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Effects of Red Raspberry Polyphenols and Metabolites on Biomarkers of Inflammation and Insulin Resistance in Prediabetes and Type 2 Diabetes

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EFFECTS OF RED RASPBERRY POLYPHENOLS AND METABOLITES ON BIOMARKERS OF INFLAMMATION AND INSULIN RESISTANCE IN PREDIABETES AND TYPE 2 DIABETES

A Dissertation

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by
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This work is dedicated to my wonderful wife Mardeli and my beloved family in Honduras, Peru and the United States.

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LIST OF ABBREVIATIONS

ACN	anthocyanins
ADME	absorption, distribution, metabolism and excretion
AGEs	advanced glycation end-products
APPs	acute phase proteins
AR	aldose reductase
ATP	adenosine triphosphate
BETA2	beta-cell E box transactivator
BMI	body mass index
BSL	baseline
CML	Nε-carboxymethyllysine
CRP	c-reactive protein
CVD	cardiovascular diseases
C3G	cyanidin-3-glucoside
C3S	cyanidin-3-sophoroside
DN	diabetic nephropathy
DOPAC	3,4-dihidroxyphenylacetic acid
DR	diabetic retinopathy
DAG	diacylglycerol
dAGEs	dietary advanced glycation end products
ELISA	enzyme-linked immunosorbent assay
EF	endothelial dysfunction
eNOS	endothelial nitric oxide synthase
ER	endoplasmic reticulum

ETs	ellagitannins
ESI	electrospray ionization mass spectrometry
FFA	free fatty acid
FPG	fasting plasma glucose
GDM	gestational diabetes mellitus
GH	growth hormone
GLUT2	glucose transporter 2
GM-CSF	granulocyte-macrophage colony-stimulating factor
GPx	glutathione peroxidase
GSH	reduced glutathione
GSIS	glucose stimulated insulin secretion
GSSG	glutathione
HbA_{1C}	hemoglobin A1C
HNF-1α	hepatocyte nuclear factor 1 alpha
HNF-4α	hepatocyte nuclear factor 4 alpha
HNF-1β	hepatocyte nuclear factor 1 beta
HOMA-IR	homeostasis model assessment of insulin resistance
hsCRP	high sensitivity c-reactive protein
HSP	hexosamine synthesis pathway
HTs	hydrolysable tannins
HUVEC	human umbilical vascular endothelial cells
IAPP	islet amyloid polypeptide
IFG	impaired fasting glucose
IFN-γ	interferon gamma
IGF-1	insulin-like growth factor-1

IGT	impaired glucose tolerance
IL-1	interleukin-1
IL-6	interleukin-6
iNOS	inducible nitric oxide synthase
IPF-1	insulin promoter factor 1
IL-1β	interleukin-1 beta
IL-2	interleukin 2
IL-4	interleukin 4
IL-8	interleukin 8
IL-10	interleukin 10
IL-12p70	interleukin 12p70
IL-13	interleukin 13
IR	insulin resistance
IRS-1	insulin receptor substrate-1
JNK	c-jun amino terminal kinase
K_{ATP}	ATP-sensitive K+ channels
MIP-1A	macrophage inflammatory protein-1 alpha
MCP-1	monocyte chemoattractant protein-1
MODY	maturity-onset diabetes of the young
MS	metabolic syndrome
MS/MS	mass spectrometry/mass spectrometry
NAD+	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
NOX	nicotinamide adenine dinucleotide phosphate oxidase
NeuroDI	neurogenic diabetes insipidus

NF-κB	nuclear factor kappa β
NLRP3	NOD-like receptor pyrin domain containing protein 3 inflammasome
NOD	nucleotide oligomerization domain
NO	nitric oxide
OGTT	oral glucose tolerance test
PAI-1	plasminogen activator inhibitor 1
PCA	protocatechuic acid
PF	post-feeding
PI3-K	phosphatidylinositol 3-kinase
PKC	protein kinase c
PPARγ	peroxisome proliferator-activated receptor-gamma
QTOF	quadrupole time of flight
RAGE	receptor of advanced glycation end product
RNS	reactive nitrogen species
ROS	reactive oxygen species
RPMI	Roswell Park Memorial Institute medium
RR	red raspberries
SAA	serum amyloid A
SAS	statistical analysis system
T1DM	type 1 diabetes mellitus
T2DM	type 2 diabetes mellitus
TGF-β	transforming growth factor beta
TLR	toll-like receptors
TNF-α	tumor necrosis factor-α
UM	urolithin metabotype

UPLC	ultra-performance liquid chromatography
Uro-A glur	urolithin A glucuronide
Uro-A sulf	urolithin A sulfate
VDCCs	voltage-dependent Ca2+ channels
VEGF-A	vascular endothelial growth factor-A
VEGFR2	vascular endothelial growth factor receptor-2
WHO	world health organization

ABSTRACT

Berry fruits are rich sources of polyphenolic compounds (PCs), which can promote health benefits. Anthocyanin concentrations of red raspberry (RR)(*Rubus idaeus*) extracts were determined to be a total of 887.6 ± 262.8 µg/g cyanidin-3-sophoroside (C3S) equivalents with C3S being the most prevalent. Ellagitannin analysis by MALDI-TOF indicated sanguiin H-6 and lambertianin C were the major ellagitannins present in RR. To explore the efficacy of RR in modulating diabetes, seven type 2 diabetes mellitus (T2DM) and two pre-diabetic patients were given drinks delivering one RR serving (123 g) per day in a smoothie for two weeks. Blood samples were drawn at baseline (BSL) and post-feeding (PF) days. The samples were analyzed for phenolic metabolites, and for both inflammation and insulin resistance biomarkers. Two urolithin conjugates, i.e. urolithin A glucuronide (Uro-A glur) and urolithin A sulfate (Uro-A sulf) were found in 7 of the 9 patients' plasma samples at nanomolar concentrations on PF day whereas anthocyanin-derived metabolites such as protocatechuic acid (PCA) and 3,4-dihydroxyphenylacetic acid (DOPAC) were present at higher but not statistically significant levels on both groups at PF day when compared to BSL. Results indicated significant reductions in hsCRP (p= 0.01), and on insulin resistance where a statistical trend on HOMA-IR (p=0.0584) for T2DM patients was seen. DOPAC, a metabolite from anthocyanin and quercetin consumption in RR, when incubated at 1-100 µM did not stimulate insulin secretion in INS-1 rat pancreatic cells. Increases and decreases were observed on the cytokines analyzed by multiplex assay, yet, none was significant on either group. This study demonstrates the potential of RR to modulate levels of biomarkers of inflammation and insulin resistance in diabetic prediabetic patients .

CHAPTER 1. LITERATURE REVIEW

1.1. Diabetes Overview

The prevalence of type 2 diabetes globally is a concern and a significant burden due to its many health complications [1]. Diabetes is one of the most common chronic conditions and was the seventh leading cause of death in the United States (US) as of 2015 [2]. According to the International Diabetes Federation, 382 million people were affected by this disease in 2013 and the prevalence of the disease is expected to rise to 592 million by 2035 [3]. The World Health Organization (WHO) on its 2015 report discloses that cardiovascular diseases (CVD) are the main cause of death globally and prevalence of diabetes among adults over 18 years of age has increased from 4.7% in 1980 to 8.5 % in 2014. Projections indicate diabetes will still be the 7th leading cause of death in 2030 [4]. In the US, recent data indicate that 30.3 million people of all ages had the condition in 2015 (~ 9.4 % of the population) of which 30.2 million were adults aged 18 or older and of which 7.2 million were not aware or did not report they had diabetes [2]. An additional 84.1 million adults aged 18 years or older had prediabetes and nearly half (48.3%) of the adults aged 65 or older were prediabetics [2]. Regardless of technical and technological progress achieved to date, which has evolved with therapeutic tools and public health plans, it has not been possible to stop the progression of diabetes along with its complications [5]. Cardiovascular diseases are the major cause of death and disability among diabetic patients [6]. Diabetic vascular complications constitute a serious problem in diabetes which leads to additional functional decline of different organs and cause micro- and macro-angiopathy [7].

Diabetes as a disease has been recognized for several thousand years in various medical systems and cultures ranging from ancient Egypt, to classical India, China, Greece and Rome. Its meaning in Sanskrit: *Madhumedha*, shares the descriptive denotation as its Latin version, *diabetes mellitus*, which is sweet urine. In India, for instance, a classical diagnostic test was performed by pouring the patient's urine on the ground close to an ant colony and if ants swarmed the urine, the patient was deemed a diabetic [8].

1.1.1. Diabetes Types, Etiology, and Symptoms

Diabetes mellitus is a metabolic disorder marked by elevated levels of blood glucose (hyperglycemia) with disturbances on carbohydrate, fat and protein metabolism as a result of insufficient or inefficient insulin secretion, action, or both [9, 10]. Characteristic symptoms that accompany the disease are thirst, polyuria, blurred vision, and weight loss with severe symptoms leading to ketoacidosis or non-ketotic hyperosmolar state which can derive in stupor, coma or even death when not treated properly [10]. Based on the pathogenesis or etiology of the disease, diabetes can be classified in type 1 diabetes mellitus (T1DM), type 2 diabetes mellitus (T2DM), other specific types of diabetes and gestational diabetes. T1DM makes up 5-10% of the diabetic patients [11]. It is a less common form of the disease and is associated with autoimmune destruction of the pancreatic β-cells and therefore complete or near-total insulin deficiency. T1DM has been further sub-classified as Type 1A and Type 1B. Type 1A or autoimmune diabetes can sometimes be associated with other autoimmune conditions that include Hashimoto's thyroiditis, Addison's disease, vitiligo and others [12]. Because genetic factors alone do not predict the development of type 1 diabetes, therefore, environmental factors that may be involved include viral infections, toxins from food, cow milk intake during

childhood (instead of breast feeding) or vitamin D deficiency [13]. Type 1B or idiopathic diabetes on the other side, does not present an autoimmune basis to β-cell destruction and patients who suffer this type of diabetes are mainly of African or Asian ancestry [11]. T2DM, on the other hand, accounts for 90-95% of diabetic patients and its onset is dictated by a typically relative, not absolute, β-cell failure to produce insulin. This production becomes insufficient and leads to insulin resistance. Individuals with T1DM or T2DM have an increased risk of developing a number of chronic diseases, which affect the heart and blood vessels, eyes, kidneys and nerves often referred as microvascular complications. Furthermore, effects on the heart and blood vessels can cause fatal complications such as coronary artery disease and stroke which constitute the macrovascular complications that accompany the disease, with cardiovascular disease being the number one cause of death in people with diabetes mellitus [14].

Other specific types of diabetes include maturity-onset diabetes of the young (MODY) which is characterized by early age (< 25 yrs.) onset and autosomal dominant mode of inheritance. The cause of hyperglycemia in MODY is due to mutations in certain genes yet insulin resistance appears to be extremely rare [15]. Six types of MODY have been described and affect specific genes such as HNF-4α, Glucokinase, HNF-1α, lPF-1, HNF-1β, NeuroDI or BETA2 and this modality of diabetes can encompass 2-5 % of all T2DM cases [13].

Other types of diabetes can occur due to genetic defects of insulin action, due to diseases of the exocrine pancreas (pancreatitis, small pancreatic carcinomas) and in patients with cystic fibrosis, diabetes is the most common comorbidity occurring in 20% of adolescents and 40–50% of adults [16]. Endocrinopathies on which excessive production of certain hormones such as cortisol, glucagon and

catecholamines is present, the use of certain drugs and chemicals, i.e. rat poison, pentamidine, glucocorticoids and infections with viruses like rubella, are among other reasons that may cause diabetes [17, 18].

Gestational diabetes mellitus (GDM) is also a fairly common form of this disease and it is defined as a clinical condition observed in pregnant women as a result of insulin resistance and subsequent high blood glucose levels but also due to prevalence of overweight and obesity in women of childbearing age [19]. This state develops as a result of insulin action being blocked, presumably by placental hormones [20, 21].

Normally, gestational diabetes ends after the baby is born; however, women with gestational diabetes are at high risk of having the same condition in following pregnancies and are at higher risk of developing T2DM later in life but risk for T1DM is also present [22].

1.1.2. Disease Diagnosis

Several criteria can be used to diagnose whether an individual suffers from diabetes. One of them is through hemoglobin A1c (HbA_{1C}). HbA_{1C} is a test that reflects long-term blood glucose [23]. Hemolysates of red blood cells can be chromatographed, resulting in three or more small peaks named hemoglobin A1a, A1b, and A1c. These peaks (referred to as “fast” peaks) are eluted before the main hemoglobin A peak, and are formed by the irreversible attachment of glucose to hemoglobin in a two-step reaction [24]. The percentage of hemoglobin glycosylated depends on the average glucose concentration the red cell is exposed to over time, and due to the average 4-month life of a red cell, the percentage of glycosylated hemoglobin indicates accurately the degree of blood sugar control over the preceding weeks. Hemoglobin A1c is quantifiably the largest peak so that it is mostly

selectively measured, but occasionally all “fast" hemoglobins can be measured. An $HbA_{1C} \geq 6.5$ % will deem a positive diagnostic for diabetes. Another way is through plasma glucose criteria by measurement of either fasting plasma glucose (FPG) or the 2-h plasma glucose (2-h PG) value by means of an oral glucose tolerance test (OGTT) after taking a 75-g glucose containing drink. FPG ≥126 mg/dL (7.0 mmol/L) or 2-h PG ≥200 mg/dL (11.1 mmol/L) during an OGTT will also indicate the person being evaluated is indeed a diabetic [25].

“Prediabetes” is the term used for individuals with impaired fasting glucose (IFG, defined as FPG levels 100–125 mg/dL or 5.6–6.9 mmol/L) and/or impaired glucose tolerance (IGT, defined as 2-h PG after 75-g OGTT levels 140–199 mg/dL or 7.8–11.0 mmol/L). These conditions indicate pre-disposition for future development of diabetes. IFG and IGT should be considered risk factors for diabetes and CVD, and are associated with obesity, dyslipidemia (high triglycerides and/or low HDL cholesterol), and hypertension [25], a cluster of conditions similar as those observed on the metabolic syndrome (MS) [26]. Besides IFG and/or IGT, individuals with an A1C of 5.7–6.4% are considered at increased risk for diabetes and CVD and should adopt effective strategies to lower their risks [25].

