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Effects of Niacin and Vitamin D on Endothelial Cell Angiogenic Function and Vascular Regeneration During Lipotoxicity

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Graduate Program in Physiology and Pharmacology
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Abstract

Observational studies have suggested an association between low levels of niacin and vitamin D and increased cardiovascular disease risk. Both vitamins have been shown to improve endothelial functions and vascular regeneration following vascular injury, however, it appears vitamin D may promote or inhibit neovascularization in a context-dependent manner. We hypothesized that supplementation of vitamin D alone and in combination with niacin, would improve endothelial cell function under lipotoxic conditions and promote revascularization and functional recovery in obese mice with ischemic injury. Matrigel assays, mRNA microarray analyses and growth rate assays were used to investigate angiogenic function of endothelial cells exposed to the saturated fatty acid, palmitate. Supplementation with vitamin D, niacin, and the combination improved endothelial tube formation and stability in high palmitate. Supplementation with vitamin D markedly decreased expression of cell cycle regulators, where niacin induced stable expression of miR126, a known regulator of angiogenesis. In diet-induced obese mice with acute ischemic injury, treatment with niacin, but not vitamin D or the combination, improved hind limb functional recovery. No significant improvements in revascularization, regeneration, inflammation, or fibrosis were observed. In conclusion, although both vitamins promoted *in vitro* endothelial cell angiogenic function, only niacin improved functional recovery following ischemic injury.

Keywords

Metabolic syndrome, angiogenesis, lipotoxicity, endothelial cells, peripheral vascular disease, 1,25-dihydroxyvitamin D₃, revascularization, hind limb ischemia

Summary for Lay Audience

In Canada, obesity, diabetes and metabolic syndrome are on the rise, especially among Canada's Indigenous population. These diseases often lead to damaged blood vessels and cardiovascular diseases such as heart attack, stroke, hypertension, atherosclerosis and loss of blood flow to the lower legs, which can lead to amputation. These complications occur during obesity and diabetes due to the high levels of fats and sugars in the blood stream that damage endothelial cells, which make up the inner lining of blood vessels and maintain vessel function. One of the biggest challenges to our health care system is finding effective treatment options for patients with vascular disease. Both niacin (vitamin B3) and vitamin D have potential as alternative or complementary treatment options for vascular disease as they have been show to improve and maintain the function of endothelial cells. This project investigated whether vitamin D alone or in combination with niacin, could improve the ability of blood vessels to repair and regrow following damage caused by high fats. Supplementation with vitamin D, niacin, and the combination improved the ability of endothelial cells to form blood vessels in an experimental dish. However, in obese mice with hind leg blood vessel injury, supplementation with niacin alone, but not vitamin D or the combination, improved functional recovery of the hind leg. It is possible that vitamin D may limit the growth of new blood vessels during obesity as it was also found that vitamin D decreased the growth and expansion of endothelial cells when exposed to high fat. These findings raise questions as to effectiveness of vitamin D supplementation to promote cardiovascular health in a high fat setting such as obesity or metabolic syndrome. Ultimately, better understanding of the effects of nutritional compounds on blood vessels will help guide therapeutic and dietary recommendations for the promotion of vascular health.

Co-Authorship Statement

Rachel Wilson assisted with some of the RNA extractions and completed the final qRT-PCR experimental repeat for MIR126 time course data presented in Figure 3.3 B-D.

Plasma, liver lipid and enzyme measurements in Table 3.3 were performed through the Metabolic Phenotyping Laboratory in Robarts Research Institute by Cindy Sawyez and Brian Sutherland, and the London Health Science Center Core Facility.

Brian Sutherland assisted in Catwalk data collection presented in Figure 3.4.

Dissection and immunostaining of the tibialis anterior muscle presented in Figures 3.5 A and 3.7 A were performed by Zengxuan Nong, Hoa Yin and the Molecular Pathology Core Facility in Robarts Research Institute.

Capillary and arteriole densities, arteriole diameter, myofiber areas and fibrosis location score analyses presented in Figures 3.5, 3.6 and 3.7 were performed with the help of Peter Park, Richard Zhang, and Dr. Nica Borradaile.

Richard Zhang assisted in the development of all customized, automated ImageJ protocols used for image analysis presented in figures 3.5, 3.6 and 3.7.

