysaccharide-induced RAW264.7 macrophages treated with red raspberry crude extracts and anthocyanins demonstrated reduced expression if iNOS and COX2 compared to the control group (Li et al., 2014). Blood samples of healthy donors were anti-thrombotic when exposed to red raspberry extract (1mg/mL) (Torres-Urrutia et al., 2011). Aortic rings exposed to a single dose of raspberry extract increased vasodilation response (Mullen et al., 2002).

In conclusion, a plethora of studies demonstrate the positive relationship between red raspberry and health. However, these studies do not investigate the benefits of WRR related to the MetS in a valid in vivo model.

2.7 The Obese Zucker Rat a model of the metabolic syndrome

The obese Zucker rat (OZR) is an ideal model for MetS due to its similar multifactorial conditions to that of human MetS and has been cited and used as an experimental model in several studies. The OZR has a mutation in the leptin receptor gene (Lepr) (fa/fa). The fa mutation was discovered by Lois Zucker in 1961, using a cross between Sherman and Merck
stock M rats (Kava, Greenwood, & Johnson, 2017). Leptin is produced by the adipose tissue and regulates energy balance (Aleixandre de Artiñano & Miguel Castro, 2009). Leptin production correlates with the amount of lipid stored and binds to the leptin receptors of the brain, regulating food intake and energy expenditure (Aleixandre de Artiñano & Miguel Castro, 2009). Thus, this mutation causes early-onset morbid obesity, mild hypertension, reduced energy expenditure, insulin resistance, dyslipidemia, and inflammation (Clément et al., 1998). These characteristics of the MetS are present at eight weeks of age (Clément et al., 1998). The OZR is hyperphagic compared to their lean littermates as early as 17 days and continues particularly during the growth period during the first 16 weeks of life (Kava et al., 2017). Obesity is present by three weeks of age and by 14 weeks the body composition of the OZR is approximately 40% weight lipid (Aleixandre de Artiñano & Miguel Castro, 2009). The excessive fat tissue of the OZR is associated with abnormal production and over expression of pro-inflammatory mediators including IL-1, IL-6, TNF-α, CRP, and monocyte chemotactic protein-1 (Aleixandre de Artiñano & Miguel Castro, 2009).

In addition to obesity, OZR displays elevated plasma levels of triglycerides, plasma cholesterol and free fatty acids, and overproduction of hepatic VLDL, which can be observed shortly after weaning (Zucker, 1965). The increase in triglyceride concentration in the plasma is due to the accumulation of VLDL, and the increase in plasma cholesterol is due to the presence of cholesterol in the VLDL and HDL fractions (Aleixandre de Artiñano & Miguel Castro, 2009). The excess serum cholesterol in the male obese Zucker rat is transported by HDL as cholesterol ester (Lin & Lin, 1985). Adipose tissue activity is correlated with enhanced triglyceride uptake via enhanced lipoprotein lipase activity resulting in increased liver lipogenesis and hyperinsulinemia (Aleixandre de Artiñano & Miguel Castro, 2009). Whereas, the lipid profile of
LZR is similar to that of the Sprague-Dawley and Wistar rats (Aleixandre de Artiñano & Miguel Castro, 2009).

The combination of elevated pro-inflammatory mediators and altered lipid profile are major contributors to the development of endothelial dysfunction and increased vascular oxidative stress in the OZR (Sista et al., 2019). Endothelium dependent vasodilators are impaired whereas constrictors are enhanced in the OZR (Frisbee & Delp, 2006). In the OZR, these alterations can reduce blood flow under resting condition or elevate in metabolic demand (Frisbee & Delp, 2006). These documented characteristics of the OZR validate the animal as an ideal model of the MetS.
CHAPTER 3

3. MATERIALS AND METHODS

3.1 Animals

Two sets of animals were used. At 8 weeks of age, 32 obese Zucker rats (OZR) (fa/fa) and 32 lean controls (LZR) (Fa/Fa) were purchased from Charles River Laboratories (Raleigh, North Carolina). The rats were housed individually in stainless steel mesh-bottom cages in the Small Animal Facility at the University of Maine in an environmentally controlled room maintained at room temperature and light cycle (12:12 hours light:dark). Body weights were measured weekly, to monitor growth and possible weight differences among groups. The experimental protocol was approved by the University of Maine Institution Animal Care and Use Committee (IACUC: A2017-01-06). Animals were treated in accordance with Guide for the Care and Use of Laboratory Animals (8th edition, National Academies Press).

3.2 Diets

Rats from each animal model (LZR and OZR) were randomly assigned to either a whole red raspberry (Rubus idaeus)-enriched diet (WRR), or on a control diet (C), for 8 weeks. A standard rodent chow AIN93G was used as the C, while for the test diet 8% w/w WRR powder was added substituting for cornstarch (Table 4). Both diets were prepared at Dyets Inc. (Bethlehem, PA) and kept at -80°C in vacuum-packed UV protective bags until use. Freeze-dried WRR powder was provided by FutureCeuticals (Momence, IL). The raspberry powder consisted of verities: Willamette and Heritage. FutureCeuticals standardized the powder to a total 0.5% anthocyanins, 1.5% phenolics and 0.01% ellagic acid. Supplementation of red raspberry powder was equivalent to a daily human dose of approximately a cup and a half of fresh red raspberry.
Food consumption was measured daily. The WRR powder provided 365 calories/100 g, with 7.92% protein, 84.42% carbohydrates 0.36% fat, 12.12% fiber, 0.5% total anthocyanins, 1.5% total phenolics, and 0.001% ellagic acid. Water and food were provided *ad libitum*. Food consumption was measured daily to monitor possible differences between the groups.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>AIN-93G (grams/kg)</th>
<th>AIN-93 + 8% WRR-enriched (grams/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein</td>
<td>200</td>
<td>200</td>
</tr>
<tr>
<td>L-Cystine</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Sucrose</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Cornstarch</td>
<td>397.486</td>
<td>317.486</td>
</tr>
<tr>
<td>Whole Red Raspberry</td>
<td>0</td>
<td>80</td>
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<tr>
<td>Dextrose</td>
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<td>132</td>
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<tr>
<td>Soybean Oil</td>
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<td>70</td>
</tr>
<tr>
<td>t-Butylhydroquinone</td>
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<td>0.014</td>
</tr>
<tr>
<td>Cellulose</td>
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<td>50</td>
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<tr>
<td>Mineral Mix</td>
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<tr>
<td>Vitamin Mix</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Choline Bitartrate</td>
<td>2.5</td>
<td>2.5</td>
</tr>
</tbody>
</table>
3.3 Sample Collection

At the end of the 8-week experimental period, animals were fasted overnight. Rats were anesthetized with CO\textsubscript{2} for approximately 2 minutes and exsanguinated by cardiac puncture.

*Set One* (Table 5): The thoracic aorta was quickly harvested and placed in a physiological salt solution (PSS) (composed of: 118 mM NaCl, 4.7 mM KCl, 25 mM NaHCO\textsubscript{3}, 1.18 mM KH\textsubscript{2}PO\textsubscript{4}, 1.17 mM MgSO\textsubscript{4}, 11 mM dextrose, 1.25 mM CaCl), and was cleaned of blood clots and connective tissue. The middle segment of the aorta was sectioned into four rings (approximately 3mm length) and used immediately for the evaluation of vasoconstriction. The remaining end of the aorta was sectioned into three pieces (approximately 1mm length) and used for effluent measurements. The arch of the aorta was snap-frozen in liquid nitrogen and stored at -80°C until further analysis. Blood was collected in a tube containing 200μl of a 5% EDTA solution to prevent clotting, centrifuged immediately for 15 minutes at 2300g for plasma separation and stored at -80°C until subsequent analysis of inflammatory biomarkers. Liver and adipose tissue (AT) were harvested and weighed. Liver samples were fixed in 10% formalin and stored at room temperature and stained with Hematoxylin and eosin (H&E) stain for histopathology evaluation of basic structures of the tissue. Remaining liver and AT tissues were snap-frozen in liquid nitrogen and stored at -80°C for latter mRNA extraction to analyze inflammation.

*Set Two* (Table 5): The thoracic aorta was processed in the same way as set one, the four aortic rings were immediately evaluated for vasorelaxation. Blood was processed in the same way as set one, plasma was analyzed for lipid biomarkers. Liver and adipose tissue (AT) were harvested and weighed. Liver samples were fixed in 10% formalin and stored at room temperature and stained with Masson’s Trichrome stain for histopathology evaluation of collagen fibers in the
tissue. Remaining liver and AT tissues were snap-frozen in liquid nitrogen and stored at -80°C for latter mRNA extraction and triglyceride concentration to analyze lipid metabolism.

Table 5: Summary of experiments conducted with two sets of animals.

<table>
<thead>
<tr>
<th></th>
<th>Set One (n = 32)</th>
<th>Set Two (n = 32)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma Biomarkers</td>
<td>Inflammation</td>
<td>Lipid Profile</td>
</tr>
<tr>
<td>Gene Expression in Liver and Adipose Tissue</td>
<td>Inflammation</td>
<td>Lipid Metabolism</td>
</tr>
<tr>
<td>Aortic Function</td>
<td>Vasoconstriction</td>
<td>Vasorelaxation</td>
</tr>
<tr>
<td>Aortic Effluent</td>
<td>Prostanoids</td>
<td></td>
</tr>
<tr>
<td>Histology of Liver</td>
<td>Hematoxylin and eosin</td>
<td>Masson’s Trichrome</td>
</tr>
</tbody>
</table>

3.4 Biochemical Assays

C- Reactive Protein

Plasma CRP was measured using a CRP Rat ELISA kit (Invitrogen #ERCRP). This test is a solid-phase enzyme immunoassay (ELISA) that detects and quantifies the presence of CRP, by pairing with a target-specific pre-coated antibody well of a microplate. The enzyme activity was measured using an absorbance spectrophotometer Synergy 2 microplate reader (BioTek Instruments, Inc.) at λ=450nm and 550nm.

Interleukin-6

Plasma concentration of IL-6 was measured using Rat IL-6 Quantikine ELISA kit (R&D Systems). This test is an enzyme-linked immunosorbent assay (ELISA) detects and quantifies the presence of circulating IL-6 by binding to a monoclonal antibody specific coated well of a
microplate. The enzyme activity was measured using an absorbance spectrophotometer Synergy 2 microplate reader (BioTek Instruments, Inc.) at $\lambda=450$nm and 570nm.