1.2. Reactive Oxygen Species and Insulin Resistance

Oxidative stress and oxidative damage to tissues are a habitual result of chronic diseases such as atherosclerosis, diabetes and rheumatoid arthritis [27]. Oxidative stress denotes an imbalance between cellular reactive oxygen species (ROS) and antioxidants, where the former have an advantage. Examples of ROS include the superoxide anion ($\bullet$-O_2), hydrogen peroxide (H_2O_2), hydroxyl radical (OH), singlet oxygen ($^1O_2$) and ozone (O_3). Growing evidence suggests that oxidative stress has a causative role in insulin resistance [28]. To illustrate this, a

reduction on mitochondrial hydrogen peroxide emission when treating rats with a mitochondrial-targeted antioxidant or by overexpression of catalase in mouse skeletal muscle aided on preserving insulin sensitivity [29].

Highly reactive molecules include ROS and reactive nitrogen species (RNS). Among these reactive molecules superoxide ($\bullet O^{-}_{2)}$, nitric oxide ($\bullet NO$), and peroxynitrite ($ONOO^{-}$) are the most widely studied and are relevant in the diabetic cardiovascular complications. Superoxide ($\bullet O^{-}_{2}$) is produced by one electron reduction of oxygen by different oxidases such as dihydro-nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX), xanthine oxidases, cyclooxygenase and also by mitochondrial electron transport chain during normal oxidative phosphorylation, a fundamental process for generation of adenosine triphosphate (ATP) [30]. Five major pathways become activated because of increased production of superoxide, all of which are involved in the pathogenesis of diabetic complications: the polyol pathway flux, increased formation of advanced glycation end-products (AGEs), increased expression of the receptor for AGEs and its activating ligands, activation of protein kinase C (PKC) isoforms and over activity of the hexosamine pathway [31].

The polyol pathway is a pathway of glucose metabolism regarded as a key component in the pathogenesis of diabetic retinopathy, refractive changes and cataract formation in diabetic patients [32].The enzyme aldose reductase (AR) plays an important role in the polyol pathway as it reduces toxic aldehydes in the cell to inactive alcohols [33]. However, when intracellular glucose concentration becomes too high, sorbitol reduction from glucose by AR occurs as well and does this by using NADPH as a cofactor. NADPH is an important cofactor for the regeneration of antioxidants, and becomes reduced to glutathione (GSSG). A second enzyme,

sorbitol dehydrogenase oxidizes sorbitol to fructose, using nicotinamide adenine dinucleotide (NAD+) as a cofactor [32]. Sorbitol is an alcohol that is polyhydroxylated and strongly hydrophilic, and therefore does not diffuse easily through cell membranes leading to intracellular accumulation with possible osmotic repercussions[34].

Advanced glycation end products (AGEs) or glycotoxins, are highly oxidant compounds which can lead to diabetes and several other chronic diseases [35-37]. AGEs form by a nonenzymatic reaction between reducing sugars and free amino groups of proteins, lipids, or nucleic acids, a reaction also known as the Maillard or browning reaction [38]. Normal metabolism results in the formation of AGEs, however, when levels become too high in tissues and the circulation, they promote pathogenic effects through oxidative stress and inflammation. Modern diets with abundant heat-processing result in high levels of AGEs. Dietary advanced glycation end products (dAGEs) are recognized as a source of increased oxidant stress and inflammation, which are directly related to the recent epidemics of diabetes and cardiovascular disease [39]. A study with 21 patients on a high-AGE diet for 1 week resulted in weight gain and impaired insulin sensitivity [40]. These effects happen due to the binding of AGEs with cell surface receptors (such as the receptor of AGE, RAGE) or cross-linking which results in a disruption of the molecular conformation of proteins, lipids and nucleic acids and can lead to altered function [41]. Examples of widely studied AGEs are Nε-carboxymethyllysine (CML) [42] and the very reactive derivatives of methyl-glyoxal (MG) (i.e. hydroimidazolone) [43].

The diacylglycerol (DAG)-PKC pathway is one of the most studied pathways in cellular signaling induced by diabetes [44]. The protein kinase C (PKC) molecule

belongs to the serine/threonine kinase family, which catalyzes phosphorylation of proteins involved in signal transduction. Conventional and novel PKC isoforms are upregulated by (DAG) [45]. In diabetes, total DAG levels are elevated in vascular tissues such as those of retina, aorta, heart and renal glomeruli, and in nonvascular tissues such as liver and skeletal muscles, yet this is not the case in the central nervous system and peripheral nerves [46]. *In vitro* studies demonstrate DAG levels increase in a time-dependent manner as glucose levels rise from 5.5 to 22 mM in aortic endothelial cells, retinal pericytes, smooth muscle cells and renal mesangial cells [46].

PKC has been linked to vascular alterations which include increases in contractility, endothelial permeability, extracellular matrix synthesis, angiogenesis, cell growth and apoptosis, and cytokine activation and inhibition as well as leukocyte adhesion[47].

Several groups have proposed that flux through the hexosamine synthesis pathway (HSP) could have a role as a cellular nutrient sensor and be involved in the development of insulin resistance and the vascular complications of diabetes [48]. The HSP is a somewhat small component of the glycolytic pathway, accounting for ~ 3% of total glucose utilized [49]. The mode of action seems to be transcriptional regulation, possibly regulated by N-acetylglucosamine in O-linkage (O-GlcNAc) modification of transcription factors [48]. Incubation in high glucose, or with GlcNAc or overexpression of the first and rate limiting enzyme in HSP, glutamine: fructose-6-phosphate (F-6-P) amidotransferase (GFAT), enhanced the activation of the plasminogen activator inhibitor 1 (PAI-1) promoter, as well as stimulated the cytokine transforming growth factor beta (TGF-β) expression in mesangial cells or aortic endothelial cells [50, 51].

Beta cells have a high metabolic activity due to glucose oxidation-dependent stimulus secretion coupling, which derives in formation of ROS. With low expression of anti-oxidative enzymes such as catalase, super oxide dismutase (SOD) and glutathione peroxidase[52], β-cells are vulnerable to ROS and in both T1DM and T2DM, increased ROS formation results in β-cell loss [53]. ROS involvement in the progression of insulin resistance as well as pancreatic β-cell dysfunction has been demonstrated [54] and it was previously reported that ROS disrupted insulin-induced cellular redistribution of insulin receptor substrate-1 (IRS-1) and phosphatidylinositol 3-kinase (PI3-K), and thus impaired insulin-induced glucose transporter type 4 (GLUT4) translocation in 3T3-L1 adipocytes [55, 56]. Treatment with antioxidants such as N-acetyl L-cysteine and taurine appeared to prevent hyperglycemia-induced insulin resistance *in vivo* [57]. Acute and chronic administrations of the antioxidant α-lipoic acid improved insulin resistance in T2DM patients, advocating a role for ROS in the development of insulin resistance [58, 59].

1.3. Inflammation and Diabetes

Chronic inflammation is a crucial factor in the development of insulin resistance and T2DM (6). The presence of inflammation in metabolic disorders like diabetes mellitus has been studied in recent years and the inflammatory reaction is mediated by acute phase proteins and cytokines. The acute phase response is a systemic reaction to tissue injury and infection. This response leads to macrophage release of cytokines interleukin-1 (IL-1), interleukin-6 (IL-6) and tumor necrosis factor (TNF) which reach the liver and stimulate this organ to produce acute phase proteins (APPs) [60] . The two main APPs are C-reactive protein (CRP) and serum amyloid A (SAA) [61]. The term cytokine refers to an abundant number of cellular proteins that mediate pleiotropic (stimulation of numerous functions on many cell types) pro-

inflammatory and anti-inflammatory effects. In the case of diabetes, the release of such APPs and cytokines can have either diabetes-preventing or diabetes-promoting effects [62].

A distinctive feature of T2DM is the combination of both insulin resistance and pancreatic β-cell dysfunction, which leads to hyperglycemia at various levels. Some mechanisms identified to contribute to this condition include oxidative stress, nuclear factor kappa β (NF-κB) dependent production of pro-inflammatory cytokines, Toll-like receptors {TLR (Figure 1.1.)]) expression, and inflammasome [1][63-65]. T2DM has a genetic element but other factors such as over-nutrition (excessive food intake), lack of physical activity, but also exogenous factors such as medications, can intervene. Inflammatory pathways are a mean for T2DM to develop and as such, provide an important setting to develop treatments to prevent or control diabetes. NF-κB is considered the main control regulating the synthesis of many proteins critical for the activation and maintenance of inflammation [9]. IL-1 is a pleiotropic cytokine whose biologic effects include upregulated expression of adhesion molecules, cytokines and arachidonic acid metabolites as well as enhanced neutrophil accumulation, fibroblast proliferation and angiogenesis. The IL-1 family consists of two pro-inflammatory cytokines: IL-1α, IL-1β and a naturally occurring anti-inflammatory agent, the IL-1receptor antagonist (IL-1RA). IL-1β induces intra islet inflammation which in turn diminishes β-cell function and survival and more specifically, IL-1β plays a significant role on T2DM onset and insulin resistance [66]. TNF-α and IL-1β act as inducers of NF-kB activation producing elevated reactive oxygen species (ROS) levels. This suggests the involvement of ROS as common mediators of NF-kB activation [67]. Mature IL-1β is associated with the NOD (nucleotide oligomerization domain) -like receptor pyrin domain containing protein 3

(NLRP3) inflammasome subunit [1][68, 69]. NLRP3 is present in the pathogenesis of T2DM, Alzheimer's and amyotrophic lateral sclerosis [39].

Inflammation is a protective response of the host to infections and tissue damages and includes a series of reactions such as vasodilation and recruitment of immune cells and plasma proteins to the site of infection or injury. Reactive oxygen species (ROS) generated from deteriorated mitochondria activate NLRP3 inflammasome [70], and the latter promotes other inflammatory cytokines such as IL-18 and IL-33 which are involved in the pathogenesis of diabetes [71].

1.4. Insulin Resistance and β-cell Apoptosis

Increased insulin resistance (IR) is considered to be the major pathophysiological cause for T2DM in patients. In healthy individuals, insulin increases glycogen production in the liver, lipid synthesis by adipose tissue, and glucose uptake in muscle [72].

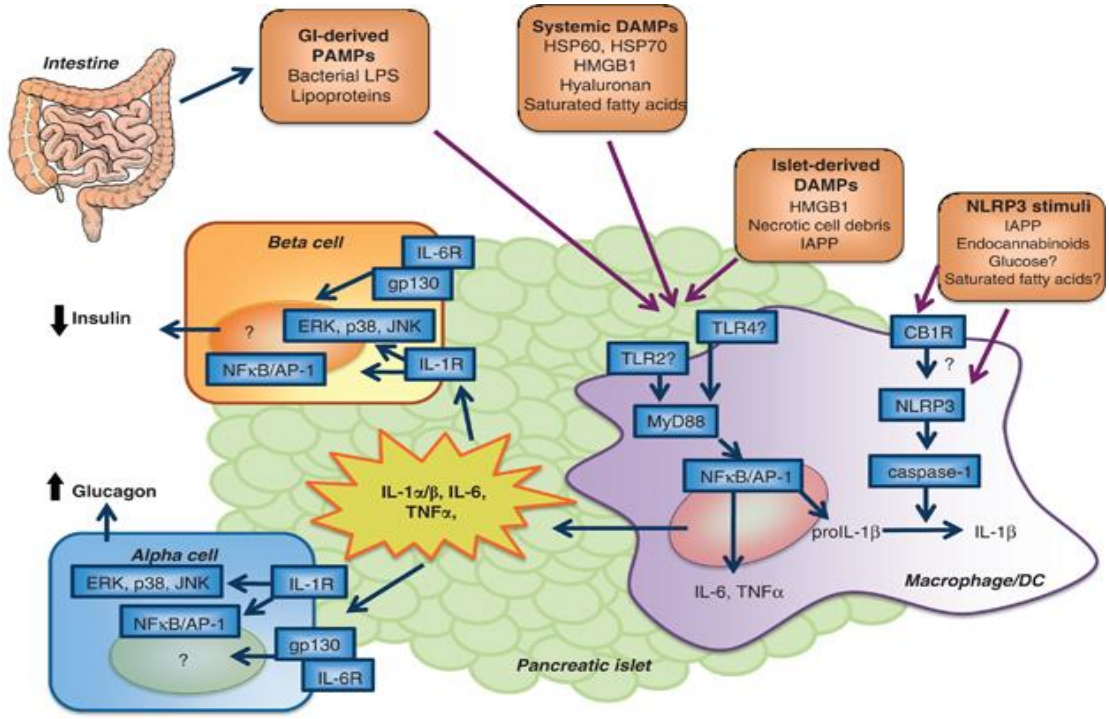

Figure 1.1. The pro-inflammatory response of islet macrophages in type 2 diabetes. (fig. cont'd)

“TLR2-and TLR4-activating PAMPs (LPS, lipoproteins) and DAMPs (HSP60, HSP70, HMGB1, hyaluronan, saturated fatty acids) are increased systemically in type 2 diabetes. Local islet-derived TLR2 and/or TLR4 DAMPs may also be increased and may include HMGB1, necrotic cell debris and IAPP deposits. These ligands may act on islet macrophages via TLR2 and/or TLR4, upregulating cytokine secretion (IL-6, TNF-α), with deleterious effects on β-cell insulin secretion and α-cell glucagon secretion. Islet-derived (IAPP, endocannabinoids) or systemically elevated (glucose, saturated fatty acids) NLRP3 stimuli also act on macrophages to facilitate cleavage of proIL-1β to mature IL-1β and secretion of IL-1β. IL-1β then acts in a paracrine manner, together with elevated IL-6 and TNF-α, to impair β-cell insulin secretion and cause dysregulated α-cell glucagon secretion. A potential role for macrophage-derived IL-1α also exists but has not been studied. AP-1, activator protein 1; DC, dendritic cell” [65].

IR is many times the result of reduced sensitivity of the insulin receptor that is composed of two insulin-binding α-subunits and two β-subunits [73]. A reduction of 40 % β-cell mass in young adults is enough to induce hyperglycemia, a hallmark of diabetes [74]. Problems with insulin secretion and action derive in numerous abnormalities in T2DM such as hyperglycemia due to disruption of insulin-stimulated glucose uptake, uncontrolled hepatic glucose production and dyslipidemia where impaired homeostasis of fatty acids, triglycerides and lipoproteins are usually observed [75].