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Abbreviations and Symbols

ABCA1	ATP-binding cassette transporter A family member 1
ABCG1	ATP-binding cassette transporter G family member 1
Akt	Protein kinase B
AMPK	5' adenosine monophosphate-activated protein kinase
ANOVA	Analysis of variance
Apo	Apolipoprotein
ATF6	Activating transcription factor 6
ATP	Adenosine triphosphate
BMI	Body mass index
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CD31	Cluster of differentiation 31
cDNA	Complementary DNA
CHO	Chinese hamster ovary
CEPT	Cholesteryl ester transfer protein
CHOP	CCAAT-enhancer-binding protein homologous protein
CPT1	Carnitine palmitoyl transferase I
CVD	Cardiovascular disease
CYP2A4	Cytochrome P450, family2, subfamily A, polypeptide 4
DGAT	Diacylglycerol acyltransferase
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EC	Endothelial cell
EDTA	Ethylenediaminetetraacetic acid
EGM-2MV	Endothelial growth media-2 microvascular
ELISA	Enzyme-linked immunosorbent assay
ET-1	Endothelin-1
eNOS	Endothelial nitric oxide synthase
ER	Endoplasmic reticulum
ERK1/2	Extracellular signal–regulated kinases

FMD	Flow mediated dilation
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GO	Gene ontology
GPR109A	G-protein coupled receptor 109A
H ₂ O ₂	Hydrogen peroxide
HAEC	Human aortic endothelial cell
HDL	High density Lipoprotein
HIF-1	Hypoxia-inducible factor 1
HMVEC	Human microvascular endothelial cell
HO-1	Heme oxygenase 1
HRP	Horseradish peroxidase
HUVEC	Human umbilical vein endothelial cell
ICAM-1	Intercellular adhesion molecule 1
IKK	Inhibitor of kappa B kinase
IL-6	Interleukin 6
IL-8	Interleukin 8
i.p	Intra-peritoneal
IRE1	Inositol-requiring enzyme 1
IU	International units
KEGG	Kyoto encyclopedia of genes and genomes
LDL	Low density lipoprotein
MCP-1	monocyte chemoattractant protein-1
miRNA	micro-ribonucleic acid
MPO	Myeloperoxidase
MTT	3-(4,5-dimethylthiazol-2-Yl)-2,5-diphenyltetrazolium bromide
NA	Niacin
NAD ⁺	Nicotinamide adenine dinucleotide (oxidized)
NADH	Nicotinamide adenine dinucleotide (reduced)
NADPH	Nicotinamide adenine dinucleotide phosphate
NEFA	Non-esterified fatty acids
NF-κB	Nuclear factor κB

NLRP3	Nucleotide-Binding Oligomerization Domain, Leucine Rich Repeat and Pyrin Domain Containing 3
NO	Nitric oxide
PBS	Phosphate buffered saline
PGD ₂	Prostaglandin D ₂
PI3K	Phosphoinositide 3-kinase
PKC	Protein kinase C
PP2A	Protein phosphatase 2A
PPAR γ	Peroxisome proliferator-activated receptor γ
PTH	Parathyroid hormone
PVD	Peripheral vascular disease
qRT-PCR	Quantitative reverse transcriptase polymerase chain reaction
rcf	Relative centrifugal force
RAAS	Renin-angiotensin-aldosterone system
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RXR	Retinoid X Receptor
SEM	Standard error of mean
SIRT	Sirtuin
T2DM	Type 2 diabetes mellitus
TCAM	Traditional complementary and alternative medicine
TG	Triglyceride
TLR4	Toll-like receptor-4
TNF α	Tumor necrosis factor α
TXA ₂	Thromboxane A ₂
USPSTF	United States Preventive Services Task Force
VCAM-1	Vascular cell adhesion molecule-1
VDR	Vitamin D receptor
VDRE	Vitamin D response element
VEGF	Vascular endothelial growth factor
VLDL	Very low density lipoprotein
VSMC	Vascular smooth muscle cell

Chapter 1 ¹

1 Introduction

1.1 Obesity, Metabolic Syndrome and Ischemic Peripheral Vascular Disease

Worldwide prevalence of obesity has nearly tripled since 1975, with its prevalence only expected to increase in years to come. As of 2016, the World Health Organization estimated that more than 650 million people worldwide are obese (World Health Organization, 2016). More shockingly, the prevalence of obesity among children and adolescents has risen from 4% to 18% in the span of 41 years (World Health Organization, 2016). Historically, obesity has been regarded as a high-income country problem, with the highest prevalence observed in the Americas; however it is currently on the rise in low- and middle-income countries (World Health Organization, 2016). Increased prevalence of obesity is largely attributed to the increased consumption of calorie dense, highly palatable foods that are high in fat and sugar (Crino *et al.*, 2015). Increased calorie consumption, coupled with decreased physical activity due to an increasingly sedentary lifestyle further drive this increased prevalence of obesity in the western world (Unger & Scherer, 2010; Sturm & An, 2014).

The World Health Organization defines obesity as having abnormal or excess accumulation of adipose tissue that may be detrimental to health (World health organization, 2016). In order to be classified as obese, a body mass index (BMI = weight [kg] / height² [m²]) of >30 kg/m² is required. Although BMI has been used clinically for centuries to classify obesity, its use has been scrutinized and found to be an ineffective tool to measure associations between weight and cardiometabolic health. Rather, medical practitioners should consider ectopic adipose tissue distribution within the body as an indicator of weight and cardiometabolic health (Tomiyaama *et al.*, 2016). Specifically, abdominal obesity, or excess fat accumulation around the waist or abdomen, is a key risk factor for the development of metabolic syndrome and other chronic diseases such as type 2 diabetes mellitus (T2DM) and cardiovascular disease (CVD) (Mokdad *et al.*, 2003;

¹ A version of this thesis has been accepted to the Journal of Nutritional Biochemistry [K.M. Peters *et al.* (2019) *J. Nutr. Biochem.* 70: 65–74].