**Adiponectin**

Circulating plasma adiponectin was measured using Rat Total Adiponectin Quantikine ELISA kit (R&D Systems). This test is a solid-phase enzyme immunoassay (ELISA) that detects and quantifies the presence of adiponectin, by binding with a target-specific pre-coated antibody well of a microplate reader. The enzyme activity was measured using an absorbance spectrophotometer Synergy 2 microplate reader (BioTek Instruments, Inc.) at $\lambda=450$nm and 570nm.

**Triglycerides**

Fasted plasma triglycerides were measured using the Triglyceride Colorimetric Assay kit (Cayman Chemical #10010303). This enzymatic assay converted triglycerides to glycerol and free fatty acids by lipase. The glycerol released was measured using an absorbance spectrophotometer Synergy 2 microplate reader (BioTek Instruments, Inc.) at $\lambda=530$nm.

**Total Cholesterol**

Fasted plasma total cholesterol was measured using the Cholesterol/Cholesteryl Ester Assay Kit (abcam #65359). The enzyme activity was measured using an absorbance spectrophotometer Synergy 2 microplate reader (BioTek Instruments, Inc.) at using $\lambda=570$nm.
High-Density Lipoprotein Cholesterol

HDL was measured using the HDL and LDL/VLDL quantification Colorimetric kit (BioVision. #K613). The enzyme activity was measured using an absorbance spectrophotometer Synergy 2 microplate reader (BioTek Instruments, Inc.) at using λ=570nm.

Non-High-Density Lipoprotein Cholesterol

Plasma non-HDL (VLDL + LDL cholesterol) was calculated by subtracting HDL-cholesterol from total cholesterol.

Nitric Oxide

Plasma samples were analyzed for NO concentration using Total Nitric Oxide and Nitrate/Nitrite Parameter Kit (R&D Systems, KGE001), following the instructions provided by the manufacturer. The colorimetric assay is based on a spectrophotometric detection of a Griess Reaction performed at λ=540nm and 690nm on a Synergy 2 microplate reader (BioTek Instruments, Inc.).

Prostacyclin I2

Aortic effluent levels of PGI2 were determined using the competitive ELISA kit 6-keto-PGF1α (Cayman Chemicals, #515211). Following manufactures instructions, concentration of the secondary metabolite of PGI2 was measured using an absorbance spectrophotometer Synergy 2 microplate reader (BioTek Instruments, Inc.) at using λ 412nm. Results were normalized to the dry weight of the aorta section used.
**Thromboxane A2**

Aortic effluent levels of TXA₂ were determined using the competitive ELISA kit Thromboxane B₂ (Cayman Chemicals, #501020). Following manufactures instructions, concentration of the secondary metabolite of TXA₂ was measured using an absorbance spectrophotometer Synergy 2 microplate reader (BioTek Instruments, Inc.) at using λ=412nm. Results were normalized to the dry weight of the aorta section used.

**3.5 Gene Expression**

Messenger RNA from frozen liver, adipose tissue and aorta were isolated, retro-transcribed to cDNA subjected to two-step, real time, reverse transcription polymerase chain reaction (PCR) amplification using rat-specific primer sequences (Qiagen). Relative expression of the genes of interest were determined by the ΔΔCt method as described by Livak and Schmittgen (Livak & Schmittgen, 2001), relative to housekeeping gene beta-actin (Qiagen) and expressed as fold-variation following WRR treatment compared to the control animals.

**Messenger RNA extraction from liver**

From each animal, mRNA was isolated from liver sample using RNeasy Mini Kit (Qiagen, #74104). Frozen liver samples (50 mg) were homogenized with buffer (provided with the kit) using a TissueRuptor (Qiagen) and centrifuged. Ethanol (96-100%) was added to the lysate and added to the column membrane. Following the manufactures protocol from the mini kit the RNA was purified with a series of wash steps and eluted with 50μl of RNase-Free water.
**Messenger RNA extraction from adipose tissue**

From each animal, mRNA was isolated from adipose tissue sample using RNeasy Lipid Tissue Mini Kit (Qiagen, #74804). Frozen adipose tissue samples (100-150 mg) were homogenized with buffer (provided with the kit) using a TissueRuptor (Qiagen) and centrifuged. Ethanol (96-100%) was added to the lysate and added to the column membrane. Following the manufactures protocol from the mini kit the RNA was purified with a series of wash steps and eluted with 50\(\mu\)l of RNase-Free water.

**Messenger RNA Extraction from aorta**

From each animal, mRNA was isolated from aorta tissue sample using RNeasy Fibrous Tissue Mini Kit (Qiagen, #74704). Frozen aorta samples (10 mg) were homogenized with buffer (provided with the kit) using a TissueRuptor (Qiagen) and centrifuged. Ethanol (96-100%) was added to the lysate and added to the column membrane. Following the manufactures protocol from the mini kit the RNA was purified with a series of wash steps and eluted with 40\(\mu\)l of RNase-Free water.

**RNA Purity and Concentration**

The purity and concentration of the purified RNA was measured using a NanoDrop™ Spectrophotometer (ThermoFisher Scientific). The purity the RNA was assessed using A260/A280 ratio. RNA that read between 1.8 and 2.1 was considered pure. The ratio of A260/A280 and A260/A230 was used to determine the concentration of RNA recovered.
Reverse Transcription

cDNA was synthesized from mRNA using the RT² First Strand Kit (Qiagen, #330404). Following the manufactures instructions, genomic DNA was eliminated for each RNA sample (0.5 µg total) then incubated at 42°C to initiate reverse transcription. The cDNA was stored at -20°C for real-time PCR analysis.

Quantitative PCR Analysis

The cDNA product was used as a template for real-time PCR for RT² Profiler PCR Custom Array (Qiagen, #330231) using specific targeted rat specific primer sequences in combination with RT² SYBR Green Master Mix (Qiagen, #330502) and amplified on a quantitative PCR system (Bio-Rad CFX96). For each primer (Table 6) and tissue, in duplicates, a total of 25µl of the PCR component mix (SYBR green, cDNA, and RNase-Free water) was added to each well of the customized 96-well plate. The cycling condition for the PCR system was as followed: 1 cycle at 95°C for 10 minutes for enzyme activation and 40 cycles at 15 seconds at 95°C, 1 minute at 60°C for amplification finishing with a melting curve at 65-90°C, 2-minute increments.
### Table 6: Genes of interest used to evaluate gene expression.

<table>
<thead>
<tr>
<th>Gene of Interest</th>
<th>Gene</th>
<th>Primer Reference ID (Qiagen)</th>
<th>Target Tissue(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Inflammation</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C- Reactive Protein</td>
<td><em>Crp</em></td>
<td>RefSeq NM_017096, Rn. 16463</td>
<td>Liver</td>
</tr>
<tr>
<td>Adiponectin</td>
<td><em>Adipoq</em></td>
<td>RefSeq NM_144744, Rn. 24299</td>
<td>Adipose Tissue</td>
</tr>
<tr>
<td>Nuclear Factor-kB</td>
<td><em>NFkB1</em></td>
<td>RefSeq NM_001276711, Rn. 2411</td>
<td>Liver, Adipose Tissue</td>
</tr>
<tr>
<td>Tumor Necrosis Factor-Alfa</td>
<td><em>Tnf</em></td>
<td>RefSeq NM_012675, Rn. 2275</td>
<td>Liver, Adipose Tissue</td>
</tr>
<tr>
<td>Interleukin-6</td>
<td><em>Il6</em></td>
<td>RefSeq NM_012589, Rn. 9873</td>
<td>Liver, Adipose</td>
</tr>
<tr>
<td><strong>Lipid Metabolism</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fatty Acid Synthase</td>
<td><em>Fasn</em></td>
<td>RefSeq NM_017332, Rn. 9486</td>
<td>Liver, Adipose Tissue</td>
</tr>
<tr>
<td>Microsomal Triglyceride Transfer Protein</td>
<td><em>Mttp</em></td>
<td>RefSeq NM_001107727, Rn. 81556</td>
<td>Liver, Adipose Tissue</td>
</tr>
<tr>
<td>Peroxisome Proliferator-Activated Receptor Alpha</td>
<td><em>Ppara</em></td>
<td>RefSeq NM_013196, Rn. 9753</td>
<td>Liver, Adipose Tissue</td>
</tr>
<tr>
<td>Peroxisome Proliferator-Activated Receptor Gamma</td>
<td><em>Pparg</em></td>
<td>RefSeq NM_013124, Rn. 23443</td>
<td>Liver, Adipose Tissue</td>
</tr>
<tr>
<td>ATP Binding Cassette Transporter 1</td>
<td><em>Abca1</em></td>
<td>RefSeq NM_178095, Rn. 148916</td>
<td>Liver, Adipose Tissue</td>
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<tr>
<td>Lipoprotein Lipase</td>
<td><em>Lpl</em></td>
<td>RefSeq NM_012598, Rn. 3834</td>
<td>Liver, Adipose Tissue</td>
</tr>
<tr>
<td>Acyl-CoA Oxidase 1</td>
<td><em>Acox1</em></td>
<td>RefSeq NM_001106508, Rn. 112005</td>
<td>Liver</td>
</tr>
<tr>
<td>Low Density Lipoprotein Receptor-Related Protein 1</td>
<td><em>Lrp1</em></td>
<td>RefSeq NM_001130490, Rn. 22436</td>
<td>Liver</td>
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<tr>
<td>Acetyl-CoA acetyltransferase</td>
<td><em>Acat1</em></td>
<td>RefSeq NM_017075, Rn. 4054</td>
<td>Liver</td>
</tr>
<tr>
<td><strong>Vascular Function</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Inducible Nitric Oxide</td>
<td><em>Nos2</em></td>
<td>RefSeq NM_012611, Rn. 10400</td>
<td>Aorta</td>
</tr>
<tr>
<td>Endothelial Nitric Oxide</td>
<td><em>Nos3</em></td>
<td>RefSeq NM_021838, Rn. 44625</td>
<td>Aorta</td>
</tr>
<tr>
<td>Cyclooxygenase-2</td>
<td><em>Ptgs2</em></td>
<td>RefSeq NM_017232, Rn. 44369</td>
<td>Aorta</td>
</tr>
</tbody>
</table>
3.6 Hepatic Analysis

Liver Weight

Liver tissue was collected, weighed, and flash-frozen in liquid nitrogen within 1-2 minutes of euthanasia. The percentage of liver weight was calculated by dividing the total body weight of the animal at the end of the experimental period by the total liver weight.

Liver Histopathology

Liver samples were collected and fixed in 10% formalin. Samples were processed on Leica TP1020 (Leica, Buffalo Grove, IL) using a 16.5-hour overnight schedule. Tissue Prep2 (Fisher Scientific) paraffin blocks were prepared and cut at 5µm thickness. Sections were mounted on glass slides, deparaffinized and stained following either the hematoxylin and eosin (H&E) or Masson’s Trichrome protocol. Samples were examined through a light microscope.