Significant evidence suggests that obesity activates inflammatory signaling pathway and can induce endoplasmic reticulum (ER) stress with particular activation of serine/threonine kinases IκB kinase (IKK) and c-jun amino terminal kinase (JNK) [76]. Figure 1.2., shows how IKK and JNK pathways are activated in response to stimuli during metabolic dysregulation. This includes ligands for TNF-α, IL-1, Toll, RAGE, ROS and ER stress, ceramide, as well as several PKC isoforms [9]. JNK plays a central role in the cell stress response and recent evidence points at JNK1 and JNK2 isoforms as promoters of obesity and insulin resistance, whereas JNK3 activity seems to protect from excessive adiposity. Moreover, evidence indicates that

JNK activity can promote cell tolerance to the stress associated with obesity and type-2 diabetes in certain cell types at specific disease stages [77]. Even though IR is characteristic of T2DM, disease progression depends on insufficient insulin production, in all probability as result of reduced β-cell mass due to apoptosis [78].

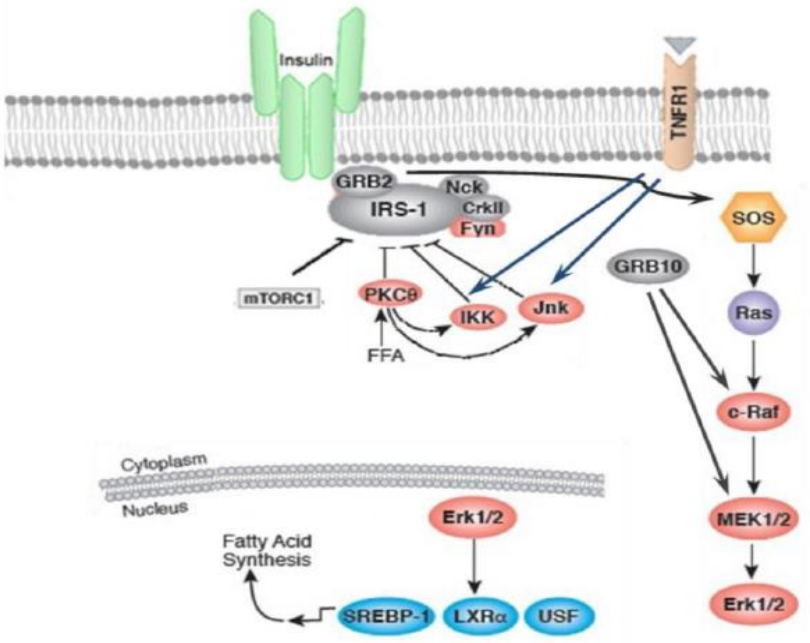

Figure 1.2. **“**The insulin action can be inhibited by inflammatory signaling pathways. Inflammation and stressful stimuli activates c-jun amino terminal kinase (JNK), IκB kinase (IKK), and protein kinase Cθ (PKC-θ) which result in inhibition of insulin signaling. The activation of sterol regulatory element binding protein-1c (SREBP-1C), upstream stimulatory factor 1 (USF1), and liver X receptor (LXR) induces fatty acid synthesis”[9].

Himsworth and Kerr were the first to recognize the relation between insulin resistance and diabetes in 1939 based on blood glucose responses to exogenous insulin [79]. They demonstrated that obese patients with diabetes could be classified into two categories: those who were insulin-sensitive (T1DM) and those who were insulin-resistant (T2DM) [79].Current information advocates at IR being an acquired issue vastly related to unhealthy lifestyles with impaired insulin secretion being the major genetic factor [80].

Overweight and obesity increase risk for diabetes and CVD, mainly through IR [81]. Weight loss has been proven beneficial in obese individuals with IR, yet, the same benefits have not been observed in overweight individuals [81]. McLaughlin *et al*. [81] administered a hypocaloric diet for 14 weeks and 2 weeks of weight maintenance to a group of healthy volunteers with BMI 25-29.9 kg/m2. They performed detailed metabolic phenotyping which included insulin-mediated-glucose disposal and fasting/daylong glucose, among others. To gauge adipose cell size on the individuals, subcutaneous fat biopsies were performed as well. The metabolic phenotype provides a description of the metabolic state of an individual and derives from the genetic and environmental (diet, lifestyle, gut microbial activity) contributions under a specific set of circumstances [82]. The researchers found the patients had weight loss (4.3 kg) which yielded significant improvements in insulin resistance and all cardiovascular risk markers except glucose, HDL-C, and LDL-C. They reported that insulin sensitivity was greater among those with < 2 vs > 2 cardiovascular risk factors at baseline. Decrease in adipose cell size and waist circumference, but not weight or body fat, independently predicted improvement in insulin resistance [81]. Their results suggested that IR overweight individuals, regardless of the absence of potential established CVD risk markers, can yield benefits from dietary weight loss, and that both, reduction in adipose cell size and waist circumference, are better predictors of metabolic response than weight loss *per se [81]*.

There is controversy on regards to the role of factors such as obesity, insulin resistance, insulin secretory dysfunction, and excess hepatic glucose production in the development of T2DM. Lilioja *et al*. [83] conducted a prospective study to determine which of these factors predicted the development of the disease in a

group of Pima Indians. The Pima Indians, a Native American population in Arizona, have the highest reported prevalence of diabetes of any population in the world [84]. Researchers assessed potential development of the disease through oral and intravenous glucose-tolerance tests, and a hyperinsulinemic-euglycemic clamp study. These were performed in 200 nondiabetic Pima Indians (87 women and 113 men; mean [±SD] age, 26 ±6 years). The subjects were followed yearly thereafter for an average of 5.3 years. Their results showed the development of diabetes in 38 subjects during follow-up. Obesity, insulin resistance (independent of obesity), and low acute plasma insulin response to intravenous glucose (with the degree of obesity and insulin resistance taken into account) were predictors of T2DM. The six-year cumulative incidence of T2DM was 39 percent in persons with values below the median for both insulin action and acute insulin response, 27 percent in those with values below the median for insulin action but above that for acute insulin response, 13 percent in those with values above the median for insulin action and below that for acute insulin response, and 0 in those with values originally above the median for both characteristics. Through these results, the researchers concluded IR was a major risk factor for the development of T2DM with low acute insulin response to glucose being an additional but weaker risk factor [83].

Besides being a storage place for fat, adipocytes perform an important endocrine role by secreting several relatively recently identified hormones and cytokines [80]. These molecules are hypothesized to play relevant functions in insulin action and metabolism of glucose and fat. The factor resistin, for instance, is an adipocyte-secreted peptide hormone that has been observed to impair glucose homeostasis and insulin action in rodents [85]. Work done previously shows circulating resistin levels are increased in diabetic rodent models and that the

administration of antiresistin antibody improves blood glucose and insulin sensitivity [85].

Thiazolidinediones that improve insulin sensitivity reduce resistin production [86] yet, the role of resistin in humans is not clear since reports of resistin protein levels and gene expression in obese humans remain inconsistent [87-89].

The hormone adiponectin is another promising and interesting bridge between IR and increased fat tissue. Studies show adiponectin plasma levels decrease proportionally with the accumulation of adipose tissue, especially visceral one [90] as well as with the development of insulin resistance and T2DM. Administration of adiponectin to mice enhanced insulin sensitivity and glucose tolerance, reduced food intake, lowered plasma glucose and triglyceride levels, and increased free fatty acids (FFA) oxidation in muscle [91-93].

Predisposition of some individuals to IR and T2DM from birth due to consistent IR has been found in first degree relatives and offspring of patients with T2DM. Hence, the underlying view is that IR is at least partially determined by a role played by genetic traits [94]. Warram *et al.* [95] reported that IR predicts the development of type 2 diabetes in the offspring of diabetic parents. This is reflected in young and healthy offspring of diabetic parents who exhibit IR and impaired muscle insulin signaling and glycogen synthesis many years before onset of evident T2DM [96]. Recent large-scale genome-wide association (GWA) studies, however, have failed to detect polymorphisms of genes involved in insulin action or insulin signaling which contribute to the development of T2DM [97]. Contrary to that, several genes thought to be involved in the control of insulin secretion have shown polymorphisms that grant minor but statistically significant increased susceptibility to T2DM [97]. Therefore, reports lead to speculate that IR in offspring of diabetic

mothers to some unknown degree may obey to the presence of obesity and/or mild glucose intolerance during pregnancy itself, and not necessarily to genetics [98]. Vaag [98] suggests that even though many genes involved in the development of T2DM remain to be discovered, the chance of 'genetic insulin resistance' due to intrauterine programming should remain open.

Data from several sources points out that insulin-like growth factor-1 (IGF-1) seems to play a role in progressive decline in insulin sensitivity observed in T2DM [99]. Obese individuals display abnormalities in the growth hormone (GH)/IGF-1 axis resulting in low basal GH levels and reduced IGF-1 levels compared with nonobese individuals. These levels are improved when weight loss is attained [100, 101].

In higher organisms, GH controls growth by regulating IGF-1 concentrations, but another major function of GH is to provide a mechanism for surviving periods of food deprivation. GH stimulates lipolysis, providing FFAs and glycerol as substrates for energy metabolism, and also inhibits insulin-induced suppression of hepatic gluconeogenesis. These effects counteract insulin action and reduce the need for a dietary source of carbohydrate [102].

IGF-1 shares 48% amino acid sequence identity with proinsulin. IGF-1 enhances insulin sensitivity in both experimental animals and human subjects and its primary insulin-sensitizing action is believed to be mediated through skeletal muscle [103]. A problem, however, when interpreting human studies of IGF-1 has been that, in addition to enhancing insulin action, it also suppresses GH secretion. This fact has made difficult to determine the relative roles of the direct actions of IGF-1along with those mediated by suppression of GH [103].

Besides IR, defects in pancreatic β-cell function constitute one of two major pathophysiologic abnormalities that lie behind most cases of T2DM [104]. Genetic and epigenetic components have been identified.

While the epigenome may change due to environmental exposure, variations may also be stable and inherited, making epigenetics a hypothetical important pathogenic mechanism. Impaired intrauterine environment from an environment that may alter the pancreatic islet epigenome and possibly affect β-cell function and diabetes pathogenesis is shown in human and animal studies. Resulting low birth weight, increased risk for postnatal metabolic disease, decreases in β-cell proliferation mass, and insulin secretion are some documented epigenetic modifications occurring at key β-cell genes [105, 106]. Epigenetic mechanisms include DNA methylation and histone modifications which can be active during fetal, postnatal and adult life [107].

Many physiologic stressors may influence β-cell function in the environment of metabolic overload and IR commonly found in human obesity-linked T2DM. Pathologic conditions associated with beta cell demise include ER stress, metabolic and oxidative stress, amyloid plaques, inflammation and disruption of islet integrity/organization (Figure 1.3.) [104].

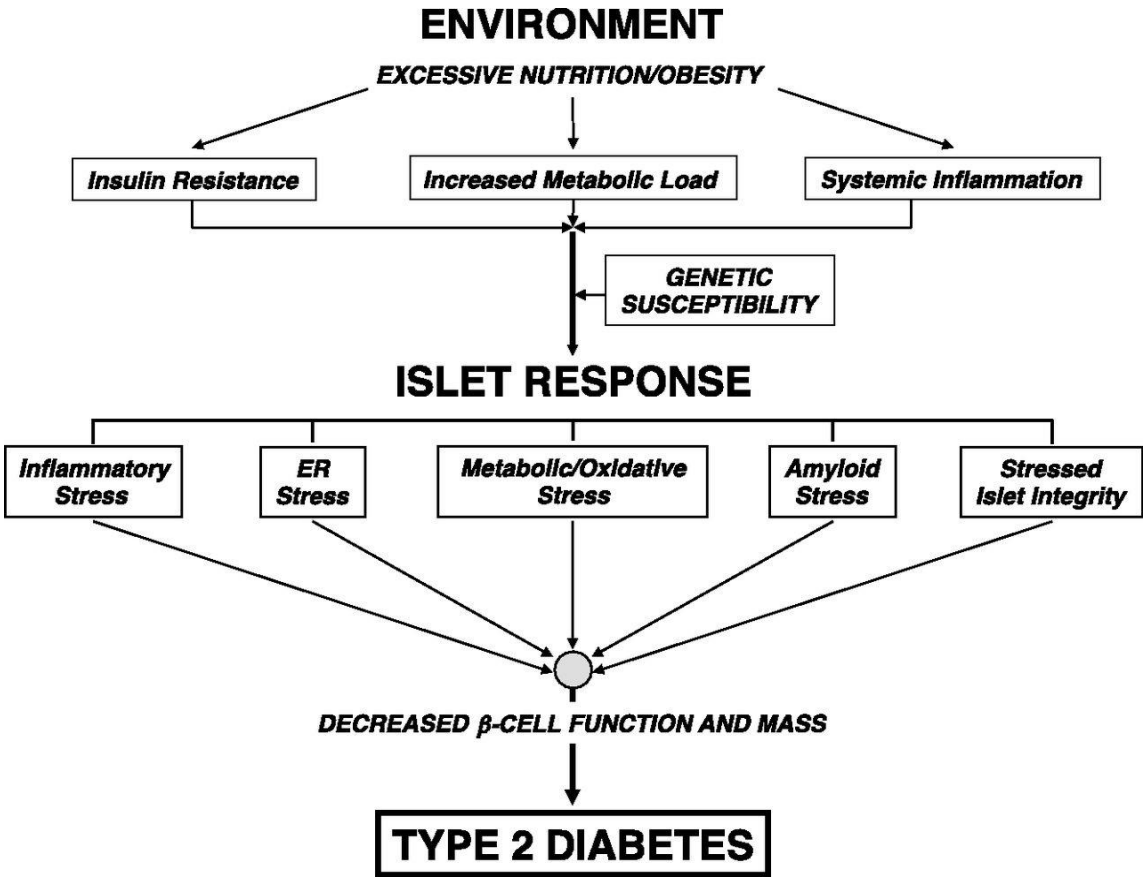

Figure 1.3. Stressors on the β-cell in the pathogenesis of T2DM [104].

Apoptosis or programmed cell death, is a complex biological process where events such as cell shrinkage, chromatin condensation, DNA fragmentation and finally disassembly into vesicles called apoptotic bodies are manifested [108]. Deposits of amyloid are characteristic of islets in T2DM [109] . These deposits are derived from amylin or islet amyloid polypeptide (IAPP) [110]. Excess nitric oxide (NO) production, when obtained through inducible nitric oxide synthase (iNOS), has implications in insulin resistance, markedly when obesity is present [111]. This may be explained by hyperglycemia and hyperlipidemia, as both conditions are common to T2DM, and can induce iNOS expression in islet tissue in healthy animals [112].