Després & Lemieux, 2006). The most common and prevalent downstream complication associated with abdominal obesity is metabolic syndrome. Due to the many physiological and metabolic disturbances that occur during metabolic syndrome, there is an increased risk for the development of other co-morbidities such as CVD, T2DM and all-cause mortality (O'Neill & O'Driscoll, 2015). Metabolic syndrome is diagnosed when a patient presents with at least 3 of the following 5 criteria: elevated blood pressure >130/85 mmHG, elevated fasting blood glucose level >5.6 mmol/L, high triglycerides (TG) >1.7 mmol/L, low levels of high density lipoprotein (HDL) <1.0 mmol/L in men or <1.3 mmol/L in women or an increased waist circumference >102 cm in men or >88 cm in women (Metabolic Syndrome Canada, 2019).

Under normal physiological conditions, adipocytes are able to compensate for caloric excess and inactivity by expanding their lipid depots (adipose hypertrophy) and through adipogenesis (adipose hyperplasia) to maximize storage of diet-derived plasma lipids in the form of TG (Virtue & Vidal-Puig, 2010). During periods of fasting, stored TG can undergo lipolysis to provide energy to other organs, in the form of free fatty acids (Unger & Scherer, 2010). Adipocytes are both an endocrine and paracrine organ and secrete various factors, or adipokines, that can influence different metabolic and vascular processes. In the setting of obesity, concomitant with adipocyte hypertrophy, is a proportional increase in leptin secretion from adipocytes that targets the hypothalamus to reduce food consumption (Unger & Scherer, 2010; Harman S. Mattu, 2013). Leptin also has a physiological role, via leptin-induced fatty acid oxidation, to minimize the accumulation of lipids in cell types other than adipocytes (Unger & Scherer, 2010). Hyperleptinaemia may contribute to the pathophysiology of metabolic syndrome and CVD during obesity, as it is associated with insulin resistance, platelet aggregation and arterial thrombosis (Van Gaal *et al.*, 2006). Adipocytes can further contribute to the pathophysiology of obesity and metabolic syndrome through the secretion of proinflammatory molecules such as tumour necrosis factor alpha (TNF α), interleukin-6 (IL-6), and monocyte chemoattractant protein-1 (MCP-1), leading to a sustained pro-inflammatory state (Van Gaal *et al.*, 2006; Campia *et al.*, 2012), which has been shown to induce insulin resistance and increase the risk for the development of metabolic syndrome (Odegaard & Chawla, 2013).

Under sustained conditions of increased caloric intake and decreased physical activity, the ability of adipocytes to store excess lipids is exceeded, resulting in uncontrolled TG lipolysis and subsequent increase in circulating fatty acids (van Herpen & Schrauwen-Hinderling, 2008). Adipose hypertrophy, rather than adipose hyperplasia, is associated with impaired adipocyte function, inflammation, insulin resistance, metabolic disease and cardiovascular risk (Skurk *et al.*, 2007; Sun *et al.*, 2011). An increased flux of fatty acids from dysfunctional adipocytes can partially account for hypertriglyceridemia seen in patients with metabolic syndrome, as the liver generates more very low density lipoproteins (VLDL) in response to increased substrate availability (van Herpen & Schrauwen-Hinderling, 2008). Increased plasma levels of fatty acids and lipoproteins during chronic hyperlipidemia, can lead to steatosis, or the abnormal accumulation of fatty acids in tissues, such as the liver, heart, skeletal muscle, and vasculature, that are not metabolically programmed to store these excess lipids (van Herpen & Schrauwen-Hinderling, 2008). Steatosis can disruption cellular signaling and tissue homeostasis, leading to cell death in various tissues and organs, through a process termed lipotoxicity (Wende *et al.*, 2012; Dalan *et al.*, 2014).

Cardiovascular diseases are some of the most prevalent downstream complications associated with obesity and metabolic syndrome (Campia *et al.*, 2012; Brostow *et al.*, 2012). Vascular complications arise in multiple tissue sites and vascular beds in response to excess circulating fatty acids. In addition to fatty acid accumulation within vascular endothelium, the inflammatory response induced by vascular steatosis results in macrophage recruitment and subsequent plaque formation in large and medium sized arteries (Talayero & Sacks, 2011). Plaque development occludes arteries contributing to the hypertensive state observed during metabolic syndrome. Over time, plaques may become unstable and are at risk of rupture that can lead to myocardial infarction and stroke (Libby, 2012; Manduteanu & Simionescu, 2012). Beyond large and medium sized arteries, plaques may also develop in peripheral vascular beds, disrupting blood flow to the extremities, and lead to the development of peripheral vascular disease (PVD) (Brostow *et al.*, 2012; Teodorescu *et al.*, 2013). Left untreated, patients can develop end stage PVD, or critical limb ischemia, and may require limb amputation (Teodorescu *et al.*, 2013). Metabolic syndrome is associated with increased incidence of CVD

morbidity, mortality and all-cause mortality (Galassi *et al.*, 2006), as well as increased incidence of CVD endpoints, including ischemic stroke (Chen *et al.*, 2006), PVD (Garg *et al.*, 2014) and end stage PVD (Gardner *et al.*, 2006). Therefore, prevention and intervention strategies protecting against vascular damage during obesity and metabolic syndrome are warranted.