Liver Triglycerides

Following manufacturer’s instructions, liver homogenates were used to extract and quantify triglycerides by the use of Triglyceride Colorimetric Assay Kit (Cayman Chemical #10010303). This enzymatic assay converted triglycerides to glycerol and free fatty acids by lipase. The glycerol released was measured using an absorbance spectrophotometer microplate reader (BioTek) at λ=530nm.

3.7 Endothelial Function

Vascular studies were conducted on isolated aortic ring preparations to measure Phe-induced vasoconstriction and Ach-induced vasodilation (Figure 7). The four aortic rings were
suspended between two stainless-steel triangles and submerged in 20mL Radnoti tissue baths (Radnoti Glass Technology Inc., Monrovia, CA) that contained PSS at 37°C aerated with 95% O₂/5% CO₂ gas mix (pH 7.4). The bottom triangle was fixed to the tissue bath and the top triangle was connected to a tissue force analyzer (TFA Model 410; Micro-Med Inc., Louisville KY) that recorded the force (g) developed by the aortic ring using DMSI-450 software (version 1.01: Micro-Med Inc.).

The isolated rings were pre-loaded with a baseline tension of 1.50 ± 0.01g and pre-conditioned with a single dose of Ach (10⁻⁸ M) and Phe (10⁻⁸ M). Each aortic ring was randomly assigned to either a treatment that inhibits nitric oxide (NO) synthase with L-N-monomethyl-arginine (L-NMMA) or a treatment which inhibits cyclooxygenase (COX1 and COX2) with mefenamic acid (MFA); or a treatment with no inhibitor.

Figure 7: Method used to evaluate endothelial function using the aorta.

The maximum force of contraction (Fmax), the effective concentration at 50% of maximum response (EC₅₀), and the vessel sensitivity (pD2, -log10 EC₅₀) were determined (Table 7).
### Table 7: Individual dose-response curve.

<table>
<thead>
<tr>
<th>Parameters Measured</th>
<th>Biological Interpretation</th>
<th>Measured by</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fmax</td>
<td>Maximal Force of vessel (contraction/relaxation)</td>
<td>A tissue force analyzer, used to calculate % increase/decrease of force at each Phe/Ach dose</td>
</tr>
<tr>
<td>EC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Dose to inhibit 50% of vessel response</td>
<td>Transformation of contraction/relaxation dose-response curves to semilog curves</td>
</tr>
<tr>
<td>pD&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Vessel reactivity (receptor-agonist interaction)</td>
<td>-log EC&lt;sub&gt;50&lt;/sub&gt; to give normal data distribution</td>
</tr>
</tbody>
</table>

**Phe-induced vasoconstriction**

Animals from set 1, the four thoracic aortic rings were used to generate a Phe dose-response curve to evaluate vasoconstriction (Figure 8). The aortic rings were exposed to six cumulative Phe doses (from $10^{-8}$ to $3 \times 10^{-6}$ M) and were allowed to reach maximum vasoconstriction force for 6 minutes. A single dose of Ach ($3 \times 10^{-6}$ M) was added following the last dose of Phe to determine endothelial integrity through the vasorelaxation response. For each ring, the maximum force of contraction (Fmax), the effective concentration at 50% of maximum response (EC<sub>50</sub>), and the vessel sensitivity (pD2, $-\log 10$ EC<sub>50</sub>) were determined.
Ach-induced vasodilation

Animals from set 2, the four thoracic aortic rings were used to generate an Ach dose-response curve was generated to evaluate vasodilation (Figure 9). Each aortic ring was exposed to single dose of Phe (3 x 10⁻⁶ M) for 10 minutes which allowed the vessel to reach maximum vasoconstriction. Then six cumulative Ach doses (from 10⁻⁸ to 3 x 10⁻⁶ M) were introduced to each of the tissue baths, to allow the aortic rings to reach maximum vasorelaxation for 6 minutes after each dose. The maximum relaxation value at each dose of Ach was expressed as a percentage from the Phe-induced preconditioned force (0% vasorelaxation). The effective concentration at 50% of vasorelaxation response (EC 50) and vessel sensitivity (pD2, -log10 EC 50) were determined.
Aortic Effluent

The remaining segment of the aorta from each animal was incubated in a 2mL Radnoti tissue bath under the same conditions mentioned above (Figure 10). The aorta pieces were allowed to equilibrate for 20 minutes before being exposed to a single dose of Phe (10⁻⁶ M) for 10 minutes followed by Ach (10⁻⁶ M) for 10 minutes to stimulate prostanoid release. The medium was collected and stored at -80°C until further analysis. The aortic tissues were dried at room temperature and weighed. Estimated levels of PGI₂ (competitive ELISA kit 6-keto- PGF₁₀₂ (6kPGF₁₀₂) (Cayman, 515211)) and TXA₂ (competitive ELISA kit Thromboxane B₂ (TXB₂) (Cayman, 501020)) were determined. Results were normalized to the dry weight of the aorta section used.
3.4 Statistical Analysis

A two-way analysis of variance (ANOVA) was used to examine the effect of dietary treatment (C vs. WRR) and animal model (LZR vs. OZR). The differences between means were further analyzed using the Tukey’s Honestly Significant Difference (HSD) test. Results were expressed as ± SEM and considered significant at P < 0.05. Statistical analysis was performed using GraphPad Prism 7 version 7.0d statistical software (GraphPad Software, La Jolla, CA).
CHAPTER 4

4. RESULTS

4.1 Animal Weight

In Figure 11, the average weight of the animals at 8-weeks of age was significantly higher in the OZR (394 ± 47g) compared to their litter mates (265 ± 36g). The average weight of the animals at 16 weeks of age was significantly higher in the OZR (608 ± 74 g) compared to the LZR group (434 ± 68 g). There was no significant difference within each diet group between the C and WRR animals.

![Figure 11: Average animal weight.](image)

The values are expressed as means ± SEM (n= 8 rats per treatment group)

*Significant effect of model, LZR-C vs OZR-C (p < 0.05)

4.2 Food Intake

In Figure 12, average daily food consumption was significantly higher in the OZR (36.3 ± 1.054 g) compared to the LZR group (26.5 ± 0.729 g). Within each diet group (C vs. WRR) the daily food consumption was not significant.
Figure 12: Average daily food consumption.

The values are expressed as means ± SEM (n= 8 rats per treatment group)

\(^a\) Significant effect of model, LZR-C vs OZR-C (p < 0.05)

4.3 Inflammation

Circulating markers of inflammation in the plasma are reported in Table 8.

\textit{Tumor Necrosis Factor-Alpha}

Levels of TNF-\(\alpha\) was undetectable in the LZR (Table 8). With the consumption of WRR, no changes were observed in plasma TNF-\(\alpha\) in the OZR.

\textit{Interleukin-6}

The levels of IL-6 was higher in the OZR compared to the LZR (Table 8). Following the consumption of WRR, IL-6 concentration decreased in the OZR (50.50 ± 3.66 pg/ml vs. 20.69 ± 1.4 pg/ml, \(p<0.05\)).
C-Reactive Protein

The levels of CRP were higher in the OZR compared to the LZR (Table 8). Plasma concentration of CRP decreased in the OZR consuming a WRR-enriched diet from 4250 ± 1120.12 pg/ml to 1024 ± 108.74 pg/ml ($p<0.05$).

Adiponectin

No significant difference was detected in adiponectin concentrations with animal type or diet (Table 8).

<table>
<thead>
<tr>
<th></th>
<th>IL-6 (pg/ml)</th>
<th>TNF-α (pg/ml)</th>
<th>CRP (µg/ml)</th>
<th>Adiponectin (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LZR-C</td>
<td>8.37 ± 1.07&lt;sup&gt;a&lt;/sup&gt;</td>
<td>n.d.</td>
<td>786.80 ± 80.83&lt;sup&gt;a&lt;/sup&gt;</td>
<td>79.24 ± 20.98</td>
</tr>
<tr>
<td>LZR-WRR</td>
<td>15.13 ± 1.53</td>
<td>n.d.</td>
<td>967.50 ± 281.11</td>
<td>66.60 ± 5.72</td>
</tr>
<tr>
<td>OZR-C</td>
<td>50.50 ± 3.66&lt;sup&gt;b&lt;/sup&gt;</td>
<td>25.44 ± 3.92</td>
<td>4250 ± 1120.12&lt;sup&gt;b&lt;/sup&gt;</td>
<td>69.52 ± 4.87</td>
</tr>
<tr>
<td>OZR-WRR</td>
<td>20.69 ± 1.41</td>
<td>27.00 ± 4.56</td>
<td>1024 ± 108.74</td>
<td>52.19 ± 5.39</td>
</tr>
</tbody>
</table>

The values are expressed as means ± SEM ($n=8$ rats per treatment group)
<sup>a</sup> Significant effect of model, LZR-C vs OZR-C ($p < 0.05$)
<sup>b</sup> Significant effect of diet, OZR-C vs OZR-WRR ($p < 0.05$)
Gene Expression of Inflammatory Markers of the Liver

Gene expression of inflammatory markers in the liver is represented in Figure 13.

Interleukin-6

Hepatic gene expression of IL-6 was not significantly different between OZR-C and their lean littermates. Following consumption of WRR, expression of IL-6 (Figure 13A) in the OZR liver was significantly down-regulated at -45.69% (p<0.05).

Tumor Necrosis Factor-Alpha

Significant increase in hepatic TNF-α (Figure 13B) expression was observed in the OZR compared to the LZR (p<0.05). Hepatic expression of TNF-α (Figure 13B) in the OZR liver was significantly down-regulated at -13.50% with the consumption of WRR (p<0.05).

Nuclear Factor-κB

Hepatic gene expression of NF-κB was not significantly different between OZR-C and their lean littermates. Hepatic expression of NF-κB (Figure 13C) in the OZR liver was significantly down-regulated at -20.97% with the consumption of WRR (p<0.05).

C-Reactive Protein

Hepatic expression of CRP (Figure 13D) was significantly down regulated in the OZR compared to the LZR (p<0.05). No significant difference of CRP expression was observed with the consumption of WRR in the OZR (Figure 13D).
Figure 13 (A-D): Gene expression of inflammatory markers in the liver.