1.5. INS-1 Cells and Insulin Secretion

Glucose represents the main physiological stimulus for the secretion of insulin from pancreatic β-cells. As glucose enters the cells through glucose transporter 2

(GLUT2), it becomes phosphorylated to glucose-6-phosphate by the enzyme glucokinase and is then metabolized to generate ATP. Production of ATP induces the closure of ATP-sensitive K^+ channels (K_{ATP}) which induces membrane depolarization and the opening of voltage-dependent Ca^{2+} channels (VDCCs). This results in an increase in cellular Ca^{2+} influx and serves as a primary driver of the insulin secretory mechanism [113, 114].

Insulinoma-derived INS-1 and INS-1E cells are trustworthy beta-cell surrogates which display electrophysiological properties, secretagogue-induced electrophysiological activity, Ca_2+ signaling, stimulus-secretion coupling and sulfonylurea and diazoxide-sensitivities similar to those found in native islets [115]. Glucose stimulated insulin secretion (GSIS) is important for the control of metabolic fuel homeostasis and defective GSIS is a critical part of β-cell failure that leads to T2DM [116]. Geniposide, an iridoid glucoside derived from *Gardenia jasminoides* or gardenia fruit [117] was found to enhance GSIS in response to the stimulation of low or moderately high concentrations of glucose, and promoted glucose uptake and intracellular ATP levels in INS-1 cells. Experiments performed by Liu *et al.* [109] demonstrated that geniposide modulated pyruvate carboxylase expression and the production of intermediates of glucose metabolism suggesting this compound has potential to improve insulin secretion in β-cells challenged by high glucose concentrations. Kittl *et al.* when using quercetin to test insulin secretion on INS-1 cells [108], concluded that quercetin acutely stimulated insulin release, presumably by short-term K_{ATP} channel inhibition and simultaneous short-term stimulation of voltage-sensitive Ca^{2+} channels.

1.6. Economic Burden of Diabetes

Diabetes along with its complications represents a substantial economic loss to not only people who suffer from it, but also to their families, health systems and national economies through medical costs and loss of work and wages [118]. Recent systematic reviews estimate the world's annual cost of diabetes to be more than US $827 billion [119, 120]. The International Diabetes Federation (IDF) estimates that total global health-care spending on diabetes more than tripled between 2003 to 2013 as a result of increases in the number of people with the disease and increases in per capita diabetes spending [121]. The medical costs associated with T2DM continue to increase and there is a dire need for new options to treat the disease.

1.7. Therapeutic Approach

The major classes of oral antidiabetic medications include biguanides such as metformin, sulfonylureas, meglitinide, thiazolidinediones (TZD), dipeptidyl peptidase 4 (DPP-4) inhibitors, sodium-glucose cotransporter (SGLT2) inhibitors, and α-glucosidase inhibitors [122]. Even though they can be effective in ameliorating symptoms present in diabetes, a downside to these agents is that they may cause a myriad of disturbances or side effects. These disturbances include gastrointestinal ones such as diarrhea, nausea, and dyspepsia and may also be contraindicated in patients with renal failure [64].

1.8. Dietary Approach

An important recommendation by the American Association of Clinical Endocrinologists consists of consuming a plant-based diet which is high in fiber, low in carbohydrates and calories, and high in phytochemicals/antioxidants [123].

Phenolics encompass a numerous group of natural and anthropogenic compounds. Most natural phenolic compounds are secondary metabolites in plants

and trees, therefore, being present in foods but are also used as supplements, additives and nutraceuticals. Chinese physicians, for instance, have been using plant phenolics for many years to treat various diseases and disorders. Currently, more than 8,000 phytochemicals are known of which more than 5,000 are flavonoids.

These phytochemicals can be divided into at least 10 types depending on their basic structure and those are: phenols, phenolic acids, hydroxycinnamic acids, coumarins/isocoumarins, naphthoquinones, xanthones, stilbenes, anthroquinones, flavonoids and lignins [124]. Flavonoids can further be divided into flavones, flavonols, flavanones, flavan-3-ols, anthocyanidins and isoflavones. Phenolics are able to act as antioxidants through several routes.

The hydroxyl groups present in phenolics are good hydrogen donors: hydrogen-donating antioxidants, which can react with both reactive oxygen and reactive nitrogen species [125-128]. A variety of assays can be employed to measure the potential antioxidant and free radical scavenging capacity of polyphenolic containing foods. Those assays include: i) oxygen radical absorbance capacity or ORAC, which is based on hydrogen-transfer ability ii) trolox equivalent antioxidant capacity (TEAC) a method based on the scavenging ability of antioxidants to the long-life radical anion ABTS•+ and results from test compounds are expressed relative to trolox, a water soluble analog of vitamin E and iii) ferric reducing antioxidant power (FRAP) based on the ability of a compound which measures reduction of ferric 2,4,6-tripyridyl-s-triazine (TPTZ) to a colored product [129].

1.8.1. Dietary Flavonoids

Flavonoids represent a large class of phenolic compounds found in numerous food products such as fruits, vegetables, cocoa, chocolate, tea, red wine, as well as

other plant food and beverage products [130]. The Mediterranean Diet (MD) is a diet characterized by an abundance of vegetable foods (bread, pasta, vegetables, legumes, fruits and nuts) where flavonoids are considered to be important bioactive compounds which impart health benefits [131]. In terms of glucose homeostasis, experiments performed at both *in vitro* and *in vivo* levels demonstrate they can regulate carbohydrate digestion, insulin secretion, insulin signaling, and glucose uptake in insulin-sensitive tissues by means of several intracellular signaling pathways [132].

Regardless of the significant progress obtained in the fields of flavonoid bioavailability and their effect at the cellular level, this topic remains a complex one. Part of the problem arises from the fact that these dietary compounds are not drugs that possess clear pharmacokinetics and pharmacological targets [133].

Flavonoids are only moderately bioavailable and highly metabolized by intestinal, hepatic and bacterial cells and considered to be fairly reactive due to their phenolic nuclei which grants them a reducing character as well as affinity for proteins[133].

After the ingestion of red raspberries (RR), for instance, the polyphenols are at least partially bioavailable to systemic organs, through absorption, distribution, metabolism and excretion (ADME) [134]. Anthocyanins are widely reported to have low bioavailability, with most of the studies recording peak plasma concentrations (Cmax) ranging from 1 to 120 nmol/L [135] and urinary recoveries < 2% of intake [136] [137]. Yet, a recent study shows extensive colonic microbiota mediated degradation of $^{13}C_5$-labeled cyanidin-3-O-glucoside which resulted in the production of many phenolic metabolites over a 0-48 h period. The relative bioavailability of the

$^{13}C_5$-labeled was 6.9 % in breath as CO_2, 5.4 % in urine and 32% in feces in the form of $^{13}C_5$-labeled phenolic and aromatic compounds [138] [139].

Regarding ellagitannins and ellagic acid, also very significant polyphenolics in RR, their bioavailability is considered to be very low. These molecules are subject to extensive metabolism by the gut microbiota to produce urolithins that are much better absorbed. Urolithins circulate in plasma as glucuronide and sulfate conjugates at concentrations usually ranging from 0.2–20 μM [140]. Hence, it can be implied that the health effects of ellagitannin-containing products can be associated with these gut-produced urolithins, therefore, evaluation of the biological effects of these metabolites is essential [140].

Berries are distinguished from other fruits and vegetables containing phenolics by having high concentrations of anthocyanins, with strong antioxidant capacities, up to 4 times greater than non-berry fruits and 40 times that of cereals [141]. Berries can be considered as small fruits that can be eaten whole and include true berries such as black currant, red currant and gooseberry whereas false or epigenous berries include cranberry and blueberry. RR falls within the aggregate berries, which also include blackberry and hybrid boysenberry and the multiple berry mulberry.

RR are becoming more appreciated due to their culinary versatility and multiple applications [35] and are singular among berries due to their attractive red color, low glycemic index, low caloric value, high dietary fiber, good flavor, and high concentrations of hydrolysable and condensed tannins, flavonoids, phenolic acids, carotenoids including lutein and zeaxanthin, choline, potassium, and vitamin C and K1 [142]. Raspberries place very high when performing assays such as ORAC, TEAC and FRAP on the ranking of antioxidant fruits and vegetables. This allows

raspberries to be considered as one of the richest sources of dietary antioxidants overall [143]. The antioxidant capacity of raspberries is believed to derive from their vitamin C (~20%), anthocyanins (~25%) and ellagitannins (more than 50%) content [143].

RR are native to northern North America and Eurasia. Raspberries can be easily grown in numerous areas around the world and are relevant in both the fresh fruit market and for processing into frozen products, juices or dried fruit. Fruits of the raspberry tree are typically red colored but can also be black or yellow. Cultivated RR were introduced into the United States as long ago as 1771. RR are the third most consumed fresh berries in US households, accounting for ~ 3-4 % of total berry production and are considered delicacies which are cultivated to deliver more than 70 million pounds per year in leading producing regions such as Washington, Oregon and California. Raspberries can be consumed raw or as a processed (frozen, pureed) ingredient in a number of dishes, sauces, salads, and drinks [35].

1.8.2. Phytochemical Profile of Red Raspberry

Phenolic phytochemicals are abundant in plants and serve a plethora of biological functions such as roles in growth and development of the plant and in defense mechanisms to counter insects and UV radiation. The phenolic profile in RR consists mainly of anthocyanins and hydrolysable tannins. A serving of fresh RR contained the following biologically active compounds [43]:

(A) Ellagitannins: Sanguiin H-6 and lambertianin C [144] are the major ones. Ellagitannins (ETs) are hydrolysable tannins (HTs) which when compared to condensed tannins are more stable [43]. Berries are considered to be the major contributors to the ET intake in westernized countries [145]. ETs are known to be

present in large amounts in berry fruits. Sanguiin H-6 (Figure 1.4.) is the main ET in raspberry and strawberry [146].

Ellagitannins are capable of expressing exceptional biological activities such as the potentiation of antibacterial activity, the inhibition of mutagenicity off carcinogens and tumor promotion, host-mediated antitumor and highly potent antiviral effects [147, 148].

However, the therapeutic potential of ellagitannins remains unexploited in conventional (occidental) pharmaceutical approaches [149, 150] . *In vitro*, sanguiin H-6 has been found to inhibit cell viability in a concentration-dependent manner and it increased the rates at which MCF-7 and MDA-MB-231 human breast cancer cells underwent apoptosis [151]. An *in vitro* enzymatic study by McDougall *et al*. [152] strongly suggested that ellagitannins in raspberry were the main active components for amylase inhibition, an approach considered to have the potential to be used as a therapeutic agent to control non-insulin dependent diabetes mellitus or T2DM.

Ellagitannins are not absorbed per se but rather are subjected to the action of colonic microbiota which yields ellagic acid which is further converted to urolithins and the latter are absorbed into the circulatory system mainly as sulfate and glucuronide phase II metabolites [153] [154]. Urolithins and pyrogallol deter the formation of advanced glycation end products (AGEs) [155] which are highly responsible for diabetes and its complications [156].

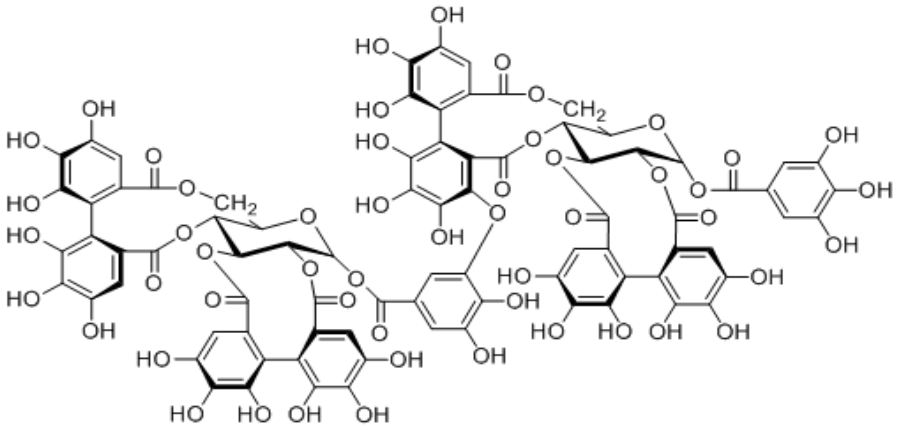

Figure 1.4. Chemical structure of the ellagitannin Sanguiin-H6.

(B) Proanthocyanidins: Also known as condensed tannins, proanthocyanidins (PA) are oligomeric and polymeric flavan-3-ols [157].

(C) Anthocyanins (ACN): C3S (Figure 1.5.) and cyanidin-3-glucosyl rutinoside are the major ones [158] with some RR varieties having cyanidin-3-glucoside in significant amounts. In general, anthocyanins with more hydroxyl groups or less sugar moieties are considered to have greater antioxidant capacity. Alzaid *et al.* [159] performed an *in vitro* study, which showed that acute exposure (15 min) to berry extract (derived from blueberry, bilberry, cranberry, elderberry, raspberry seeds and strawberry- 0.125%, w/v- the ACN content consists of cyanidins 44.5%; delphinidins 26.1%; petunidins 14.4%; malvidins 8.9%) significantly decreased both sodium-dependent (total uptake) and sodium-independent (facilitated uptake) [3H]-D-glucose uptake in human intestinal Caco-2 cells.

In vivo in humans, raspberry ACN are metabolized into phenolic acids such as hippuric acid, 4'-hydroxyphenylacetic acid, 3'4'-dihydroxyphenylacetic acid (DOPAC) [160] and 4'-hydroxyhippuric acid [161]. Analyses of fecal samples collected over 24 h in human patients showed that after ingesting 140 µM of cyanidin-3-glucoside (C3G), amounts recovered for C3G and protocatechuic acid (PCA) were 0.28 and 41.6 µmol, respectively [162].

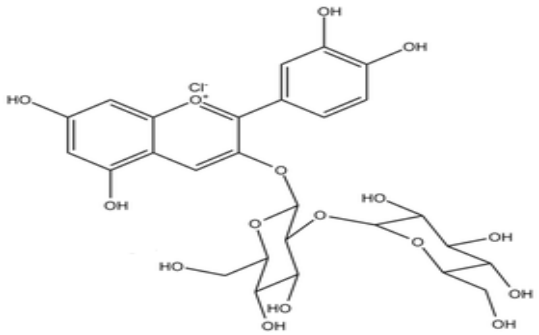

Figure 1.5. Chemical structure of the anthocyanin cyanidin-3-sophoroside (C3S).

PCA may help to reduce diabetes development because it can upregulate adiponectin and GLUT4 and exert insulin-like activity by activating PPAR-γ in human omental adipocytes [163]. When supplementing to mice at 2 % of the diet PCA lowered levels of several markers of the disease (plasma C-reactive protein, TNF-α) reducing diabetic complications [164].