1.2 Endothelial Cell Damage during Obesity and Metabolic Syndrome

Vascular endothelial cells (EC) serve many physiological functions, playing a critical role in the maintenance of vascular homeostasis. The endothelium lines the entire circulatory system acting as a physical barrier between the lumen and vessel wall. EC have both a sensory and effector capacity to regulate thrombosis, platelet and leukocyte activity, vascular permeability, fibrinolysis and immune modulation (Bonetti *et al.*, 2003). In addition, EC secrete a number of mediators that regulate vascular tone, such as endothelin-1 (ET-1) and thromboxane A2 (TXA2) for vasoconstriction, and nitric oxide (NO) for vasodilation (Rajendran *et al.*, 2013). Furthermore, EC play a critical role in the maintenance of vascular structure through angiogenesis and are responsible for vascular regeneration and repair following injury (Imrie *et al.*, 2010). Therefore, disruption of EC function can play a critical role in the pathogenesis of many vascular diseases common to obesity and metabolic syndrome.

Endothelial dysfunction, in a broad sense, is characterized by a shift in EC physiology that results in an imbalance of vascular tone regulators leading to disrupted vascular homeostasis (Endemann & Schiffrin, 2004). More specifically, there is an observed decrease in the important vasodilator, NO, with a corresponding increase in vasoconstrictor factors (Campia *et al.*, 2012; Symons & Abel, 2013). It is also understood that beyond the imbalance in vasoregulators, EC dysfunction comprises EC activation that induces a pro-inflammatory, proliferative and pro-coagulator state. Endothelial dysfunction is associated with obesity and metabolic syndrome (Steinberg *et al.*, 1996) and has been identified as a surrogate marker for vascular disease onset and progression, including the common and often underdiagnosed, condition of PVD (Vita & Hamburg, 2010; Campia *et al.*, 2012). More interestingly, endothelial dysfunction has been shown

to precede overt symptoms of metabolic syndrome and obesity, suggesting that it may contribute to the development of both the vascular and metabolic disturbances associated with these diseases (Campia *et al.*, 2012; Grandl & Wolfrum, 2018).

Initially, EC respond and adapt to vascular stress and changes in their microenvironment by modulating their constitutive functions. However, under continued or prolonged insults, such as hyperlipidemia, hyperglycemia or hemodynamic stress, endothelial dysfunction arises, leading to EC injury and eventual apoptosis (Simionescu, 2007; Kim *et al.*, 2012). An early adaptation of EC function in response to hyperlipidemia during obesity and metabolic syndrome involves altered EC permeability, such that LDL and VLDL can accumulate in the subendothelium (Simionescu, 2007). This shift in permeability is accompanied by alteration in EC secretory phenotype, where EC synthesize surface adhesion molecules, such as selectins, including vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1), chemotactic factors, particularly MCP-1 and IL-8, and vasoactive substances (Simionescu, 2007). Expression of these molecules facilitates leukocyte-endothelial interactions, promoting monocyte recruitment and generation of a chronic inflammatory state (Grover-Páez & Zavalza-Gómez, 2009; Rajendran *et al.*, 2013). Endothelial NO has been identified as an important molecule for maintenance of vascular function under normal physiological conditions. Insults to EC can reduce or even abolish NO function. Reduced NO bioavailability further contributes to the upregulation of VCAM-1 in EC via the nuclear factor κ B (NF- κ B) pathway augmenting the inflammatory response (Kuldo *et al.*, 2005).

Pathogenesis of endothelial dysfunction and progression of vascular disease is largely dependent on vessel type and location within the human body. Presentation of endothelial dysfunction in large arteries represents a crucial role in the early development of lipid-rich foam cell formation, lesion evolution and eventual atherosclerotic plaques formation. Conversely, at the level of microvessels, endothelial dysfunction results in disruption of blood flow and vasoreactivity (Abularrage *et al.*, 2005; Avogaro & de Kreutzenberg, 2005). Microvascular endothelial dysfunction is more closely related to development and progression of PVD rather than atherosclerosis. Furthermore, microvascular endothelial dysfunction precedes atherosclerotic plaque formation making it an important early marker of vascular disease during obesity and metabolic syndrome (Verma *et al.*, 2003).