A. interleukin-6 (IL-6), B. tumor necrosis factor-alpha (TNF-α), C. nuclear factor kappa-light-chain-enhancer of activated B-cells (NF-κB), D. C-Reactive Protein (CRP)

Note: A. interleukin-6 (IL-6), B. tumor necrosis factor-alpha (TNF-α), C. nuclear factor kappa-light-chain-enhancer of activated B-cells (NF-κB), D. C-Reactive Protein (CRP)

The values are expressed as means ± SEM (n= 8 rats per treatment group)

* Significant effect of model, LZR-C vs OZR-C (p < 0.05)

** Significant effect of diet, OZR-C vs. OZR-WRR (p < 0.05)
Gene Expression of Inflammatory Markers of the Adipose Tissue

Gene expression of inflammatory markers in the AT is represented in Figure 14.

Interleukin-6

Pro-inflammatory molecule IL-6 (Figure 14A) were upregulated in the OZR-C compared to the LZR-C ($p<0.05$). With the consumption of WRR, AT expression of IL-6 (Figure 14A) was significantly down-regulated at -49.53% respectively in the OZR compared to OZR-C ($p<0.05$).

Tumor Necrosis Factor-Alpha

Pro-inflammatory molecule TNF-α (Figure 14B) were upregulated in the OZR-C compared to the LZR-C ($p<0.05$). With the consumption of WRR, AT expression of TNF-α (Figure 14B) was down-regulated at 18.26%, in the OZR compared to OZR-C ($p<0.05$).

Nuclear Factor-κB

No significant difference was detected in expression of NF-κB in the AT with animal type or diet (Figure 14C).

Adiponectin

Expression of adiponectin (Figure 14D), an anti-inflammatory molecule, was significantly down-regulated in the OZR-C compared to the LZR-C ($p<0.05$). No significant difference of adiponectin expression in the AT was observed with the consumption of WRR in the OZR (Figure 14D).
Figure 14 (A-D): Gene expression of inflammatory markers in the adipose tissue.

A. **IL-6**

B. **TNF-α**

C. **NF-κB**

D. **Adiponectin**

**Note:** A. interleukin-6 (IL-6), B. tumor necrosis factor-alpha (TNF-α), C. nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), D. adiponectin.

The values are expressed as means ± SEM (n = 8 rats per treatment group)

a Significant effect of model, LZR-C vs OZR-C (p < 0.05)

b Significant effect of diet, OZR-C vs OZR-WRR (p < 0.05)
4.4 Lipid Metabolism

*Circulating Plasma Lipids*

The concentration of blood lipids is presented in Table 9.

*Triglycerides*

Plasma concentration of TGs was significantly higher in the OZR compared to their lean littermates ($p<0.05$) (Table 9). Following consumption of WRR the concentration of plasma TGs increased in the OZR ($p<0.05$).

*Total Plasma Cholesterol*

Total plasma cholesterol was significantly higher in the OZR compared to the LZR ($p<0.05$) (Table 9). With the consumption of WRR the concentration of plasma total cholesterol was attenuated ($p<0.05$).

*High-Density Lipoprotein Cholesterol*

Plasma concentration of HDL-C was significantly higher in the OZR compared to their lean littermates ($p<0.05$) (Table 9). Following the consumption of WRR the concentration of HDL-C was decreased in the OZR.

*Non-High-Density Lipoprotein*

The concentration of non-HDL-C was significantly higher in the OZR compared to the LZR ($p<0.05$) (Table 9). With the consumption of WRR plasma concentration of non-HDL-C decreased.
### Table 9: Plasma concentration of lipids.

<table>
<thead>
<tr>
<th></th>
<th>Triglycerides (mg/dl)</th>
<th>Total Plasma Cholesterol (µg/µl)</th>
<th>HDL-C (µg/µl)</th>
<th>Non-HDL (µg/µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>LZR-C</strong></td>
<td>96.17 ± 12.83&lt;sup&gt;a&lt;/sup&gt;</td>
<td>66.1 ± 5.42&lt;sup&gt;a&lt;/sup&gt;</td>
<td>31.40 ± 2.45&lt;sup&gt;a&lt;/sup&gt;</td>
<td>34.69 ± 3.55&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>LZR-WRR</strong></td>
<td>76.22 ± 4.18</td>
<td>52.44 ± 1.68</td>
<td>30.52 ± 1.49</td>
<td>21.92 ± 1.73</td>
</tr>
<tr>
<td><strong>OZR-C</strong></td>
<td>1227.20 ± 146.14&lt;sup&gt;b&lt;/sup&gt;</td>
<td>221.91 ± 18.34&lt;sup&gt;b&lt;/sup&gt;</td>
<td>111.31 ± 5.61&lt;sup&gt;b&lt;/sup&gt;</td>
<td>110.60 ± 14.69</td>
</tr>
<tr>
<td><strong>OZR-WRR</strong></td>
<td>2149.49 ± 304.70</td>
<td>156.12 ± 6.68</td>
<td>80.36 ± 6.10</td>
<td>75.77 ± 8.76</td>
</tr>
</tbody>
</table>

The values are expressed as means ± SEM (n= 8 rats per treatment group)

<sup>a</sup> Significant effect of model, LZR-C vs OZR-C (p < 0.05)

<sup>b</sup> Significant effect of diet, OZR-C vs. OZR-WRR (p < 0.05)

### Hepatic Weight

Total body weight, liver weight and percentage of liver to total body weight was significantly higher in the OZR compared to the LZR (p<0.05) (Table 10). With the consumption of WRR, liver weight and percentage of liver to total body weight significantly decreased in the OZR.

### Table 10: Hepatic weight.

<table>
<thead>
<tr>
<th></th>
<th>Body Weight (g)</th>
<th>Liver Weight (g)</th>
<th>% Liver</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>LZR-C</strong></td>
<td>434.30 ± 68&lt;sup&gt;a&lt;/sup&gt;</td>
<td>13.73 ± 0.95&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.15 ± 0.15&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>LZR-WRR</strong></td>
<td>419.50 ± 50</td>
<td>12.86 ± 0.42</td>
<td>3.10 ± 0.11</td>
</tr>
<tr>
<td><strong>OZR-C</strong></td>
<td>607.60 ± 74</td>
<td>36.58 ± 1.73&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.10 ± 0.36&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>OZR-WRR</strong></td>
<td>594.20 ± 65</td>
<td>26.12 ± 1.55</td>
<td>4.40 ± 0.27</td>
</tr>
</tbody>
</table>

The values are expressed as means ± SEM (n= 8 rats per treatment group)

<sup>a</sup> Significant effect of model, LZR-C vs OZR-C (p < 0.05)

<sup>b</sup> Significant effect of diet, OZR-C vs. OZR-WRR (p < 0.05)

### Gross Evaluation

A single animal representation of livers from OZR-C (Figure 15C) appeared more yellow in color and were significantly heavier (Figure 15A) than those of the LZR-C (Figure 15A). With WRR consumption, liver weight (Figure 15D) was lower and the yellow color was attenuated in the OZR, coming closer in appearance to the liver of their lean littermates (Figure 15A and 15B).
**Hepatic Histopathology**

In Figure 16 and Figure 17, a single animal representation of livers from the LZR group showed normal hepatic architecture (Figure 16A and 16B), whereas livers from the OZR-C group (Figure 16C) showed severe accumulation of fat in the hepatocytes. Fat accumulation in the hepatocytes diminished in the OZR with the WRR consumption (Figure 16D).
Figure 16 (A-D): Representation of liver sections stained by hematoxylin and eosin.

A. LZR-C
B. LZR-WRR
C. OZR-C
D. OZR-WRR
**Hepatic Concentration of Triglycerides**

Hepatic concentration of TGs was significantly elevated in the OZR compared to the LZR, as reported in Table 11. Supplementation with WRR significantly decreased TG accumulation in the liver of the OZR.

**Table 11: Hepatic concentration of triglycerides (TG).**

<table>
<thead>
<tr>
<th></th>
<th>TG (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LZR-C</td>
<td>$14.14 \pm 0.91^a$</td>
</tr>
<tr>
<td>LZR-WRR</td>
<td>$12.81 \pm 1.32$</td>
</tr>
<tr>
<td>OZR-C</td>
<td>$33.45 \pm 3.38^b$</td>
</tr>
<tr>
<td>OZR-WRR</td>
<td>$21.41 \pm 2.10$</td>
</tr>
</tbody>
</table>

The values are expressed as means ± SEM (n= 8 rats per treatment group)

$^a$ Significant effect of model, LZR-C vs OZR-C (p < 0.05)

$^b$ Significant effect of diet, OZR-C vs. OZR-WRR (p < 0.05)
Gene Expression of Lipid Metabolism in the Liver

Gene expression of lipid metabolism in the liver are represented in Figure 18.

Fatty Acid Synthase

As reported in Figure 2, hepatic gene expression of FAS (Figure 18A) was significantly upregulated in the OZR compared to the LZR, independent of diet ($p<0.05$). With the consumption of WRR, hepatic expression of FAS (Figure 18A) was down regulated in the OZR ($p<0.05$).

ATP Binding Cassette Transporter 1

Gene expression of ABCA1 (Figure 18B), was significantly down regulated in the OZR compared to the LZR ($p<0.05$). No significant difference was observed in the OZR with the consumption of WRR.

Peroxisome Proliferator-Activated Receptor Gamma

Expression of PPARγ (Figure 18C) was significantly down regulated in the OZR compared to the LZR ($p<0.05$). No significant difference was observed in the OZR with the consumption of WRR.

Lipoprotein Lipase

The expression of Lpr1 (Figure 18D) was significantly down regulated in the OZR compared to the LZR ($p<0.05$). No significant difference was observed in the OZR with the consumption of WRR.
Microsomal Triglyceride Transfer Protein

The expression of Mttp (Figure 18E) was significantly down regulated in the OZR compared to the LZR (p<0.05). With the consumption of WRR, hepatic expression Mttp was significantly upregulated in the OZR (p<0.05) (Figure 18E).

Acyl-CoA Oxidase 1

No significant difference was detected in hepatic expression of Acat1 with animal type or diet (Figure 18F). Even though hepatic expression of Acox1 (Figure 18F) was not significantly different with WRR supplementation in the OZR, a trend toward its increased expression could be observed.

Acetyl-CoA acetyltransferase

No significant difference was detected in hepatic expression of Acat1 with animal type or diet (Figure 18G).

Peroxisome Proliferator-Activated Receptor Alpha

No significant difference was detected in hepatic expression of PPARα with animal type or diet (Figure 18H).
Figure 18 (A-H): Gene expression of lipid metabolism in the liver.