(D) Phenolic acids: Gallic acid (GA) is the major phenolic acid in the group and is a trihydroxybenzoic acid. Studies show GA decreases ROS in isolated mitochondria [165] and increases antioxidant enzyme activity in a rat kidney model using diazinon to induce renal toxicity[166]. Such enzymes include superoxide dismutases, glutathione peroxidase (GPx), catalase, glutathione-s-transferase and reduced glutathione (GSH). These highlights remark the potential of GA as a redox regulator.

(E) Quercetin. A flavonol being one of the most abundant polyphenols present in fruits and vegetables [167]. The major microbial metabolite of quercetin is 3,4-dihydroxyphenylacetic acid or DOPAC (Figure 1.6.) which possesses strong antioxidant activity. DOPAC has been shown to have the highest free radical scavenging activity when tested *in vitro* along with other flavonoid metabolites and it might also reduce plasma lipid peroxidation *in vivo* [168, 169]. In a study using Min6 pancreatic β cells, DOPAC increased Nrf2 translocation to the nucleus and protected pancreatic β cells against impaired insulin secretion induced by cholesterol through

prevention of oxidative stress, apoptosis and mitochondrial dysfunction. Their findings suggest that DOPAC is a promising drug target for the prevention of development from a prediabetic to a diabetic state [170].

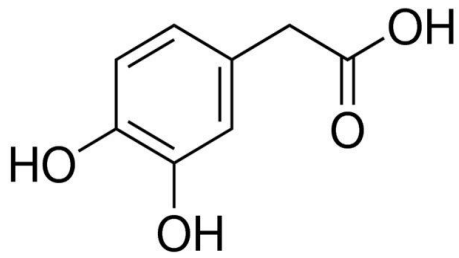

Figure 1.6. Chemical structure of 3,4-dihydroxyphenylacetic acid or DOPAC.

(F) RR also contain rheosmin or raspberry ketone (4-(4-hydroxyphenyl)-2-butanone) which is the key flavor of raspberries and has been extensively used by the food industry as flavoring agent and for other purposes in perfumery and cosmetics [171].

1.9. Research Objective and Specific Aims

The main objective of the clinical study proposed in this project is to investigate the protective effect of whole red raspberries (RR) (*Rubus idaeus*) against insulin resistance, oxidative stress, and biomarkers of inflammation in prediabetic and type 2 diabetic patients. The *in vitro* studies will elucidate how metabolites from the fruit can effect insulin resistance, oxidative stress and loss of cell function. We hypothesized that regular consumption of whole RR will be effective in improving insulin sensitivity and reducing islet cell toxicity and inflammation in T2DM patients which often accompany this condition.

The specific aims were: (1) to determine the efficacy of whole RR against insulin resistance and inflammation in prediabetic and T2DM patients. (2) To determine the molecular mechanism(s) by which whole RR prevent the destruction of pancreatic β-cells.

CHAPTER 2. EFFECTS OF RED RASPBERRY POLYPHENOLS AND METABOLITES ON BIOMARKERS OF INFLAMMATION AND INSULIN RESISTANCE IN PREDIABETES AND TYPE 2 DIABETES

2.1 Introduction

Berry fruits are excellent sources of various polyphenolic compounds, which exhibit anti-oxidative, anti-inflammatory and anti-carcinogenic activities [172]. Anthocyanins and ellagitannins are the most abundant polyphenolics found in red raspberries (RR) (*Rubus idaeus*) [2]. RR fruits also contain other beneficial compounds such as vitamin C, fiber, phenolic acids and carotenoids [172]. Anthocyanins which are responsible for the blue, purple and red color of many plants make up the largest and probably the most important group of water-soluble plant pigments [173]. Anthocyanins occur naturally in plants as glycosides of the anthocyanidin molecule [174]. Multiple health-promoting properties are attributed to anthocyanidins including antioxidant activity, anti-inflammatory activity and anticarcinogenic properties, as well as protection against heart disease, and reduction in the risk of diabetes and cognitive function disorders [5]. Similar attributes to those of anthocyanidins have been observed in ellagitannins and ellagic acid (a tannin and tannin derivative, respectively) as well [175].

Diabetes mellitus (DM) which includes type 1 (T1DM) and type 2 (T2DM) is a noncommunicable and severe endocrine metabolic disorder which reduces the ability of cells to uptake glucose and as a result induces serious complications in various organs. Diabetes Mellitus is characterized by an increase in blood glucose levels due to either deficiency of insulin secretion by pancreatic β-cells or inefficiency of cells to use insulin against glucose. Type 2 diabetes (T2DM) is a serious health threat with global impact that results from

a combination of risk factors such as genetic, environmental, and behavioral risk factors (diet, lack of exercise) [176]. Type 2 diabetes is considered a chronic inflammatory disease which results in high circulating levels of tumor necrosis factor (TNF), interleukins, and adipokines which are released from adipose tissue [177]. Additionally, insulin resistance and dysfunctions of pancreatic beta cells are primary characteristics of T2DM [178]. IL-1β, a key inflammatory mediator during T2DM, promotes insulin resistance, impairs β-cells function, and causes apoptosis. Reactive oxygen species (ROS) play a pivotal role in a variety of processes such as cell proliferation, inflammation, apoptosis, immune system and maintenance of redox balance [179]. Over accumulation of free radicals and ROS is implicated in the development of age-related diseases and chronic disorders such as DM, cancer, atherosclerosis, and neurodegenerative disorders [180-182]. In a study involving 1997 females from the United Kingdom, higher intake of anthocyanins was associated with significantly lower concentrations of high-sensitivity C-reactive protein (hsCRP), a marker of obesity and diabetes [183].

Moreover, a large cohort study of 200,994 health professionals from the United States revealed that consumption of anthocyanin-rich foods were inversely correlated with the risk of diabetes [184]. Alzaid *et al*. (2013) [159] found that acute exposure to the anthocyanin-rich extract from berry fruits significantly decreased both Na+-dependent and Na+-independent glucose uptake in Caco-2 cells.

Anthocyanins are widely reported to have low bioavailability, with most of the studies recording peak plasma concentrations (C*max*) ranging from 1 to 120 nmol/L [135] and urinary recoveries < 2% of intake [136] [137]. After the ingestion of red raspberries, the polyphenols are partially bioavailable to systemic organs, through absorption, distribution, metabolism and excretion (ADME) [17]. A recent study shows extensive colonic microbiota mediated degradation of ^{13}C5-labeled cyanidin-3-O-glucoside, which resulted in the production of many phenolic metabolites over a 0-48 h period. The relative bioavailability of the ^{13}C5-labeled was 6.9 % in breath as CO_2, 5.4 % in urine and 32% in feces in the form of ^{13}C5-labeled phenolic and aromatic compounds [138] [139]. Ellagitannins and ellagic acid also exhibit low bioavailability. These molecules are subject to extensive metabolism by the gut microbiota to produce urolithins that are much more efficiently absorbed.

Urolithins circulate in plasma as glucuronide and sulfate conjugates at concentrations usually ranging from 0.2–20 μM [140]. Hence, it can be implied that the health effects of ellagitannin-containing products can be associated with the gut-produced urolithins, therefore, evaluation of the biological effects of these metabolites is essential [140].

The aim of the present work was to determine the anthocyanin profile of mixed puree containing Meeker, Wakefield and Chemainus red raspberries, to measure the level of metabolites in the plasma of T2DM and prediabetic patients before and after 2 weeks of RR smoothie consumption, as well as to measure the levels of insulin, and glucose intolerance. DOPAC, a metabolite of quercetin and anthocyanins, was evaluated *in vitro* to try to elucidate whether it can promote insulin secretion. Pro-inflammatory and anti-

inflammatory cytokines and biomarkers in the serum of the same group of T2DM patients were measured both before and after 2 weeks consuming the fruit.

2.2. Materials and Methods

2.2.1. Materials

Whole raspberries were provided by the National Processed Raspberry Council (Lynden, WA). Cyanidin-3-sophoroside (C3S) standard was purchased from Indofine (Hillsborough, NJ). HCYTOMAG panels for 13-plex Luminex assay were purchased from EMD Millipore (Billerica, MA). Rat insulin ELISA kits and oxLDL were purchased from Mercodia (Uppsala, Sweden).

Urolithin A glucuronide (Uro-A glur), isourolithin A (IsoUro-A glur), urolithin B glucuronide (Uro-B glur), isourolithin A (IsoUro-A), urolithin A (Uro-A), urolithin B (Uro-B), were chemically synthesized and purified by Villapharma Research S.L. (Parque Tecnológico de Fuente Alamo, Murcia, Spain). Ellagic acid (EA), 3,4-Dihydroxyphenylacetic acid (DOPAC) and protocatechuic acid (PCA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Urolithin A sulfate and urolithin B sulfate were obtained as described elsewhere [185]. Stock solutions were prepared in methanol to a final concentration of 2000 ppm (11.2 mM). All solutions were stored at −20 °C. Methanol (MeOH) and acetonitrile were purchased from J. T. Baker (Deventer, The Netherlands). Formic acid and acetic acid were from Panreac (Barcelona, Spain). Milli-Q system (Millipore Corp., Bedford, MA) ultrapure water was used throughout this experiment. All chemicals and reagents were of analytical grade.

2.2.2. Cell Culture

Rat pancreatic β-cells (INS-1), courtesy of Dr. Henrique Cheng at the LSU School of Veterinary Science, were used for this study. Cells were cultured in RPMI-1640 medium (11 mM glucose and supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 1 mM sodium pyruvate and 50 µM 2-mercaptoethanol. The cells were maintained in a humidified atmosphere (5 % CO_2 at 37 ºC) and the experiments performed with cells between passages 80 and 82.

2.2.3. HPLC-PDA-MS Analysis of Anthocyanins

To determine the profile and concentration of anthocyanins and ellagitannins in liquefied RR, the method of Ludwig *et al* 2015 (2) was used. Briefly, 5 g of thawed RR were homogenized with 15 ml of MeOH/H_2O/formic acid at 75:25:1 v/v/v and ultrasonicated for 1 h at 5 ºC. Samples were then centrifuged for 10 min at 4000 x g and 20 µl of the supernatant were analyzed. Anthocyanins were evaluated by reverse phase chromatography using a Shimadzu UHPLC-PDA (Nexera-i LC-2040C 3D system) coupled to a Shimadzu MS (triple-quadrupole LCMS-8040, LC-MS/MS), a Shimadzu C18 (50 x 2.1 mm, 1.9 µm) column and solvents A: 4.5% formic acid acidified water and B: acetonitrile. Separation was achieved with a flow rate of 0.4 mL/min and a gradient of 2% B for 0-1 min, 2-22% B from 1-11 min, 22-40% 11-12 min at 40 °C. Twenty µL of each sample was injected for analysis. Spectral data was collected using a photodiode array detector from 250-700 nm. Mass spectrometry was performed under positive ion mode; data were monitored using Q3 total ion scan (SCAN, from m/z 100-1100), and selected ion monitoring was conducted for m/z 271, 287, 301, 303, and 331, representing

common anthocyanidins [186]. This analysis was performed at The Ohio State University (Columbus, OH).

2.2.4. MALDI/MS Analysis of Ellagitannins

For sanguiin H-6, the method of Kahkonen et al., 2012 [187] was used with slight variations. Briefly, 100 g of RR was thawed, homogenized, and then mixed with 833 ml of 70 % aqueous acetone solution. This mixture was then centrifuged at 3000 RPM for 15 min. A final volume of ~ 1,666 ml was recovered and placed on a rotovapor at 40 ºC to evaporate the acetone portion. A ~ 540 ml volume was recovered and freeze-dried. The ellagitannin extract was further purified using Amberlite XAD-7 (Fischer Sci, Hampton, NH) column chromatography with 100 ml of 6% CH_3CN (CH_3CN:TFA:H_2O 6:0.5:93.5 V/V/V) to wash out free sugars and organic and phenolic acids.

Elution was continued with CH_3CN (CH_3CN: TFA 99.5:0.5) to obtain a fraction containing flavonols, anthocyanins, and ellagitannins. To separate ellagitannins, a column of similar size and packed with Sephadex LH-20 (GE Healthcare, Little Chalfont, UK) (3 g) was used. The sample was introduced into the column, and flavonols and anthocyanins were eluted with 50% MeOH. Finally, ellagitannins were eluted with aqueous acetone (70:30 V/V). A fraction of this was used to do MALDI-MS analysis.

2.2.5. Clinical Study Design

Seven type 2 diabetic (T2DM) and 2 pre-diabetic subjects on no current diabetic treatment between the age of 18 and 70 who were not pregnant or nursing a child were enrolled in this study at Pennington Biomedical Research Center (PBRC). Subjects taking a medication that affects insulin sensitivity were excluded from the study. Subjects taking any anti-inflammatory

medications such as naproxen, aspirin, or ibuprofen less than 2 weeks prior to first blood test and for duration of study were excluded. Subjects with an inflammatory disease were excluded from the study. A finger stick was done to confirm fasting blood glucose was between 109 and 200 mg/dL to qualify, or have a recorded post-prandial glucose greater than 200 mg/dL. The subjects returned on day one (BSL) for an oral glucose tolerance test in which insulin and glucose were measured at all-time points (OGTT) (0, 30, 60, 120, and 180 min), and C-reactive protein (hsCRP) levels were measured at baseline. Insulin and hsCRP were measured from serum by chemiluminescent assays using an Immulite® 2000 Immunoassay System (Siemens, Munich, Germany). In an OGTT, the blood sugar rises and then returns to baseline. People with diabetes show a slower return to baseline than people without diabetes. At time 0 of the OGTT, 10 cc of blood were drawn and plasma frozen at -70 ºC for analysis of metabolites. In addition, blood was drawn for a multiplex magnetic bead-based immunoassay. The subjects returned the following day for a blood draw. The participants were asked to come to the clinic every day for two weeks including weekends to drink one serving (123 g) of a raspberry smoothie containing 9.67 % erythritol as sweetener. Other than the smoothie, no dietary changes were made. At the end of the two week feeding (PF), the participants underwent another identical OGTT.

2.2.6. Metabolite Analysis in Plasma

Plasma samples were obtained by centrifuging whole blood at 800 x *g* for 10 min at room temperature and was collected and separated into 1 ml aliquots and placed at -80 ºC until use. Plasma samples were defrosted, vortexed and 200 µL were thawed and extracted with 600 µL

acetonitrile:formic acid (98:2, v/v) by vortexing for 2 min and ultrasonic bath for 10 min. Next, the mixture was centrifuged at 14000 x *g* for 10 min, and the supernatant was lyophilized.