Therefore, prevention and intervention strategies for PVD should target endothelial dysfunction. However, currently available pharmacotherapies for PVD are limited and generally focus on symptom relief for thrombosis and claudication pain management rather than promoting endogenous revascularization of the ischemic tissues (Farber & Eberhardt, 2016). Patients who develop end stage PVD, have the option to undergo surgical revascularization to increase chances of limb salvage, but this method is rather invasive and further highlights the need for more therapeutic treatments targeting the promotion of endothelial function and endogenous vascular regeneration (Saghiri *et al.*, 2017).

1.3 Endothelial Cell Lipotoxicity

High levels of circulating fatty acids are key endothelial stressors during metabolic diseases. Lipotoxicity is the process by which lipid accumulation results in alteration of tissue homeostasis that leads to cell dysfunction and eventual apoptosis (Imrie *et al.*, 2010; Kim *et al.*, 2012). EC are especially vulnerable to lipotoxicity as they form the inner most layer of the vasculature and are constantly exposed to high levels of circulating lipids (Wende *et al.*, 2012). Furthermore, EC's main energy source is derived from aerobic glycolytic ATP, and therefore EC lack the metabolic programming to efficiently process excess lipids through β -oxidation. Due to the inability of EC to process large quantities of lipids, metabolic stress pathways are activated and resulting in the generation of reactive oxygen species (ROS) that contribute to endothelial dysfunction (Wende *et al.*, 2012). Figure 1.1 provides a summary of EC lipotoxicity and related pathways during obesity and metabolic syndrome, which are discussed in detail below.

Fatty acids are classified as either saturated or unsaturated fatty acids. It is largely recognized that saturated fatty acids are responsible for CVD, where unsaturated fatty acids are well tolerated by cells and may actually protect against CVD (Ghosh *et al.*, 2017). Experimental work in EC has demonstrated that saturated fatty acids, such as palmitate, induce cellular apoptosis in a dose-dependent manner, where unsaturated fatty acids do not (Staiger *et al.*, 2006; Ciapaite *et al.*, 2007). Palmitate is one of the most

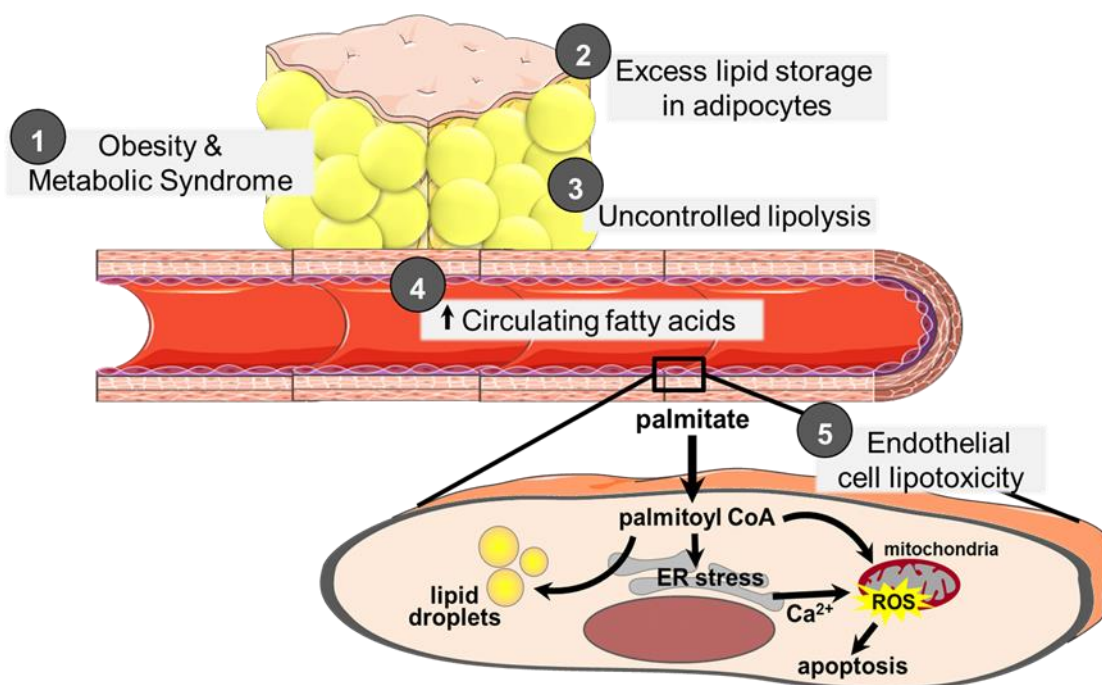


Figure 1.1 Summary of endothelial dysfunction and related pathways during obesity and metabolic syndrome.