A. fatty acid synthase (FAS), B. ATP-binding cassette transporter (ABCA1), C. peroxisome proliferator-activated receptor gamma (PPARγ), D. LDL receptor protein (Lrp1), E. microsomal triglyceride transfer protein (Mttp), F. Acyl-CoA oxidase 1 (Acox1), G. Acyl-CoA (Acat1), H. (PPARα).

Note: A. fatty acid synthase (FAS), B. ATP-binding cassette transporter (ABCA1), C. peroxisome proliferator-activated receptor gamma (PPARγ), D. LDL receptor protein (Lrp1), E. microsomal triglyceride transfer protein (Mttp), F. Acyl-CoA oxidase 1 (Acox1), G. Acyl-CoA (Acat1), H. (PPARα).

The values are expressed as means ± SEM (n= 8 rats per treatment group)

* Significant effect of model, LZR-C vs OZR-C (p < 0.05)

* Significant effect of diet, OZR-C vs. OZR-WRR (p < 0.05)
Gene Expression of lipid metabolism in the Adipose Tissue

Gene expression of lipid metabolism in the AT are represented in Figure 19.

Fatty Acid Synthase

As reported in Figure 19, the AT gene expression of FAS (Figure 19A) was significantly down regulated in the OZR compared to the LZR, independent of the diet. Expression of FAS was not significantly different with the consumption of WRR in the OZR.

Lipoprotein Lipase

Gene expression of LPL (Figure 19B) was significantly down regulated in the OZR compared to the LZR. Expression of LPL was not significantly different with the consumption of WRR in the OZR.

Peroxisome Proliferator-Activated Receptor Alpha

Expression of PPARα (Figure 19C) was significantly down regulated in the OZR compared to the LZR. Expression of PPARα was not significantly different with the consumption of WRR in the OZR.

Peroxisome Proliferator-Activated Receptor Gamma

Expression of PPARγ (Figure 19D) was significantly down regulated in the OZR compared to the LZR. Expression of PPARγ was not significantly different with the consumption of WRR in the OZR.
ATP Binding Cassette Transporter 1

No significant difference in ABCA1 expression between animal type or diet (Figure 19E).

Figure 19 (A-D): Gene expression of lipid metabolism in the adipose tissue.

A. fatty acid synthase (FAS), B. lipoprotein lipase (LPL), C. peroxisome proliferator-activated receptor alpha (PPARα), D. PPARγ, E. ATP-binding cassette transporter (ABCA1).

Note: A. fatty acid synthase (FAS), B. lipoprotein lipase (LPL), C. peroxisome proliferator-activated receptor alpha (PPARα), D. PPARγ, E. ATP-binding cassette transporter (ABCA1).

The values are expressed as means ± SEM (n= 8 rats per treatment group)

a Significant effect of model, LZR-C vs OZR-C (p < 0.05)

b Significant effect of diet, OZR-C vs. OZR-WRR (p < 0.05)

4.5 Endothelial Function

Phenylephrine-induced Vasoconstriction

The Phe-induced vasoconstriction dose response curve is shown in Figure 20. In the absence of inhibitors, the maximal force of contraction induced by Phe was significantly lower in the OZR-C (Fmax 0.75 ± 0.05 g) compared with the LZR-C (Fmax 1.15 ± 0.05 g, p<0.05)
Consumption of WRR partially restored Phe-induced vasoconstrictor responses in OZR, with a significant increase in the maximal force of contraction ($F_{max}$ $0.98 \pm 0.03$ g $p<0.05$) (Figure 20A). The overall maximal tension of aorta rings with the pre-treatment of NO inhibitor, L-NMMA increased (Figure 20B). The Phe-induced vasoconstrictor response significantly increased the maximal force of the OZR assigned to a WRR diet compared to OZR-C ($F_{max}$ $2.03 \pm 0.12$ g and $F_{max}$ $1.82 \pm 0.14$ g, $p<0.05$ respectively) (Figure 20B). Following pre-treatment of rings with MFA, a COX pathway inhibitor, the vasoconstrictor response was significantly lower in the OZR-C ($F_{max}$ $0.95 \pm 0.14$ g) compared to LZR-C ($F_{max}$ $1.34 \pm 0.09$ g, $p<0.05$) (Figure 20C). No significant differences were observed between treatment group in the OZR (C vs WRR).

**Figure 20 (A-C):** Phenylephrine (Phe)-induced vasoconstriction dose response curve.

<table>
<thead>
<tr>
<th>A. PSS</th>
</tr>
</thead>
</table>

![Graph showing dose response curve for phenylephrine-induced vasoconstriction](image)
B. L-NMMA

![Graph showing the effect of L-NMMA on vasoconstriction response]

C. MFA

![Graph showing the effect of MFA on vasoconstriction response]

**Note:** A. Phe-induced vasoconstriction response in the absence of the inhibitor. B. Phe-induced vasoconstriction response with the pre-treatment of L-N-monomethyl-arginine (L-NMMA). C. Phe-induced vasoconstriction response with the pre-treatment of Mefenamic Acid (MFA).

The values are expressed as means ± SEM (n=8 rats per treatment group).

a. Significant effect of animal type, LZR-C vs OZR-C (p < 0.05)

b. Significant effect of diet, OZR-C vs. OZR-WRR (p < 0.05)
Vessel sensitivity (pD2), shown in Table 12, was similar between animal type and was unaffected by diet. Pre-treatment with L-NMMA significantly increased the vessel sensitivity in both LZR and OZR (p<0.05). No significant difference was observed either with animal type or diet with MFA pre-treatment.

<table>
<thead>
<tr>
<th>Table 12: Vasoconstriction Vessel Sensitivity (pD2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
</tr>
<tr>
<td>PSS</td>
</tr>
<tr>
<td>PSS + L-NMMA</td>
</tr>
<tr>
<td>PSS + MFA</td>
</tr>
</tbody>
</table>

Note: No inhibitor (PSS); L-N-monomethyl-arginine (L-NMMA); Mefenamic Acid (MFA). PSS (physiological salt solution); L-NMMA (L-N-monomethyl-arginine); MFA (mefenamic acid)

The values are expressed as means ± SEM (n=8 rats per treatment group).

*Significant effect between treatment, PSS vs L-NMMA (p < 0.05)

Acetylcholine-induced Vasorelaxation

The Ach-induced vasorelaxation dose response curve is illustrated in Figure 21. In the absence of inhibitors, the OZR-C exhibited exaggerated relaxation response to Ach compared to the LZR-C at the first two doses, 10⁻⁷ and 3x10⁻⁸M (61.64 ± 4.88 % and 92.30 ± 1.98 % vs. 29.19 ± 3.84 % and 71.40 ± 4.58 %, p<0.05, respectively) (Figure 21A). An attenuated Ach-induced vasorelaxation response was observed in the OZR-WRR at 10⁻⁷ and 3x10⁻⁸M (42.12 ± 5.02 % and 80.69 ± 4.41 %, p<0.05, respectively) compared with OZR-C in the absence of inhibitors (Figure 21A). Following pre-treatment with L-NMMA, the maximum relaxation response was higher in the OZR-C compared to the LZR-C (80.37 ± 2.95 % vs. 60.65 ± 4.67 %, p<0.05, respectively) (Figure 21B). The maximal vasorelaxation response in OZR-WRR (70.99 ± 3.02 %, p<0.05) was lower compared to the OZR-C in the presence of L-NMMA (Figure 21B). An exaggerated vascular response of the OZR-C was observed compared to the LZR-C at
$10^8, 3 \times 10^8$ and $10^7$M (26.09 ± 3.44 %, 56.30 ± 2.03 % and 82.76 ± 1.17 % vs 45.65 ± 6.13 %, 76.53 ± 4.04 % and 95.90 ± 1.44 %, $p<0.05$, respectively) following pre-treatment of MFA (Figure 21C). Consumption of WRR in the OZR partially attenuated the exaggerated response observed in the OZR-C (30.08 ± 3.66 %, 55.58 ± 4.28 % and 82.66 ± 2.78 %, $p<0.05$, respectively) (Figure 21C).

Figure 21 (A-C): Acetylcholine (Ach)-induced dose response curve.

A. PSS

![Dose response curve](image)
B. L-NMMA

C. MFA

**Note:** A. Phe-induced vasoconstriction response in the absence of the inhibitor. B. Phe-induced vasoconstriction response with the pre-treatment of L-N-monomethyl-arginine (L-NMMA). C. Phe-induced vasoconstriction response with the pre-treatment of Mefenamic Acid (MFA).

The values are expressed as means ± SEM (n=8 rats per treatment group).

\[ a \] Significant effect of animal type, LZR-C vs OZR-C (p < 0.05)

\[ b \] Significant effect of diet, OZR-C vs OZR-WRR (p < 0.05)

Shown in Table 13, the pD₂ of Ach-induced vasorelaxation was higher in the OZR compared with LZR, in the absence of the inhibitors (7.63 ± 0.58 vs 7.28 ± 0.04, p<0.05) and following L-NMMA treatment (7.09 ± 0.11 vs 6.45 ± 0.16, p<0.05). The pD₂ was significantly
higher in the absence of the inhibitors compared with the pre-treatment of L-NMMA in all groups \((p<0.05)\). Additionally, the OZR assigned to a WRR diet had a significantly lower \(pD_2\) with the pre-treatment of MFA \((7.15 \pm 0.08, p<0.05)\) compared to the treatment with no inhibitors.

### Table 13: Vasorelaxation Vessel Sensitivity (pD2)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>LZR-C</th>
<th>LZR-WRR</th>
<th>OZR-C</th>
<th>OZR-WRR</th>
</tr>
</thead>
<tbody>
<tr>
<td>PSS</td>
<td>7.28 ± 0.04(^a, b)</td>
<td>7.27 ± 0.06(^b)</td>
<td>7.63 ± 0.58(^b)</td>
<td>7.48 ± 0.09(^b, c)</td>
</tr>
<tr>
<td>PSS + L-NMMA</td>
<td>6.45 ± 0.16</td>
<td>6.48 ± 0.10</td>
<td>7.10 ± 0.11</td>
<td>6.90 ± 0.10</td>
</tr>
<tr>
<td>PSS + MFA</td>
<td>7.21 ± 0.10</td>
<td>6.94 ± 0.12</td>
<td>7.39 ± 0.52</td>
<td>7.15 ± 0.08</td>
</tr>
</tbody>
</table>

**Note:** No inhibitor (PSS); L-N-monomethyl-arginine (L-NMMA); Mefenamic Acid (MFA); PSS (physiological salt solution); L-NMMA (L-N-monomethyl-arginine); MFA (mefenamic acid)

The values are expressed as means ± SEM \((n=8\) rats per treatment group).