The lyophilized residue was redissolved in 100 µL of MeOH and filtered through a 0.22 µm PVDF filter before analysis by ultra-performance liquid chromatography quadrupole time of flight mass spectrometry using electrospray ionization (UPLC-ESI-QTOF-MS/MS). This analysis was performed at CEBAS-CSIC center in Murcia, Spain.

Plasma samples were analyzed using an Agilent 1290 Infinity UPLC system coupled to the 6550 Accurate-Mass Quadrupole Time-Of-Flight (QTOF) mass spectrometer (Agilent Technologies, Waldbronn, Germany) through an electrospray interface with Jet Stream technology. Separation was achieved as previously reported [188]. Briefly, a reverse phase column Poroshell 120 EC-C18 column (3 × 100 mm, 2.7 µm) (Agilent) operating at 30 °C was used. The mobile phases were water:formic acid (99.9:0.1 v/v; Phase A) and acetonitrile:formic acid (99.9:0.1 v/v; Phase B). Gradient was as follows: 0–3 min, 5–15% B; 3–11 min, 15–30%; 11–15 min, 30–50%, 15–21 min, 50–90%. The flow rate was set constant at 0.4 mL/min and the injection volume was 3 µL. The optimal conditions of the electrospray interface were as follows: Gas temperature 280 °C; drying gas 9 L/min, nebulizer 45 psi, sheath gas temperature 400 °C, sheath gas flow 12 L/min. Spectra were acquired in the m/z range of 100–1100, in a negative mode and with an acquisition rate of 1.5 spectra/s. Internal mass calibration by simultaneous acquisition of reference ions and mass drift compensation was used to obtain low mass

errors. Data were processed using the Mass Hunter Qualitative Analysis software (version B.06.00, Agilent).

A target screening strategy was applied to all plasma samples searching for a list of target compounds after MS full-acquisition as well as the direct comparison with authentic standards was performed. The quantification of ellagitannins derived metabolites as well as DOPAC and PCA were determined in plasma by interpolation in the calibration curve obtained with their own available standards in the plasma matrix. All metabolites were quantified in MS by peak area integration of its extracted ion chromatograms.

2.2.7. Effect of DOPAC on Insulin Secretion

INS-1 cells, an insulinoma cell line, were cultured following Suantawee *et al*. [189] method. In brief, cells were cultured on 24-well plates at a density of 5 X 10^5 cells/well. When confluency was achieved following ~ 72 h, the cells were incubated for 30 min in modified Krebs-Ringer bicarbonate buffer (KRB) containing 136 mM NaCl, 4.8 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 5 mM $NaHCO_3$, 10 mM HEPES, 4 mM glucose, and 0.1 % bovine serum albumin (BSA), pH 7.4. A second incubation was performed for 30 min with KRB containing DOPAC at different concentrations (1-100 µM) or 20 mM KCl (positive control). After incubation, the supernatant was collected and stored frozen at -80 ºC for insulin determination with ELISA.

2.2.8. Analysis of Levels of Inflammatory Cytokines in Serum

The levels of pro-inflammatory and anti-inflammatory cytokines in serum including interleukin1 beta (IL-1β), interleukin 2 (IL-2), interleukin 4 (IL-4), interleukin 6 (IL-6), interleukin 8 (IL-8), interleukin 10 (IL-10), interleukin 12p70 (IL-12p70), interleukin 13 (IL-13), interferon gamma (IFN-γ),

granulocyte-macrophage colony-stimulating factor (GM-CSF), monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1 alpha (MIP-1A), tumor necrosis factor alpha (TNF-α), and plasminogen activator inhibitor-1 (PAI-1) were measured by a multiplex magnetic bead-based immunoassay (Luminex®) system. This analysis was performed at PBRC using a Luminex 200 (Luminex Co., Austin, TX). Oxidized Low density lipoprotein (oxLDL) was measured by ELISA using plasma.

2.2.9. Statistics

Results are expressed as means ± SD with the exception of AUC curves for diabetics which were expressed using standard error of the mean (S.E.M). Differences between weeks were analyzed by paired t-test whereas levels of inflammatory cytokines in serum were analyzed using Wilcoxon signed rank test using SAS 9.4 (SAS Institute, Cary, NC). Results for insulin secretion were expressed as mean ± S.E.M from two independent experiments, each experiment having n=6. Basal control and the different DOPAC concentrations were compared using unpaired student t-test using GraphPad Prism v8.0 (GraphPad Software, San Diego, CA). Significance was taken at $p < 0.05$.

2.3. Results

2.3.1. Quantitative Analysis of Cyanidin-3-Sophoroside (C3S) in Raspberry Purée

C3S, an anthocyanin and one of the bioactive compounds of interest was analyzed to determine anthocyanin profile by UHPLC-PDA-MS. C3S with 79% area under the peak (AUP), cyanidin-3-glucoside (15% AUP), and cyanidin-3-sambubioside (4% AUP) were the major anthocyanins identified by PDA/MS-MS data and by comparison to literature at 520 nm ([190] [191])

(Figure 2.1.). Small traces of additional anthocyanin derivatives, tentatively assigned identities of pelargonidin, peonidin and malvidin, accounted for the remaining percentage area under the peak of the pigments, but were not clearly identified due to presence of co-eluting compounds (Table 2.2.). These peaks were assigned aglycone identities based on select ion monitoring.

Quantitative values of the anthocyanins in the extracts were calculated after production of a standard curve using a standard of C3S as a reference material. The curve showed a good fit by linear regression (R^2 = 0.9964) with injections of C3S amounts of 0.1 – 10 µg. Anthocyanin concentrations of the extracts were determined to be a total of 887.6 ± 262.8 µg/g C3S equivalents, as determined by a HPLC calibration curve (detection at 520 nm, Table 2.2.). The most prevalent anthocyanin of the extracts was C3S (626.0 ± 179.8 µg/g), accounting for the majority of the total anthocyanins.

2.3.2. Qualitative Analysis of Ellagitannins in RR

Raspberry extraction of ellagitannin compounds including casuarictin (936.64 M.W.), sanguiin H-6 (1871.27 M.W.) and lambertianin C (2,805.81 M.W.) was performed. These polyphenols are responsible for some of the beneficial health effects in raspberry [192-194], and were analyzed by MALDI-MS. Results showed strong peaks for two of these compounds which corresponded to sanguiin H-6 (1893.22 M.W.) being the highest followed by lambertianin C (2,827.25 M.W.) (Figure 2.2.). Quantification of these compounds in the extract was not performed due to lack of a standard for sanguiin H-6, therefore this analysis covered the qualitative aspect only.

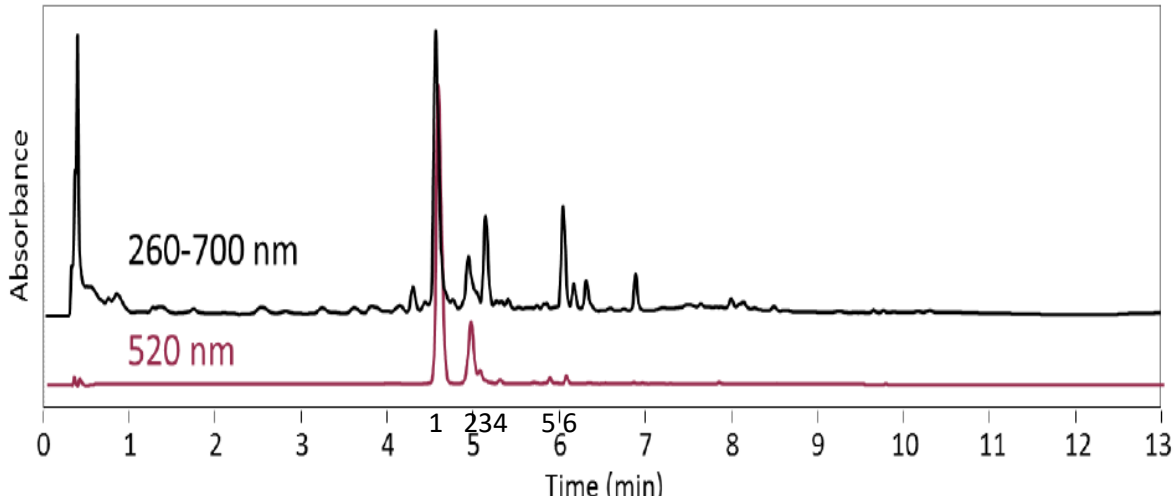

Figure 2.1. UHPLC chromatogram of RR extract compounds, detection at 520 nm with the six peaks identified and 260-700 nm (max plot).

2.3.3. Baseline Characteristics of the Study Participants

Samples from ten patients who successfully completed the study were collected and plasma, serum and PBMCs extracted from peripheral blood, however one of them was determined to be a type 1 diabetic and was, therefore, excluded from the study. Out of the 9 patients left, 7 were type 2 diabetic and 2 pre-diabetic based on their fasting glucose values during screening. Table 2.3. lists the anthropometrics of the participants and results observed between test day 1 (BSL) and test day 2 (PF). Values for HOMA-IR and hsCRP decreased on PF for the type 2 diabetics after 2 weeks of feeding with the reduction having statistical trend for HOMA-IR (p=0.0584) and statistical significance for hsCRP (p=0.01). Values for HOMA-IR and hsCRP numerically increased on PF in the pre-diabetic group, but with only 2 subjects further studies are needed to judge significance. Glucose at 0 min on the OGTT increased in both groups, but the area under the curve (AUC) was 1,243 ± 878.97 mg/dl/minute less on PF for the type 2 diabetics and 2,497.5 ±

1,766 mg/dl/minute less on the pre-diabetics, yet, neither difference was significant (Figures 2.3.A and 2.3.B). AUC analysis for insulin was 1,842.3 ± 1,302.7 µU/ml/minute less on PF for the type 2 diabetics and 534.8 ± 378.1 µU/ml/minute more on the pre-diabetics, yet, neither difference was significant, but with a statistical trend at 0 minutes for the diabetic group (p=0.0759) (Figures 2.3.C and 2.3.D). In the diabetic group, HOMA-IR and hsCRP were calculated with n=7 and n=6, respectively, since one patient had an infection and the hsCRP value was a statistical outlier. For the pre-diabetics, HOMA-IR and hsCRP were calculated with n=2 and n=1, respectively, since one of the patients had a high hsCRP value, so statistical significance could not be determined.

2.3.4. Metabolite Analysis in Plasma

Two urolithin conjugates, i.e. urolithin A glucuronide (Uro-A glur) and urolithin A sulfate (Uro-A sulf) were identified and quantified in 7 patients (n=5 for diabetics and n=2 for pre-diabetics) at PF but not BSL, indicating they were derived from RR smoothie feeding (Table 2.5.). Uro-A glur and Uro-A sulfate were quantified after consumption at PF in the range of high and low nM range, respectively (Table 2.5.). Uro-A sulfoglucuronide was also identified but not quantified, (because no standard was available), in the samples PF of 6 subjects (n=5, diabetics and n=1, prediabetic).

In these patients no other urolithin conjugates such as Uro-B or IsoUro-A glur were found suggesting a metabolism of ellagitannins according to their metabotype A [195]. Therefore, among 9 patients, IsoUro-A glur and its sulfate derivate were identified and quantified in only one of the diabetic patients, suggesting metabolism of ellagitannins according to their metabotype

B [195]. Uro-B sulfate was also identified in the PF samples of this diabetic patient. Finally, no urolithin conjugates were identified in a different diabetic patient; however, dimethyl ellagic acid (DMEA) glucuronide was identified but not quantified in this patient suggesting a lack of metabolism of ellagitannins (metabotype 0). DMEA glucuronide was also found in the PF samples of one of the pre-diabetic patients.

All urolithin conjugates were quantified above the limits of detection (LOD) and quantification (LOQ) previously reported in García-Villalba *et al*. [196]. Moreover, high concentration (low µM range) of DOPAC and PCA were identified and quantified in all volunteers of both BSL and PF samples (before and after RR intake, respectively). However, no significant differences were found over the course of the study. Figure 2.5. shows structures for the metabolites found at highest concentrations.

2.3.5. DOPAC and Insulin Secretion

Incubation with KRB containing DOPAC from 1-100 µM did not stimulate insulin secretion from INS-1 cells. Decreases were observed on all treatments with those at 1 and 3 µM not being significant, yet those at 10, 30 and 100 µM had statistically significant decreases (Figure 2.6.). The only significant increase was elicited by 20 mM KCl (positive control) with a 2.6-fold increase over basal.

2.3.6. Levels of Inflammatory Cytokines in Serum

Table 2.6. lists results for multiplex magnetic bead-based immunoassay. Levels on 7 cytokines (IL-1β, IL-2, IL-4, IL-6, IL-12p70, IL-13, and GM-CSF) were not determined as they were beneath the LOD. Increases and decreases

were observed on the rest of cytokines analyzed on both T2DM and pre-diabetic patients but none were statistically significant.

2.4. Discussion

The quantification of total ACN in the RR purée extract yielded 88.8 ± 26.3 mg/100g of fresh fruit of which 91.9% or 81.7 ± 23.7 mg/100 g corresponded to cyanidin-based ACN content. This is in agreement with Wu *et al*. [197] who found 90.2 ± 19.2 mg/100 g cyanidin-based ACN content and total ACN of 92.1 ± 19.7 mg/100 g when measuring ACNs in 5 different RR samples by means of HPLC-DAD-ESI/MS/MS. The high standard deviation observed could be explained by potential heterogeneous distribution of the 3 varieties that constituted the analyzed sample.