During obesity and metabolic syndrome (1) there is accumulation of lipids in adipocytes (2). Once adipocyte capacity is exceeded uncontrolled, dysregulated lipolysis occurs (3), releasing excess fatty acids into circulation (4). Endothelial cells are continuously exposed to high levels of circulating lipids as they comprise the inner most layer of vasculature. Endothelial cells lack the metabolic programming to process excess fatty acids. Excess palmitate, an abundant dietary saturated fatty acid, can overwhelm the ability of a cell to store fatty acids in lipid droplets or to catabolize them through β -oxidation in mitochondria. This can result in production of reactive oxygen species (ROS) and ER stress, resulting in endothelial dysfunction and eventual death, through a process termed lipotoxicity. Palmitate can also readily be incorporated into the ER lipid membrane, resulting in impaired membrane structure and integrity. Oxidative stress and ER stress result in release of calcium from the ER further contributing to mitochondrial-mediated cell death (5).

abundant saturated fatty acids occurring in a typical western-diet, and has been identified as a strong contributing factor to atherosclerosis development (Yamagishi *et al.*, 2002; Lu *et al.*, 2013). Several studies have shown that animals fed a western style diet high in saturated fats resulted in various metabolic and vascular disturbances, including atherosclerosis and PVD (Breslow, 1996; Coenen *et al.*, 2009; Wang *et al.*, 2009).

During exposure to excess fatty acids, especially saturated fatty acids, toxic bioactive lipid metabolites can accumulate, contributing to impaired metabolic homeostasis and endothelial dysfunction. Ceramides are precursors for sphingolipids in cells, both of which have been recognized as initiators of cell stress response and eventual apoptosis following cellular injury (Chaurasia & Summers, 2015). Inhibition of ceramide biosynthesis has been demonstrated to prevent various cardiovascular events, including endothelial dysfunction. Ceramides are produced in response to saturated fatty acid metabolism and have been shown to decrease eNOS activity and contribute to endothelial dysfunction (Xiao-yun *et al.*, 2009). Using aortic EC, Zhang *et al.* (2012) showed that increased *de novo* ceramide production was associated with reduced eNOS activation and NO bioavailability by initiating colocalization of eNOS with protein phosphatase 2A (PP2A) and preventing Akt-associated activation. Furthermore, treatment of EC with ceramide elicited signalling events that led to increased ROS generation (Zhang *et al.*, 2012b), which has been shown to occur upstream of eNOS inhibition and mediated in an NADPH dependent manner (Mugabo *et al.*, 2011). Ceramide production has also been associated with impaired EC functions such as decreased cell proliferation and migration (Bocci *et al.*, 2012). Ceramide accumulation in response to excess palmitate outlines one mechanism by which lipotoxicity contributes to endothelial dysfunction and eventual cell death.

Saturated fatty acids, such as palmitate, have been shown to readily induce EC inflammatory pathway activation that has been shown to contribute to endothelial dysfunction (Kim *et al.*, 2005; Liu *et al.*, 2012). Several studies have reported increased activation of the NF- κ B inflammatory pathway in response to fatty acids, resulting in decreased NO production and a dysfunctional phenotype (Staiger *et al.*, 2006; Iwata *et al.*, 2011; Li *et al.*, 2011). Kim *et al.* (2007) demonstrated that palmitate signals via toll-like receptor-4 (TLR4) in EC to upregulate the expression of inflammatory genes, such as

IL-6 and TNF α . Increased expression of these inflammatory genes is associated with impaired insulin signalling and reduced NO production in vascular EC (Kim *et al.*, 2007). Follow-up studies from this group have demonstrated that palmitate-induced TLR4 activation was associated with NADPH-dependent ROS production in human microvascular endothelial cells (HMVEC), which was required for the inflammatory response elicited by palmitate (Maloney *et al.*, 2009). Fatty acids have also been shown to induce the expression of inflammasomes, which are large cytoplasmic complexes from the NOD-like receptors family of pattern-recognition receptors (Abderrazak *et al.*, 2015). Inflammasomes are responsible for the activation of caspases, which leads to the activation of pro-inflammatory cytokines, such as IL-1 β and IL-18 (Martinon *et al.*, 2002). NLRP3 is the most versatile and clinically relevant inflammasome as several inflammatory diseases such as obesity, atherosclerosis and T2DM have been linked to NLRP3 activation (Leemans *et al.*, 2011). A recent study by Wang *et al.* (2016) demonstrated that treatment of microvascular EC with palmitate activated NLRP3, which was associated with decreased expression of endothelial tight junction proteins and the promotion of a dysfunctional EC phenotype.

Oxidative stress and increased ROS production have been identified as a key player in the development EC dysfunction during lipotoxicity. Mitochondria are a major source of ROS and oxidative stress. Exposure to palmitate has been shown to increase ROS production in skeletal muscle cells (Jheng *et al.*, 2012) and in vascular EC (Szewczyk *et al.*, 2015; Broniarek *et al.*, 2016) due to incomplete mitochondrial β -oxidation. Another major source of ROS production in EC that has grown much attention is NADPH oxidase. More specifically, treatment of EC with palmitate has been shown to increase ROS production in an NADPH-oxidase dependent manner, which was associated with endothelial dysfunction in obese, diabetic rats (Chinen *et al.*, 2007). Production of ROS in EC can impair NO bioavailability via disrupted oxidant/antioxidant balance. Excess ROS has been shown to react with NO to form peroxynitrite and lead to a disruption in eNOS activity and decreased NO bioavailability (Zou *et al.*, 2002; Molnar *et al.*, 2005; Fonseca *et al.*, 2010). Furthermore, various pathways contributing to ROS production can synergistically interact to generate an increasingly dysfunctional phenotype. For example, NADPH-oxidase-derived ROS can enhance mitochondrial ROS production resulting in a

vicious cycle of increased ROS production, reduced NO bioavailability and increased endothelial dysfunction (Imrie *et al.*, 2010; Zorov *et al.*, 2014).