\(^a\) Significant effect of animal type, LZR-C vs OZR-C \((p < 0.05)\)

\(^b\) Significant effect between treatment, PSS vs L-NMMA \((p < 0.05)\)

\(^c\) Significant effect between treatment, PSS vs MFA \((p < 0.05)\)

**Aortic Effluent Prostanoids**

Aortic effluent prostanoids concentrations are reported in Table 14. In the aortic effluent, \(6kPGF_1\alpha\) was significantly higher in the OZR compared to the LZR group \((p<0.05)\). The concentration of \(6ketoPGF_1\alpha\) significantly decreased in the OZR assigned a WRR diet \((p<0.05)\).

The aortic effluent concentration of \(TXB_2\) were unaffected among animal type or diet groups.
Plasma Nitric Oxide

In Table 14, levels of plasma NO were unaffected among animal type or diet groups.

<table>
<thead>
<tr>
<th>Table 14: Concentration of aortic effluent.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
<tr>
<td>----------------</td>
</tr>
<tr>
<td>6kPGF(\alpha) (pg/mg of aorta)</td>
</tr>
<tr>
<td>TXB(_2) (pg/mg of aorta)</td>
</tr>
<tr>
<td>Plasma NO (µmol/L)</td>
</tr>
</tbody>
</table>

**Note:** 6kPGF\(\alpha\) (6-keto-prostaglandin F\(_\alpha\)); TXB\(_2\) (Thromboxane B\(_2\)); NO (Nitric Oxide)

The values are expressed as means ± SEM (n=8 rats per treatment group).

\(^a\) Significant effect of animal type, LZR-C vs OZR-C \((p<0.05)\)

\(^b\) Significant effect of diet, OZR-C vs OZR-WRR \((p<0.05)\)

Gene Expression in the aorta

In Figure 22A, there was no effect on aortic iNOS expression with animal type or diet intervention. The aortic expression of eNOS (Figure 22B) and COX-2 (Figure 22C) was upregulated in OZR-C compared with LZR-C \((p<0.05)\). Following consumption of WRR, aortic expression of eNOS (Figure 22B) and COX-2 (Figure 22C) were downregulated in the OZR-WRR \((p<0.05)\).
Figure 22 (A-C): Relative gene expression of vascular function in the aorta.

A. iNOS
B. eNOS
C. COX-2

**Note:** A. inducible nitric oxide (iNOS), B. endothelial nitric oxide (eNOS) and C. cyclooxygenase-2 (COX-2).

The values are expressed as means ± SEM (n=8 rats per treatment group).

\(^a\) Significant effect of animal type, LZR-C vs OZR-C \((p < 0.05)\)

\(^b\) Significant effect of diet, OZR-C vs OZR-WRR \((p < 0.05)\)
CHAPTER 5
5. DISCUSSION

5.1 Red raspberry consumption on inflammation in the obese Zucker rat

Our results demonstrate that the OZR had significantly higher levels of plasma IL-6 and CRP compared to the LZR. Elevated concentrations of IL-6 and CRP have been shown to be associated with systemic inflammation. It has been documented that CRP can increase in the plasma as much as 1,000-fold after acute inflammatory stimuli (Black, Kushner, & Samols, 2004). The induction of CRP by the hepatocytes is predominately regulated at the transcriptional level by IL-6 (Black et al., 2004). As acute inflammation changes to chronic inflammation, IL-6 becomes detrimental, favoring the continuous secretion of chemokines, angio-proliferation, and anti-apoptotic functions on T-cells (Gabay, 2006). This leads to an increase in serum levels of IL-6 and amplifies chronic inflammation (Gabay, 2006). This amplified chronic inflammation results in elevated plasma levels of IL-6, CRP and TNF-α in patients with the MetS (Christiana, Casimir, Nicholas, Christian, & Obiefuna, 2016).

Consumption of WRR in the OZR attenuated the plasma levels of IL-6 and CRP. Similarly, 5.3% freeze-dried raspberry consumption attenuated plasma IL-6 levels in obese diabetic mice (G. D. Noratto et al., 2017). Male Zucker fatty rats had decreased IL-6 plasma levels and no change in CRP levels with consumption of a 2% red raspberry-enriched diet compared to the control diet group (Kirakosyan et al., 2018). Male obese Zucker rat consuming a wild blueberry diet (8% (w/w)) for 8 weeks had lower CRP and IL-6 levels in the plasma compared to the control group (Vendrame, Daugherty, Kristo, Riso, & Klimis-Zacas, 2013).
These results suggest that WRR may have effective therapeutic benefit by attenuating the pro-inflammatory status associated with the MetS.

In our study, hepatic mRNA level of TNF-α was significantly up-regulated in the OZR compared to the LZR. Liver is a major site of production of inflammatory molecules. Patients and animal models with MetS, have an increase of cytokines including IL-6, TNF-α and NF-κB in the liver (Bieghs & Trautwein, 2013). MetS contributes to hepatic inflammation which leads to non-alcoholic fatty liver disease (NAFLD) and tissue damage (Bieghs & Trautwein, 2013). Previously reported, elevated levels of TNF-α promote insulin resistance and liver inflammation in patients with NAFLD (Adams, Angulo, & Lindor, 2005). It has been documented that hepatic expression of 58 out of 222 inflammatory and immune response genes including IL-6 and TNF-α was upregulated in morbidly obese patients (Bertola et al., 2010). Nuclear Factor- Kappa B activity is responsible for the production of pro-inflammatory cytokines, chemokines and adhesion molecules (Tak & Firestein, 2001). The binding of TNF-α to its receptor initiates a cascade of events that leads to the activation of NF-κB, modulating the inflammatory response (Bieghs & Trautwein, 2013). Similarly, IL-6 a pleiotropic cytokine contributes to hepatic inflammation (Bieghs & Trautwein, 2013). In the present study, consumption of WRR down-regulated hepatic expression of IL-6, TNF-α, NF-κB in the OZR compared to OZR-C. In alignment with the above results, a high-fat diet supplemented with raspberry anthocyanins (200mg/day) for 12 weeks, down-regulated the expression of hepatic IL-6, TNF-α, and NF-κB genes (Wu et al., 2018). Hepatic levels of IL-6 and TNF-α were attenuated with the consumption of quercetin in mice (C57BL/6), fed a high-fat diet (Kim, Choi, Joe, Chung, & Yu, 2016). Similarly, supplementation of fenugreek seeds (5%(w/w)) for 8 weeks modulated the expression of TNF-α and its receptor in the livers of female obese Zucker rats (Raju & Bird, 2006).
Donepudi et al. demonstrated that treatment with Jamun fruit extract, decreased NF-κB binding activity in the liver of C57B1/6 mice that had bile duct ligation (Donepudi, Aleksunes, Driscoll, Seeram, & Slitt, 2015). Supplementation of 8% wild blueberries for 8 weeks decreased hepatic expression of IL-6 and TNF-α in male obese Zucker rats (Vendrame, Daugherty, Kristo, Riso, et al., 2013). Thus, red raspberries may modify the mechanisms underlying pro-inflammatory mediator production of the liver.

Up-regulation in expression of pro-inflammatory markers IL-6 and TNF-α was observed in OZR-C compared to the LZR in the AT. As mentioned previously, obesity contributes to the MetS, increasing the risk of CVD and T2D. Obesity is characterized by an enlargement and increase in adipose tissue size (Ohashi, Shibata, Murohara, & Ouchi, 2014). Excessive fat accumulation is associated with increased levels of several inflammatory cytokines (Ohashi et al., 2014). Reduction of adipocyte size and number results in a decrease in the synthesis of TNF-α and IL-6 and an improvement in the metabolic state (Hotamisligil, 2017). In the present study, consumption of WRR downregulated expression of IL-6 and TNF-α in the AT of the OZR. Kang et al. reported that ellagic acid extracted from red raspberry seed flour, reduced expression of IL-6, IL-8, TNF-α and monocyte chemoattractant protein 1 (MCP-1) in adipose tissue of C57BL/6 mice fed a high-fat diet (Kang, Espín, Carr, Tomás-Barberán, & Chung, 2016). Vendrame et al. reported down-regulation of IL-6 and TNF-α in the adipose tissue following consumption of an 8% wild blueberry in obese Zucker rats (Vendrame, Daugherty, Kristo, Riso, et al., 2013).

Alternatively, subcutaneous adipose tissue expression of TNF-α was upregulated in post-obese subjects (Jüreis et al., 2017).
It has been well documented that conditions associated with the MetS increase TNF-\(\alpha\), IL-6 and adiposity and decrease adiponectin production resulting in a pro-inflammatory state (Aprahamian & Sam, 2011). In alignment, adiponectin gene expression in the AT was down-regulated in the OZR compared to the LZR in our study. Recent studies have suggested that consumption of polyphenols may regulate the low adiponectin levels associated with the MetS (Corrêa & Rogero, 2019). Gene expression of adiponectin was up-regulated in epididymal adipose tissue of Wistar rats fed a fructose-rich diet with chokeberry extract (200mg/kg of body weight daily) added to the drinking water for 6 weeks (Qin & Anderson, 2012). Alternatively, in the present study no change in adiponectin expression levels in the AT was observed following treatment of WRR in the OZR. Similar observations were reported in cultured human adipocytes treated with quercetin, epigallocatechin gallate, and resveratrol at 10 and 25\(\mu\)M for 24 hours and 10 and 25\(\mu\)M for 48 hours (Derdemezis et al., 2011). Even though pro-inflammatory markers IL-6 and TNF-\(\alpha\) were down-regulated in OZR-WRR group, no change in body weight between OZR-C and OZR-WRR were observed. This may explain why adiponectin expression levels in the AT and circulating plasma levels were unaffected following consumption of WRR. Reported previously, adiponectin plasma levels of individuals with MetS were unaffected with daily consumption (4 cups/day) of green tea (Basu et al., 2011). Similarly, no differences were observed in serum levels of adiponectin between the treatment group (30mg cocoa polyphenols, 80mg soy isoflavones, and 2g myo-inositol) and placebo of postmenopausal women diagnosed with MetS after 6 months of intervention (D’Anna et al., 2014). It has been documented that reduced adiponectin mRNA levels in adipose tissue were reversed after weight loss in obese subjects (Bruun et al., 2015)(W. Yang et al., 2001). Thus, further investigation is needed of the dietary role red raspberries in modulating adiponectin levels.
In conclusion, this is the first study to demonstrate that 8 weeks of WRR-enriched diet attenuates inflammation in the plasma, liver and AT of the OZR (Figure 23). The OZR exhibited elevated levels of circulating inflammatory molecules IL-6 and CRP compared to the LZR. With the consumption of WRR, circulating levels of IL-6 and CRP were attenuated in the OZR. Additionally, WRR down-regulated expression of IL-6, TNF-α, and NF-κB in the liver and IL-6 and TNF-α in the AT.