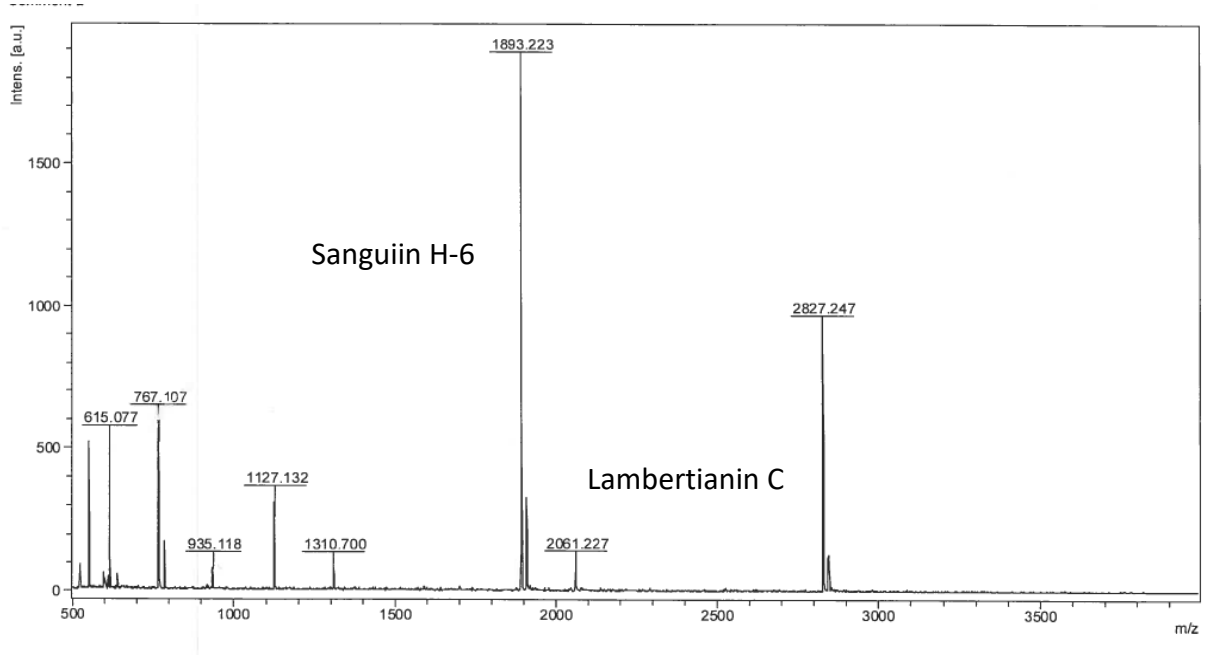

Figure 2.2. Results for ellagitannin extract using MALDI-TOF. Major peaks corresponded to sanguiin H-6 (1893.22 M.W.) and lambertianin C (2,827.25 M.W.).

Table 2.1. Concentration (µg/g of C3S equivalent) of anthocyanins in red raspberry extract identified and quantified by UHPLC-PDA-QQQ.

Peak	Identity	m/z	Mean (µg/g)	Per serving (123 g)
1	Cyanidin-3-sophoroside	611/287	626.0 ± 179.8	77.0 ± 22.1 mg
2	Cyanidin-3-glucoside	449/271	143.2 ± 44.8	17.6 ± 5.5 mg
3	Cyanidin-3-sambubioside	581/287	44.1 ± 13.0	5.4 ± 1.6 mg
4	Pelargonidin-derivative	595/271	23.8 ± 8.5	2.9 ± 1.0 mg
5	Peonidin-derivative	301	24.2 ± 8.3	3.0 ± 1.0 mg
6	Malvidin-derivative	301	26.3 ± 8.4	3.2 ± 1.0 mg
	Total anthocyanins		887.6 ± 262.8	109.1 ± 32.2 mg

Values are means ± SD, n=3

Table 2.2. Results for baseline and post-feeding characteristics of the study participants.

	Type 2 Diabetics (n=7)	Prediabetics (n=2) **
Race (African American/Caucasian) n/n	3/4	1/1
Gender (male/female) n/n	2/5	0/2
Age, y	60.9 ± 9.0	63.0
Body weight, kg	103.0 ± 16.2	101.1
BMI, kg/m^2	37.2 ± 6.3	38.6
Systolic blood pressure, mm Hg	132.6 ± 11.3	124.5
Diastolic blood pressure, mm Hg	86.7 ± 8.2	71.0
Fasting glucose, mg/dL	170.0 ± 27.8	109.0
OGTT glucose (time 0), mg/dL	BSL: 161.6 ± 32.0 PF: 164.0 ± 38.5	BSL: 93.0 PF: 100.0
HOMA-IR	BSL: 13.3 ± 7.3 PF: 9.4 ± 5.4	BSL: 6.0 PF: 8.5
hsCRP, mg/L (n=6, T2DM and n=1, PD)	BSL: 4.9 ± 2.0 PF: 4.0 ± 1.6*	BSL: 5.6 PF: 6.1

All results are expressed as the mean ± SD. Statistical analyses were performed using a paired t-test to detect differences between BSL and PF. OGTT, oral glucose tolerance test; HOMA-IR, homeostasis model of assessment of insulin resistance; hsCRP, high sensitivity C-reactive protein. HOMA index was calculated with the formula: HOMA= [serum glucose levels (mg/dl) X insulin levels (µU/ml)/22.5. * p = 0.01. ** No statistics done due to small numbers.

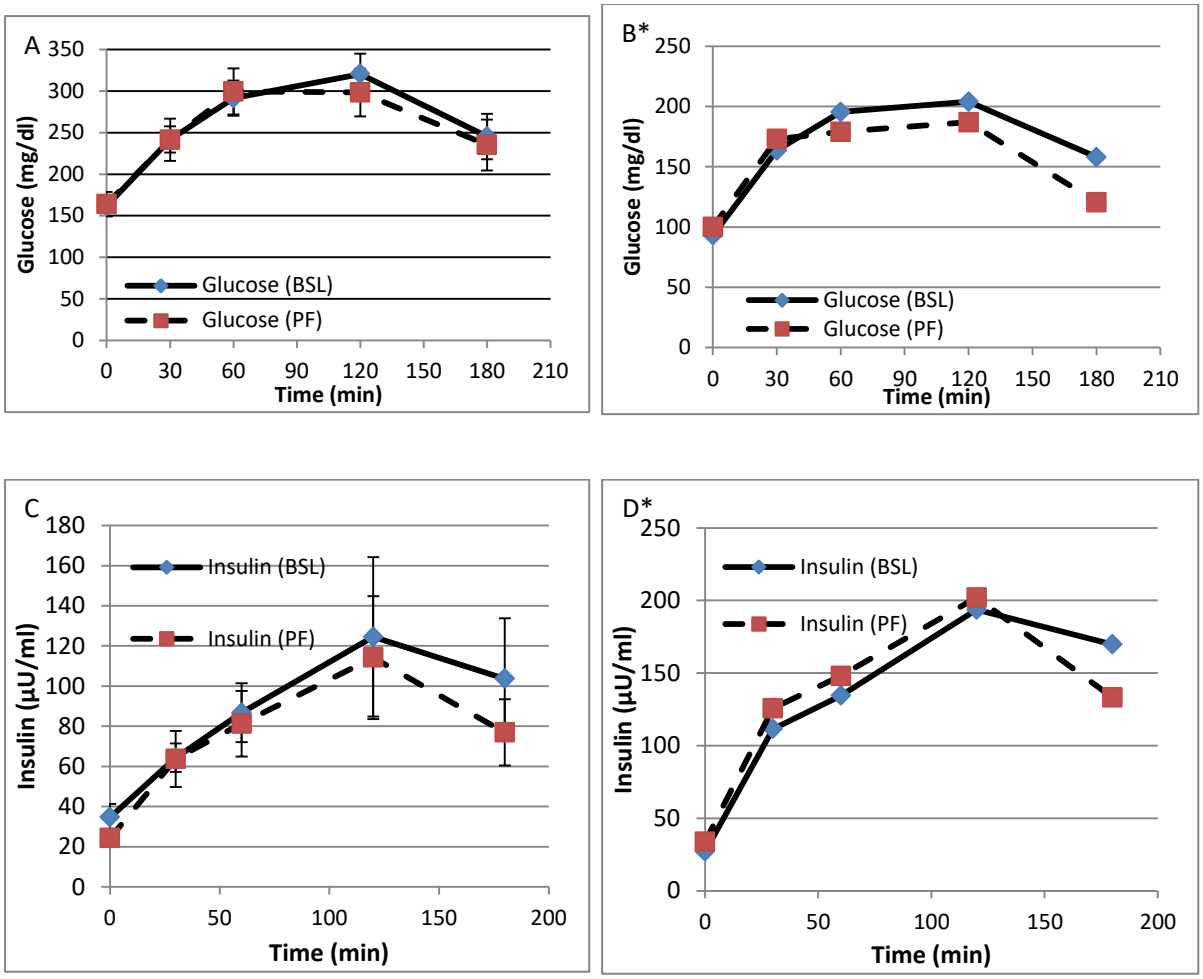

Figure 2.3. Effects of RR smoothie consumption on blood glucose and insulin levels after oral glucose tolerance test on day 1 (BSL) and day 2 (PF). Results are expressed as the mean ± S.E.M. Statistical analyses were performed using paired t-test. (A) Glucose tolerance test curve for T2 diabetic patients (n=7). (B) Glucose tolerance test curve for pre-diabetic patients (n=2). (C) Glucose tolerance test curve for insulin in T2 diabetic patients (n=7). (D) Glucose tolerance test curve for insulin in pre-diabetic patients (n=2). * No statistics done due to small numbers.

Because PBMCs contain several key inflammatory cells, higher levels of insulin resistance and inflammatory biomarkers at the baseline for T2 diabetic and pre-diabetic patients were expected to be present.

IR constitutes a crucial factor in the development of type 2 diabetes along with hypertension and cardiovascular (CV) disease [198]. Results indicated significant (inflammatory biomarker such as hsCRP) and non-significant reductions (glucose AUC, insulin resistance through HOMA-IR) after two weeks of

RR smoothie consumption. Stull *et al*. [199] found that consuming a smoothie containing blueberries for 6 weeks had a greater increase in insulin sensitivity in obese and insulin-resistant adults (prediabetics, accounting for 67%) when compared to individuals that consumed a placebo smoothie (41%) and was measured by a 10 % or greater favorable change in insulin sensitivity. For this study researchers used the hyperinsulinemic-euglycemic clamp (HEC) to assess insulin sensitivity. Even though HEC is considered the “gold standard”, HOMA-IR has proved to be a powerful proxy to assess IR [200, 201]. In our study, HOMA-IR had a *p* value of 0.0584 resulting in a statistical trend but not statistical significance. AUC analysis for both glucose and insulin showed decreases on PF day, but these were not statistically significant on either group. Losso *et. al* [202] reported that one feeding of two slices of fenugreek enriched bread (5%) to a group of eight T2DM patients yielded a significant reduction in AUC for insulin when compared to bread with no fenugreek fed to the same group. RR constitutes a high source of fiber providing 8 g per 123 g of fruit [145]. Zhao *et al*. 2018 [203] identified short chain fatty acid (SCFA) - producing bacterial strains who were promoted by dietary fibers. In their study, most other potential producers were present at either decreasing or not changing numbers in patients with T2DM. Researchers found that when the fiber-promoted SCFA producers were present in higher number, participants had better improvement in hemoglobin A1c levels. This was attributed in part due to increased glucagon-like peptide-1 production. SCFAs such as acetate and butyrate have demonstrated the ability to improve glucose homeostasis through production of glucagon-like peptide-1 (GLP-1) and peptide YY (PYY), which can stimulate insulin secretion [204-206].

However, based on the results obtained through HOMA-IR in the present study, insulin secretion in patients was reduced favoring an insulin sensitivity effect. This finding could indicate this insulin sensitivity effect could be elicited by other molecule in the fruit or perhaps, other metabolite(s).

Serum concentration of CRP is increased in both impaired glucose tolerance (IGT) and T2DM [207, 208]. Based on numerous studies, minor CRP elevation (hsCRP) has been shown to be associated with future major CV risk (hsCRP: <1 mg/L = low risk; 1–3 mg/L = intermediate risk; 3–10 mg/L =high risk; >10 mg/L _= unspecific elevation) [209]. A decrease from 4.9 ± 2 on BSL to 4.0 ± 1.6 on PF was registered in our study and found to have statistical significance on T2DM patients (p= 0.01).

The metabolite analysis found two urolithin conjugates, i.e. urolithin A glucuronide (Uro-A glur) and urolithin A sulfate (Uro-A sulf) which were found in 7 of the 9 patients' (both groups) samples, but only for PF indicating they were derived from RR smoothie feeding. These were metabolites from the ellagitannin portion of the fruit, whereas anthocyanin-derived metabolites such as DOPAC and PCA were present at similar concentrations on BSL and PF suggesting these metabolites might have come from different dietary sources as well.

An increase in IL-10 after two weeks of raspberry consumption was expected but cytokine analysis by multiplex magnetic bead-based immunoassay found the opposite. Increases and decreases were observed on the rest of cytokines analyzed, yet, none of these changes were statistically significant.

Interpatient variability was observed in plasma metabolite analysis as well as for other biomarkers and this could be due to differences in diets, individual microbiota or potential effects of erythritol used as non-caloric sweetener.

From the 9 patients evaluated in the study, six presented a urolithin metabotype (UM) A (urolithin A producers only), two (men both) a UM-B (production of urolithin A, isourolithin A and/or urolithin B) and one a UM-0 (urolithin non-producers). Cortés-Martín *et al*. [210] proposed that aging is the main factor affecting the distribution of urolithin metabotypes when they evaluated a cohort of n=839, from 5-90 yrs of age. Besides this, the researchers described a progressive increase for UM-B parallel to a decrease in UM-A, especially for individuals aged 20-40, and when considering a group of n=23 patients aged 30-63 who suffered from metabolic syndrome, both UM-A and UM-B had a similar share. In the present study, however, the dominating UM was UM-A, therefore, it is important to highlight their cohort consisted mainly of caucasian individuals only whereas in the present study African Americans constituted 44.4 % of the patients evaluated, the rest (56%) being Caucasian. Gonzalez-Sarrías *et al*.[211] observed that overweight–obese individuals with UM-B were at higher CVD risk than those with UM-A or UM-0, hence, the UM-A preponderance among the patients from our clinical trial could be taken as a positive outcome.

Regarding potential mechanisms of anti-diabetic properties of whole RR observed in patients from the clinical trial, Edirisinghe & Freeman [212], list two ways these can be addressed: insulin dependent and insulin independent. Within insulin dependent, an insulin sensitivity enhancement was observed judging by the drop on

insulin and hsCRP, an important biomarker of inflammation and CVD risk [213]. Assessment of the metabolite DOPAC (1-100 µM) to investigate if it could elicit insulin secretion on INS-1 cells showed the opposite, an even had a toxic effect at higher concentrations. This finding is congruent with what was observed on the clinical trial patients as insulin and glucose levels dropped by PF day indicating an improvement in insulin sensitivity. Work by other researchers has demonstrated stimulation on insulin secretion by compounds such as quercetin [115, 214] and cyanidin [189] when used at levels ranging from 20-100 µM. These concentrations, however, may not be feasible at physiological levels due to metabolic availability and toxicological issues.