Excess saturated fatty acids have also been shown to induce endoplasmic reticulum (ER) stress outlining another mechanism of fatty acid induced endothelial dysfunction (Li *et al.*, 2015). Several studies have shown that endothelial ER stress leads to increased NADPH-oxidase expression, decreased eNOS activity and increased ROS production (Santos *et al.*, 2014; Galán *et al.*, 2014; Li *et al.*, 2015). Palmitate has been shown to inhibit EC proliferation, increase ROS production and induce endothelial apoptosis via upregulation of the ER stress marker, C/EBP homologous protein (CHOP) (Lu *et al.*, 2013). A recent study by Tampakakis *et al.* (2016) demonstrated that elevated fatty acids following intralipid infusion in healthy patients, induced characteristics of microvascular dysfunction along with activation of activating transcription factor 6 (ATF6) and inositol-requiring enzyme 1 (IRE1) in EC, both of which are early markers of ER stress response. Lastly, excess fatty acids can induce ER stress through disruption of ER membrane. Palmitate has been shown to be rapidly incorporated into ER membrane phospholipids disrupting membrane integrity and initiate apoptosis (Borradaile *et al.*, 2006).

1.4 Niacin and the Regulation of Lipid Metabolism and Vascular Function

Niacin (vitamin B3) has been used in the clinical setting for decades to correct high lipid profiles, particularly hypertriglyceridemia, in patients at risk for CVD (Digby *et al.*, 2012b). Pharmacological doses of niacin have been shown to provide clinical benefit in the setting of metabolic disease by lowering the pro-atherogenic lipid species, VLDL and LDL, while simultaneously increasing the anti-atherogenic high-density lipoprotein (HDL) fractions, resulting in improved lipid profiles (Thoenes *et al.*, 2007; Creider *et al.*, 2012). Despite the clinical benefit of niacin, its use has been hindered by a lack of patient compliance due to its adverse, yet relatively harmless, cutaneous flushing experienced with niacin supplementation.

Niacin has been shown to elicit some effects through the cell surface receptor, GPR109A. This is a seven-transmembrane G-protein coupled receptor that has a high affinity for

niacin and was first identified in 2003 (Tunaru *et al.*, 2003; Wise *et al.*, 2003; Soga *et al.*, 2003). GPR109A is widely expressed in different cell types including immune cells (Yousefi *et al.*, 2000; Schaub *et al.*, 2001), intestinal epithelium (Thangaraju *et al.*, 2009), and epidermal Langerhans cells (Maciejewski-Lenoir *et al.*, 2006). Activation of GPR109A in Langerhans cells, and subsequent production of the vasodilatory compound prostaglandin D₂ (PGD₂), is responsible for the cutaneous flushing experienced with niacin treatment. The most robust expression of GPR109A is found on both brown and white adipocytes and accounts for the anti-lipolytic effects of niacin (Tunaru *et al.*, 2003). More importantly, research from our lab confirmed GPR109A expression in vascular EC (Hughes-Large *et al.*, 2014).

As early as 1960s, the effect of niacin on lipid profiles has largely been attributed to the partial inhibition of adipocyte lipolysis. Niacin has been shown to activate GPR109A in adipocytes to inhibit adenylyl cyclase, reduce cyclic monophosphate (cAMP) accumulation and decrease adipocyte TG hydrolysis and fatty acid release (Lukasova *et al.*, 2011; Yadav *et al.*, 2012). The resultant decrease in fatty acid efflux limits substrate availability for hepatic TG synthesis and assembly of VLDL and LDL (Zhang *et al.*, 2005; Offermanns, 2006). However, more recent findings have found that niacin causes a robust rebound in adipocyte lipolysis that actually results in increased plasma fatty acid levels over 24 h following treatment (Oh *et al.*, 2011). Moreover, in mice lacking GPR109A, niacin treatment failed to produce an anti-lipolytic effect, but retained its lipid lowering effect (Lauring *et al.*, 2012). Thus it has been suggested that inhibition of adipose lipolysis is unlikely to be the limiting factor for hepatic TG synthesis (Kamanna & Kashyap, 2008).