**Figure 23: Red raspberry consumption on inflammation in the obese Zucker rat.**

**Key:** NF-κB (Nuclear Factor Kappa); TNF-α (Tumor Necrosis Factor-Alpha); IL-6 (Interleukin-6); CRP (C-Reactive Protein)
5.2 Red raspberry consumption on lipid metabolism in the obese Zucker rat
(VandenAkker, Vendrame, Tsakiroglou, McGilvrey, & Klimis-Zacas, 2020)

The present study demonstrates that WRR enriched-diet positively modulates lipid metabolism by reducing circulating plasma total cholesterol and HDL-C levels, attenuating hepatic TG accumulation and facilitating its egress from the liver, by up-regulating hepatic expression of Mttp and down-regulating Fas.

In our study, plasma concentration of cholesterol was higher in the OZR compared to their lean littermates. Dyslipidemia is a characteristic of the MetS, and hypercholesterolemia in particular increases the risk of NAFLD (Du et al., 2016). In this study, a reduction in total plasma cholesterol levels was observed in OZR assigned to a WRR diet. Similar results were found in Zucker fatty rats supplemented with red raspberry (Kirakosyan et al., 2018) and wild blueberries (Vendrame, Daugherty, Kristo, & Klimis-Zacas, 2013). This data suggests that WRR have a cholesterol-lowering effect.

As stated in the introduction, plasma cholesterol is mainly carried in the HDL fraction and TG is mainly carried in the VLDL fraction in the male OZR (Aleixandre de Artiñano & Miguel Castro, 2009). Both plasma levels of HDL-C and non-HDL-C were significantly higher in the OZR compared to their lean littermates, independent of diet. Previous investigations, reported that the OZR exhibited an increase in VLDL-TG and HDL-C compared to their lean littermates (Schonfeld, Felski, & Howald, 1974). Also, the obese mouse model ob/ob, which has a nonsense mutation in the leptin gene similar to that of the OZR, has an increase in plasma HDL-C level (Nishina & Wang, 1994). Supplementation of WRR, significantly decreased HDL-C plasma levels in the OZR with a similar trend seen in non-HDL level. Similarly, an 8% (w/w) wild blueberry diet for 8 weeks reduced plasma HDL-C levels in the OZR (Vendrame,
We propose that WRR may improve HDL-C fraction by positively affecting its clearance (Fisher, Feig, Hewing, Hazen, & Smith, 2012).

In this study, consumption of WRR alleviated hepatic TG accumulation while elevating plasma TG levels in the OZR indicating enhanced mobilization of liver TGs. In parallel with these findings the size and weight of the liver was considerably reduced, along with a change in appearance bringing it closer to that of the LZR. This suggests that WRRs may be hepatoprotective and may aid in VLDL egress from the liver. Our results are similar to those observed in OZRs assigned to fenugreek seed (Raju & Bird, 2006), walnut oil (Fink et al., 2014), conjugated linoleic acid (Nagao, Inoue, Wang, Shirouchi, & Yanagita, 2005) and Opuntia ficus indica (Nopal) (Morán-Ramos S, Avila-Nava A, Tovar AR, Pedraza-Chaverri J, López-Romero P, 2012) enriched diets. A possible explanation of the reduction of TG accumulation in the liver with the WRR diet may be related to an enhanced export of TGs from the liver, consequently increasing levels of TG in the blood system, as other authors have also observed (Younossi, Loomba, Rinella, Bugianesi, & Marchesini, 2017). Although higher levels of TGs are normally considered unfavorable to health, in this study we documented a reduction in liver weight and hepatic TG accumulation via a decrease in de novo lipid synthesis suggesting that over a long-term treatment of WRR, a reduction of TG in the plasma may be achieved and this increase may indeed be transient. Since this study tested was an eight-week feeding period, it is not unreasonable to speculate that a longer dietary intervention may achieve a decrease in plasma TG as well. It has been documented that 12-week consumption of red raspberry powder (2%), red raspberry seed flour (equivalent to 0.03% of ellagic acid), or red raspberry anthocyanin (200mg/kg⁻¹) supplementation reduced total plasma TG levels in Zucker fatty rats and high-fat diet C57BL/6 mice (Kang et al., 2016; Kirakosyan et al., 2018; Wu et al., 2018).
Fatty acid synthase is responsible for de novo synthesis of fatty acids (Cohen, Nahari, Cerem, Neufeld, & Levi, 1996). In this study, Fas was significantly up-regulated in the liver and down-regulated in the AT of OZR compared to the LZR. In some pathological conditions such as obesity, Fas gene expression is dysregulated. It has been documented that obese patients have an increase in Fas gene expression in the liver (Wakil & Abu-Elheiga, 2009) whereas, Fas gene expression is decreased in AT (Diraison, Dusserre, Vidal, Sothier, & Beylot, 2002). Following consumption of WRR, the hepatic expression of Fas was significantly down regulated in the OZR, while no differences were observed in the AT. Previous studies have reported inhibition of Fas expression in HepG2 cells with treatment of mulberry water extract (Ou et al., 2011) and cyanidin-3-O-β-glucoside (Guo et al., 2012). Additionally, wild blueberry supplementation attenuated hepatic expression of Fas in the OZR (Vendrame, Daugherty, Kristo, & Klimis-Zacas, 2013). No alterations were observed in expression of Fas in white AT of high-fat induced C57BL/6J mice receiving whole table grape powder (5% w/w) for 16 weeks (Collins et al., 2016). Our observations suggest that WRR may decrease the synthesis of fatty acids therefore decreasing hepatic steatosis.

Microsomal triglyceride transfer protein is critical for proper folding of ApoB. The transport of TG from hepatocytes occurs through the assembly of VLDL by the endoplasmic reticulum in a two-step process (Schreuder, Verwer, van Nieuwkerk, & Mulder, 2008). The first step is the lipidation of apolipoprotein B (ApoB) which creates a pre-VLDL particle and is catalyzed by Mttp (Schreuder et al., 2008). The pre-VLDL then becomes enriched with lipids and is eventually secreted by the liver. In rodent models and in patients with NAFLD, Mttp activity is decreased and accumulation of lipids in the liver is observed (Younossi et al., 2017). In this study, Mttp expression was down-regulated in the liver of the OZR compared to the LZR,
independent of diet. Similarly, Frink et al. documented that expression of \textit{Mttp} was down-regulated in the OZR compared to their lean littermates (Fink et al., 2014). In this study, WRR diet, up-regulated hepatic \textit{Mttp} expression in the OZR, thus enabling ApoB folding and hepatic TG export enhanced or aided via ApoB-VLDL. Dietary walnut oil for 10 weeks increased hepatic \textit{Mttp} expression in the OZR (Fink et al., 2014). Similarly, fish oil supplementation for 8 weeks up regulated \textit{Mttp} expression in the liver of high-fat diet-induced Sprague-Dawley rats (Chiu, Wang, Liu, & Chiang, 2018). Thus, whole red raspberries are able to regulate lipid accumulation in the liver through modulation of \textit{Mttp} expression by increasing the secretion of lipid rich VLDL particles.

Taken together, these results suggest that WRR is able to decrease liver weight, and hepatic TG accumulation through down-regulation of hepatic \textit{Fas} and up regulation of \textit{Mttp}, indicating that WRR modulates hepatic lipid signaling, energy storage and regulation (Figure 24).

In conclusion, WRR improved the dyslipidemia, lipid metabolism and hepatic dysfunction in the OZR by decreasing lipogenesis through modulation of gene expression in the liver and AT, mobilization of hepatic TG from the liver, and decreasing liver weight. We suggest that WRR plays an anti-steatotic and hepatoprotective role in the OZR, a model of the MetS.
5.3 Red raspberry consumption on endothelial dysfunction in the obese Zucker rat
(VandenAkker, Vendrame, Tsakiroglou, & Klimis-Zacas, 2020)

These results demonstrated that 8-weeks of WRR consumption partially normalized the vascular function of the OZR aorta by restoring the impaired Phe-induced constrictor response and attenuating the exaggerated Ach-induced relaxation response. This is the first study to investigate the role of WRR on vascular function.

It has been previously demonstrated that OZRs develop a dysfunctional vasoconstriction and vasorelaxation mechanical response compared to their lean littermates independent of vessel type and age (Davidson, Coppey, Kleinschmidt, Oltman, & Yorek, 2009; Lemaster et al., 2017; Moral-Sanz et al., 2011; C. L. Oltman, 2006; Christine L. Oltman et al., 2005; Rodriguez-Rodriguez et al., 2017; Romanko & Stepp, 2005; Vendrame, Kristo, Schuschke, & Klimis-Zacas, 2014; Vessières et al., 2010). However, endothelial-derived relaxing factors (EDRF) and
endothelial-derived constricting factors (EDCF) including NO and prostanoids are inconsistent among studies.

In this study, the OZR exhibited an attenuated Phe-induced vasoconstrictor response, developing a lower maximal force compared to LZR, with no significant difference in vessel sensitivity. Studies have also demonstrated inhibition of Phe-induced vasoconstriction response in the OZR in the thoracic aorta at 16 weeks of age (Vendrame et al., 2014)(Rodriguez-Rodriguez et al., 2017), in thoracic, coronary and mesenteric arteries at 16 to 40 weeks of age (C. L. Oltman, 2006), and in pulmonary arteries at 17 to 18 weeks of age (Moral-Sanz et al., 2011). In our study, the WRR diet partially restored the impaired vasoconstrictor response in the OZR-C in the absence of inhibitors. With the consumption of WRR the maximal force of contraction increased in the OZR with L-NMMA pre-treatment, suggesting their involvement on the NO pathway. With pre-treatment of MFA no significant change was observed in the maximal vasoconstrictor response of the OZR assigned to a WRR diet. Thus, their involvement on the COX pathway may be secondary to the NO pathway.