Researchers described activation of the extracellular signal-regulated kinase (ERK) 1/2 pathway and L-type voltage-dependent Ca2+ channel (VDCC) in INS-1 cells as responsible for increases in insulin secretion. For the insulin independent part, both inhibition of digestive enzymes involved in carbohydrate breakdown as well as inhibition of glucose absorption in the GI tract can be present, however in the present study a minor increase in glucose from OGTT at 0 min on PF was observed yet non-significant reductions on the AUC for OGTT on both T2DM and prediabetics were present on PF. Work performed by other researchers highlights these findings.

Edirisinghe & Freeman, found when overweight adults were fed a freeze-dried strawberry powder that it significantly reduced postprandial insulin response and reduced postprandial inflammatory response (IL-6 and hsCRP) when the participants ate a high carbohydrate and high fat meal ($P < 0.05$) [212]. Alzaid *et al*., 2013 [159] used a berry extract containing raspberry seed and were able to significantly decrease both sodium-dependent intestinal cell

(total uptake) and sodium-independent [^{3}H]-D-glucose uptake in human intestinal Caco-2 cells. Treatment for 16 h showed SGLT1 mRNA and GLUT2 mRNA expression was significantly reduced ($P < 0.05$.) Moreover, McDougall *et al*. [215] observed amylase was inhibited when performing an *in vitro* enzymatic study using raspberry. They concluded that ellagitannins in raspberry were the main agents for this inhibition, which indicates these polyphenols in RR can promote beneficial effects just like the anthocyanin fraction does.

2.5. Conclusion

Two urolithin conjugates, (Uro-A glur and Uro-A sulf) were found in 7 of the 9 patients' plasma samples at nanomolar concentrations (1.3 - 63.2 ± 31.2 nM, Table 2.5.), whereas anthocyanin-derived metabolites such as protocatechuic acid (PCA) and 3,4-dihydroxyphenylacetic acid (DOPAC) were present at micromolar concentrations yet at similar levels on BSL (PCA= 0.5 ± 0.14, DOPAC= 1.4 ± 0.28) and PF (PCA=0.6 ± 0.07, DOPAC= 1.7 ± 0.8). Other metabolites were present in only some of the patients, illustrating that each individual's microbiome, ethnicity, age, etc., most likely plays a role on the outcome of such metabolites. Results indicated a significant reduction in hsCRP (BSL: 4.9 ± 2.0, PF: 4.0 ± 1.6 (p=0.01)) which is a very important biomarker of inflammation and heart disease risk. A reduction showed only a statistical trend for HOMA-IR when evaluating for IR. DOPAC, a metabolite from anthocyanin and quercetin in RR, when incubated at 1-100 µM did not stimulate insulin secretion in INS-1 cells. A longer feeding period with a larger group is recommended to test if the effects observed can be improved. This study demonstrated the potential of RR to modulate levels of biomarkers of

inflammation and insulin resistance in T2DM patients most likely through antioxidant activity from the polyphenolics present and from anti-diabetic effects through insulin dependent mechanisms.

Table 2.3. Results from plasma analysis for raspberry metabolites by UPLC-ESI-QTOF-MS/MS

	Uro-A-Glur [α]	*Uro-A-Sulf* [α]	*Iso-Uro-A* [α]	*Iso-Uro-A Sulf* [α]	*Uro-B- Sulf* [α]	*PCA*[b]	*DOPAC*[b]
Diabetics (n=7)							
BSL	---	---	---	---	---	0.6 ± 0.4[c]	1.2 ± 0.5[c]
PF	63.2 ± 31.2[d]	7 ± 4.2[d]	37.4 ± 1.1[f]	11.1 ± 0.6[f]	1.6 ± 0.7[f]	0.6 ± 0.4[c]	1.1 ± 0.6[c]
Pre-diabetics (n=2)*							
BSL	---	---	---	---	---	0.4[e]	1.6[e]
PF	10.3[e]	1.3[e]	---	---	---	0.5[e]	2.2[e]

All results are expressed as mean ± SD. Uro-A-Glur, urolithin-A-glucuronide; Uro-A-Sulf, urolithin-A-sulfate; *Iso-Uro-A,* isourolithin-A-glucuronide; *Iso-Uro-A Sulf,* isourolithin-A-sulfate; *Uro-B- Sulf,* urolithin-B-sulfate; *PCA,* protocatechuic acid (3,4-ihydroxybenzoic acid); *DOPAC,* 3,4-dihydroxyphenyl acetic acid. [α], nanomolar; [b], micromolar; ---, not detected; [c], detected in n=7; [d], detected in n=5; [e], detected in n=2; [f], detected in n=1. Compounds urolithin-A- sulfoglucuronide (n=5, diabetics and n=1 pre-diabetics] and dimethyl ellagic acid (n=1 for both diabetics and pre-diabetics ; both not listed in the table) were detected but not quantified on the PF day. * No statistics done due to small numbers.

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Table 2.4**.** Serum and plasma biomarkers results (pg/ml) by multiplex magnetic bead-based immunoassay and ELISA**.**

	IL-8	*IL-10*	*IFN-γ*	*TNF-α*	*MCP-1*	*MIP-1α*	*PAI-1*	*oxLDL* [β]
Diabetics (n=7)								
BSL	7.5 ± 2.9	8.1 ± 5.3	7.8 ± 4.3	15.9 ± 1.4	783.7 ± 211.3	3.8 ± 0.7	132.7 ± 20.7	75.4 ± 19.7
PF	7.7 ± 2.3	7.5 ± 4.1	7.7 ± 3.3	15.4 ± 1.3	827.6 ± 282.4	4.2 ± 0.7	133.1 ± 32.2	78.1 ± 20.2
Pre-diabetics (n=2)*								
BSL	17.8	8.3	11.9 [#]	13.4	1148.5	19	126.7	43.8
PF	14.9	7.9	8.2 [#]	11.6	1054.5	17.8	103.2	44

All results are expressed as mean ± SD. Data was analyzed using Wilcoxon signed rank test. #, n=1; β, oxLDL assay performed through ELISA using plasma samples. * No statistics done due to small numbers.

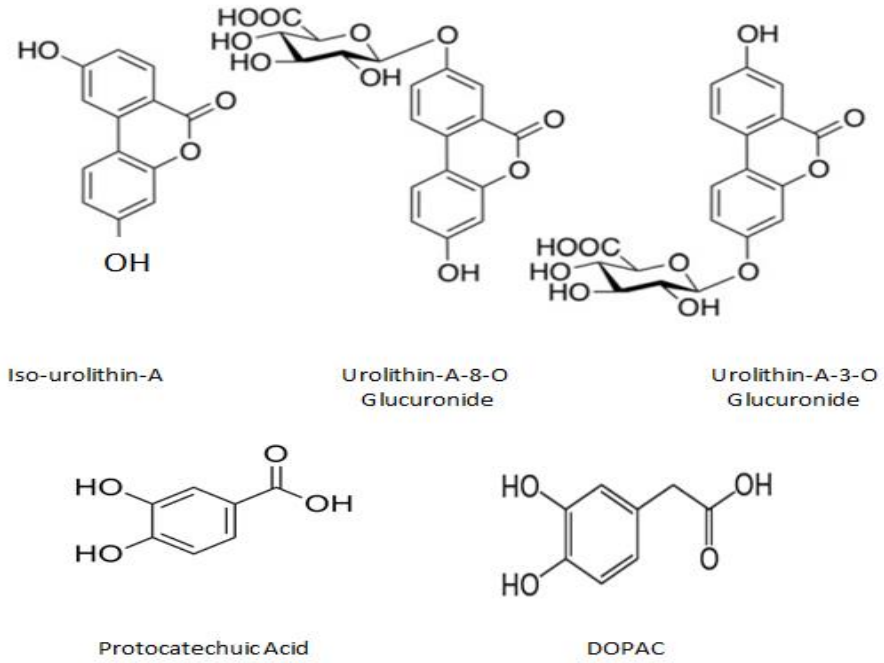

Figure 2.4. Structures of most abundant metabolites from both ellagitannins and anthocyanins in RR found in the plasma of clinical trial participants after 2 weeks of smoothie feeding.

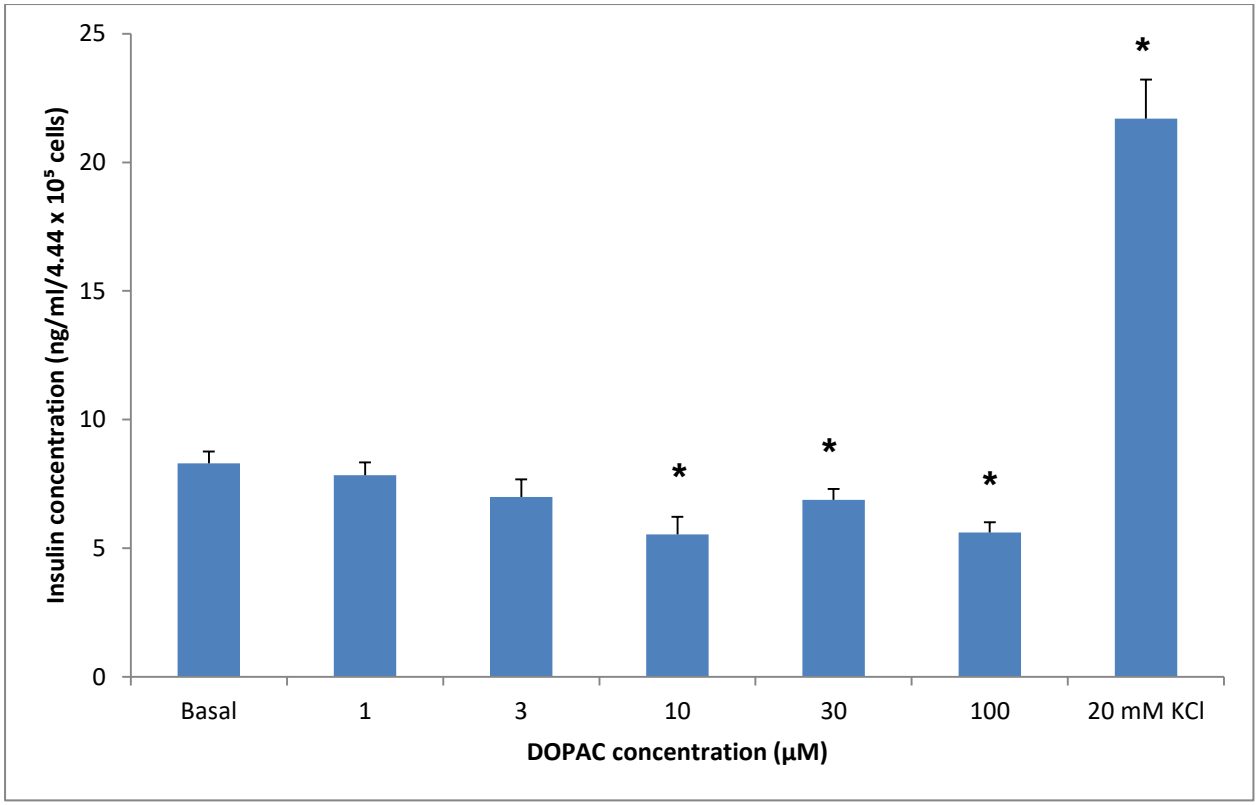

Figure 2.5. Treatment of INS-1 cells with 1-100 uM DOPAC. Results are expressed as mean ± S.E.M. from two independent experiments, each experiment having n=6; * $p < 0.05$ when compared with basal.

CHAPTER 3. CONCLUSIONS AND FUTURE WORK

It has been well established that edible berries are an excellent source of various polyphenolic compounds, which exhibit anti-oxidative, anti-inflammatory and anticarcinogenic activities [172]. The purpose of this study was to evaluate the potential of whole RR to modulate IR and inflammation in T2DM patients. Results from the clinical trial showed a significant downregulation of hsCRP, an important biomarker of inflammation and CV risk, and an improvement on insulin sensitivity based on HOMA-IR results which showed a statistical trend. These results confirm the potential that RR has to modulate IR and inflammation and we hypothesize that a longer feeding period may yield better and more representative results that could lead to further downregulation of both IR and inflammation biomarkers in T2DM patients. A trial involving more patients is recommendable too as a larger sample size would be more representative of the effects RR consumption may have and would better evaluate interpatient variability that is normally observed in clinical studies of this kind. Another aim was to determine the molecular mechanism(s) by which whole RR prevent the destruction of pancreatic β-cells. To determine if DOPAC, a metabolite of anthocyanin and quercetin, had insulin secretion effects *in vitro,* an ELISA for insulin secretion using rat pancreatic β-cells (INS-1) showed DOPAC did not stimulate insulin secretion at physiologically observed concentrations or even at higher non-physiological concentrations. An assay evaluating human β-cells is recommended to confirm these results. However, this finding complements the insulin sensitivity improvement rather than insulin secretion enhancement found in the patients from the clinical trial. It is possible that this effect of insulin sensitivity improvement may have derived from other molecules present in RR.

For instance ellagitannins, which were not evaluated in the present study due to lack of proper standards for sanguiin H-6, the major ellagitannin present in RR may have played a role The major ellagitannin-derived metabolites found in plasma of patients such as Uro-A-glur and Uro-A- sulf could have also played a role.

Therefore, studies that evaluate the effect(s) of these particular compounds may help elucidate the exact mechanism(s) being triggered to yield insulin sensitivity improvement through RR consumption.

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VITA

Reynaldo Moreno Uclés was born and raised in San Pedro Sula, Honduras. He received his bachelor's degree in Agricultural Engineering and Business Administration from Universidad de San Pedro Sula (Honduras) in 2002. He came to Baton Rouge in 2005 to pursue a bachelor's in Music at Louisiana State University which he completed in 2008. He then joined the LSU graduate program under the guidance of Dr. Giovanna Aita at the Audubon Sugar Institute in 2009. In December 2011 he completed his research titled "Identification of algal strains by PCR amplification and evaluation of their fatty acid profiles for biodiesel production" and received his Master's Degree in Food Science. He then worked in industry as Research Assistant at a facility where waste cooking oil was used to make biodiesel and as Quality Control Supervisor at Chef John Folse Inc., accumulating close to two years of industry experience. In the fall of 2014, Reynaldo returned to LSU to pursue a doctoral degree and to conduct research using red raspberries as a potential food to ameliorate symptoms that manifest in T2DM. He is expected to receive a Doctor of Philosophy degree from the School of Nutrition and Food Sciences with a minor in Biochemistry in May of 2019. He is married to Mardeli C. Saire Mendoza, a medical doctor who works at Overton Brooks VA Medical Center in Shreveport, LA.