With regard to Ach-induced vasorelaxation, the OZR-C exhibited an exaggerated relaxation response compared to LZR-C at the first two doses $10^{-8}$ and $3\times10^{-8}$M. Vendrame et al. documented similar OZR and LZR responses (Vendrame et al., 2014). A WRR diet decreased the maximal relaxation in the OZR. With the inhibition of the NO pathway with L-NMMA, the OZR that consumed a WRR-enriched diet exhibited a lower relaxation response, indicating the involvement of the NO pathway. An exaggerated vascular function of the OZR-C was observed compared to the LZR-C at the first three doses $10^{-8}$, $3\times10^{-8}$ and $10^{-7}$M following pre-treatment of MFA. With consumption of the WRR diet, vascular function in the OZR at these three doses improved but not at 100 percent, suggesting the partial involvement of the COX pathway.
Similar results to our study were reported in aortic rings of 16-weeks of age OZR with supplementation of wild blueberries (Vendrame et al., 2014). A possible explanation for the decreased vasoconstriction response to Phe and the heightened vasorelaxation response in the OZR may be due to compensatory mechanisms of the dysfunctional endothelium of the OZR that agrees with the proteomic and genomic results of this study. Ellagic acid, a compound found in abundance in red raspberries increased vasorelaxation activity of aorta of 10-12 week old male Wistar rat (Yilmaz & Usta, 2013). Moreover, ethyl acetate extract from red raspberries decreased blood pressure of spontaneously hypertensive rats at 15 weeks of age (Jia et al., 2011). With the consumption of WRR, the OZR partially restored vascular response in the presence and absence of the inhibitors suggesting a partial involvement of both the NOS and COX pathways.

Nitric oxide is a powerful vasodilator synthesized by eNOS which is expressed mainly in endothelial cells (Jones et al., 2003), inhibits platelet and leukocyte aggregation and adhesion, DNA synthesis, mitogenesis, and proliferation of vascular smooth muscle cells thus promoting blood fluidity and preventing thrombosis (Mitchell, Ali, Bailey, Moreno, & Harrington, 2008). No changes were observed in plasma levels of NO and expression of iNOS between animal type and diet group but gene expression of aorta eNOS, was significantly upregulated in the OZR compared to LZR. It has been documented that in conditions of inflammatory and oxidative stress of cardiac function, there is an overproduction of NO (Mollace, Muscoli, Masini, & Cuzzocrea, 2005). Human umbilical vein endothelial cells exposed to chronic and acute flow conditions resulted in elevated eNOS mRNA expression and protein levels (Ranjan, Xiao, & Diamond, 1995). It has been proposed that the overexpression of eNOS may enhance blood flow by attenuating the vasoconstrictor response during heart failure to increase cardiac output and survival (Jones et al., 2003). Following the consumption of WRR, expression of aortic eNOS in
the OZR was significantly down-regulated, similar to that of the LZR. This result is in line with our observations that WRR consumption improved the vasoconstriction and vasorelaxation response in the OZR. Activity of eNOS within the endothelial cells is modulated by several factors including reactive oxygen species (ROS) (Sena, Pereira, & Seiça, 2013). Previous studies have demonstrated that anthocyanin-enriched extracts of blackberry, black raspberry, blueberry, cranberry, red raspberry and strawberry possess free radical scavenging activity due to their antioxidant profiles (Ma et al., 2018). Dihydrobenzofuran-type enantiomeric neolignans isolated from red raspberries attenuated ROS generation in SH-SY5Y cells (Le Zhou, Yao, Lu, et al., 2018a). Additionally, red raspberry leaf extract has been shown to modulate blood platelet reactivity and aggregation in ADP-stimulated blood, possibly via the modulation of the redox status (Dudzinska, Bednarska, Boncler, Luzak, & Watala, 2016). These studies in addition to our data suggest that the antioxidant activity of WRR may modulate factors associated with endothelial dysfunction by positively influencing the activity of eNOS from a compensatory to a regulatory role.

Nitric oxide and prostacyclin, a product of the COX pathway, work closely together for maintenance of vascular tone (Mitchell et al., 2008). Compromised release of one of these molecular entities places results in a dysfunctional endothelium, increasing the risk of cardiovascular events (Mitchell et al., 2008). The OZR presented an increase in COX-2 activity compared to its lean littermate. Moreover, the expression of COX-2 in the OZR assigned a WRR diet was down-regulated. Anthocyanins have been shown to down-regulate expression and activity of COX-2 (Karlsson, Nnberg, Fjaeraa, & Wijkander, 2010; Mulabagal, Lang, Dewitt, Dalavoy, & Nair, 2009; Seeram, Momin, Nair, & Bourquin, 2001; Seeran, Zhang, & Nair, 2009; X. Xiao et al., 2011). In particular, the anthocyanin fraction of red raspberries containing
cyanidin-3-sophoroside, cyanidin-glucoside, cyanidin-rutinose and cyanidin-glucosylrutinose demonstrated a 47% COX-2 inhibition when tested against a COX-inhibitory assay (Seeram et al., 2001). Ellagic acid suppressed COX-2 expression in human monocytes pre-treated with lipopolysaccharide (Karlsson et al., 2010). As we expected with the shift to COX-2, the concentration of PGI₂ was elevated in the OZR-C compared to LZR in the aortic effluent. With the consumption of WRR, PGI₂ in the aortic effluent was attenuated in the OZR.

Supplementation of anthocyanin extract from black rice composed of predominately cyanidin-3-glucoside decreased serum TXA₂ and PGI₂ ratio in rats fed a high-fat diet (Y. Yang et al., 2011). In our study, aortic effluent concentration of TXA₂ did not change. Plasma concentration of TXA₂ was not altered with supplementation of 8% wild blueberries in spontaneously hypertensive rats (Kristo et al., 2013). One explanation for these observations is that the COX pathway is responsible from the production of prostanoids through COX-1 and COX-2 activity. COX-1 is responsible for the production of TXA₂, whereas the activation of COX-2 by inflammatory stimuli shifts prostanoid synthesis to favor PGI₂ and PGE₂ (Vichai, Suyarnsesthakorn, Pittayakhajonwut, Sriklung, & Kirtikara, 2005)(Nakano et al., 2007). It has been documented in numerous studies that COX-2 is upregulated with the MetS and in coronary arteries from insulin-resistant obese Zucker rat (Sánchez et al., 2010a) and HUVECs stimulated with IL-1β (Caughey, Cleland, Penglis, Gamble, & James, 2001). It has been proposed that MetS up-regulated COX-2 by enhancing the release of relaxant prostaglandins thus playing a protective role on endothelial function (Sánchez et al., 2010b).

These results of upregulation of EDRF’s, eNOS and COX-2 and increase in PGI₂ concentration, mirror our observations on vascular tone of a lessened vasoconstriction and an heighten vasorelaxation response of the OZR compared to the LZR, independent of diet.
Supplementation of WRR was able to modulate vascular tone in the OZR by down-regulating eNOS and COX-2 and decreasing PGI₂ concentration in the aortic effluent. We speculate that the observed results are due to WRR’s antioxidant and vasodilatory properties; acting as activators or inhibitors of multiple signaling pathways of the vasculature, protecting vessels against endothelial dysfunction.

Results from this study suggest that the dysfunctional aorta of the OZR is protected by COX-2 production of vasodilator prostaglandins as a compensatory response (Figure 25). In our study, the consumption of WRR improved vascular function in the OZR by down-regulating the expression of eNOS and COX-2 in the aorta. The decrease in COX-2 activity resulted in a decrease in the synthesis of PGI₂. This aligns with the mechanistic observations in this study of a heightened Phe-induced vasoconstriction response and an attenuated Ach-induced vasorelaxation response in the OZR that consumed the WRR diet.

**Figure 25: Red raspberry consumption on vascular function in the obese Zucker rat.**

**Key:** eNOS (Endothelial Nitric Oxide); NO (Nitric Oxide); COX-2 (Cyclooxygenase-2); PGI2 (Prostacyclin I2)
In conclusion, this is the first study to demonstrate that 8-week consumption of WRR improves the vascular mechanical properties of the impaired vasoconstriction and vasodilation function of the OZR and reveals that red raspberries operate through the partial involvement of both NO pathway COX pathway. These results combined with previous research document that supplementation of diets with WRR may play an important role in improving vascular dysfunction, associated with the MetS.

5.4 Significance

Metabolic Syndrome is a constellation of abnormalities such as insulin resistance, hypertension, dyslipidemia, pro-inflammatory, pro-thrombotic, and obesity that are associated with an increased risk of CVD and T2DM (Brauna, Bitton-Worms, & le Roith, 2011)(Pothiwala, Jain, & Yaturu, 2009). Associated risk factors has been diagnosed in adults and adolescents (Engelmann, Lenhartz, & Grulich-Henn, 2004) (Tailor, Peeters, Norat, Vineis, & Romaguera, 2010). The incidence worldwide have increased over the past two decades, and is estimated to rise (Eckel et al., 2010) (Misra & Khurana, 2008). Therefore, this research is imperative to increase attention and focus on non-pharmacologic treatments in efforts to reduce the risk factors of MetS. There is a great deal of literature documenting strong evidence of dietary changes as a treatment for effectively preventing and reversing MetS (de la Iglesia et al., 2016) (Calton, James, Pannu, & Soares, 2014). Especially, a diet rich in fruits and vegetables due to their health promoting properties (Vendrame, Del Bo’, Ciappellano, Riso, & Klimis-Zacas, 2016) (Cicero & Colletti, 2016) (S. Panickar, 2013). However, very little research has demonstrated the activity of whole red raspberries on human health.
Red raspberries have a unique phytochemical profile which makes this fruit an attractive subject to study (Venketeshwer Rao & Snyder, 2010). Currently, there are a limited studies that demonstrate the positive benefits of an enriched red raspberries diet in an animal model of the MetS. This work is of interest not only to the scientific community but also to health care providers and the food industry. By studying the relationship of the consumption of whole red raspberries and MetS and its associated risk factors, professionals will be able to manipulate the diet to enhance these effects. Whole red raspberries may be able to be promoted as a preventive measure for MetS and may also be included and strongly recommended for patients with MetS to incorporate whole red raspberries into their diet. These patients may be able to see improvement from a diet rich in whole red raspberries without suffering from the harsh side effects and financial burden of traditional pharmacotherapies. This research may be able to positively influence the health of our population as well as further aid economically the small red raspberry industry.
REFERENCES


BIOGRAPHY OF THE AUTHOR

Natalie Esther (Marchi) VandenAkker was born and raised in Northbridge, Massachusetts on December 16, 1990. She graduated from Northbridge High School in 2009. In 2013, she graduated from Worcester State University with a bachelor’s degree in Biology and minors in Chemistry and Business. While working full-time at a pharmaceutical company, she graduated from Worcester State University in 2015 with a master’s degree in Biotechnology. Following graduation, she worked at a biotechnology start-up before entering the doctoral program and dietetic internship at the University of Maine in 2017. Natalie is a candidate for the Doctoral of Philosophy degree in Food and Nutrition Sciences from the University of Maine in August 2021.