Independent of GPR109A, niacin can directly target the liver to exert its effects on lipid profiles. Diacylglycerol acyltransferase 2 (DGAT2) is a key enzyme involved in the final catalysis step of hepatic TG synthesis. Niacin has been shown to directly and noncompetitively inhibit microsomal DGAT2 in HepG2 cells (Ganji *et al.*, 2004; Kamanna *et al.*, 2013). Due to decreases in hepatic TG synthesis there were observed increases in apolipoprotein B (ApoB) degradation, with a resulting decrease in hepatic secretion of VLDL and LDL. In support of this concept, findings from a small human study reported reduced hepatic lipid content following 23 weeks of treatment with

extended release niacin (2 g/day). Their results suggested that the mechanism may be due to inhibition of DGAT2, as DGAT2 polymorphisms were associated with smaller reduction in hepatic lipid content (Hu *et al.*, 2012). Furthermore, rats fed a high-fat diet had decreased hepatic lipid content and displayed significant inhibition of mRNA levels, protein expression, and activity of DGAT2 following treatment with niacin (Ganji *et al.*, 2014). Results obtained in mice suggest that niacin may also inhibit hepatic peroxisome proliferator-activated receptor gamma coactivator 1- β (PGC-1 β) expression resulting in decreased ApoC3 and reduced TG synthesis (Hernandez *et al.*, 2010).

Niacin has long been recognized for its ability to raise HDL levels; however mechanisms behind this effect remain complex and rather elusive (Pang *et al.*, 2014). Kamanna *et al.* (2013) have proposed that niacin may reduce hepatic removal of HDL from circulation via down regulation of β -ATP synthase expression. Niacin may also enhance HDL formation by increasing lipidation of ApoA1 with cholesterol (Zhang *et al.*, 2012a). Further studies have revealed this effect may be a result of enhanced lipid efflux from peripheral tissues via transcription of hepatic ABCA1 (Kamanna *et al.*, 2013). Although niacin's involvement in augmenting HDL are complex and not well understood, niacin has been shown to improve the protective function of HDL on EC to limit the progression of atherosclerosis (Sorrentino *et al.*, 2010; Ganji *et al.*, 2014). Niacin's protective role may be associated with macrophage-mediated reverse cholesterol transport (Rubic *et al.*, 2004; Lukasova *et al.*, 2011). Niacin, through activation of GPR109A on macrophages can increase cholesterol efflux onto HDL particles via the ABCG1 transporter to reduce atherosclerosis progression (Lukasova *et al.*, 2011). Specific to EC, in patients with T2DM, treatment with extended release niacin showed improved HDL capacity to stimulate NO production, endothelial repair and decrease oxidative stress (Sorrentino *et al.*, 2010). More recent evidence has demonstrated that niacin may preserve HDL function by limiting HDL oxidation. Ganji *et al.* (2014) have shown that niacin decreased NADPH oxidase and ROS production, which was associated with reduced leukocyte myeloperoxidase (MPO) activity and decreased HDL oxidation.

Results from the large clinical trials HPS2-THRIVE (Haynes *et al.*, 2013) and AIM-HIGH (Albers *et al.*, 2013) have brought in to question the use of niacin for cardiovascular benefit in the treatment of dyslipidemia. These trials were designed to

assess whether the addition of niacin to statin therapy offered any additional cardiovascular benefit. Both trials were stopped due to a lack of additional cardiovascular benefit. However, many criticisms of both trials have been published and suggest either study may have been insufficient to identify the cardiovascular benefits of niacin (Digby *et al.*, 2012b; Michos *et al.*, 2012; Song & FitzGerald, 2013; Superko *et al.*, 2017). Despite the disappointing results from AIM-HIGH and HPS2-THRIVE, niacin intervention has consistently been shown to improve vascular health in several patient populations (Creider *et al.*, 2012), including those with metabolic disturbances (Thoenes *et al.*, 2007; Guyton *et al.*, 2013; Toth *et al.*, 2015). As outlined in section 1.3, vascular inflammation and oxidative stress have been identified as important contributors to endothelial dysfunction during lipotoxicity, and reductions in these pathways may drive niacin's vascular benefit. Furthermore, several lines of evidence suggest that niacin can promote vascular health and reduce the progression of atherosclerosis, independent of its lipid lowering effects as no changes in plasma lipids levels were observed (Wu *et al.*, 2010; Lukasova *et al.*, 2011; Digby *et al.*, 2012b).

Niacin treatment has been shown to reduce systemic inflammation in patients with CVD independent of changes in lipid levels, suggesting non-lipoprotein mediated anti-inflammatory effects of niacin (Ridker *et al.*, 1997, 2000; Kuvin *et al.*, 2006). Through activation of GPR109A in adipocytes, niacin has been shown to inhibit secretion of pro-inflammatory molecules such as TNF α , MCP-1, cytokines and fractaline limiting the contribution of these systemic inflammatory molecules to atherosclerosis (Digby *et al.*, 2010). GPR109A expression has been confirmed in monocytes and macrophages (Ahmed *et al.*, 2009) and niacin, via GPR109A activation, has been shown to reduce monocyte adhesion to EC, reducing the pro-inflammatory state (Tavintharan *et al.*, 2011). Niacin has also been shown to reduce the expression of MCP-1 and thus inhibit recruitment of neutrophils and macrophages to atherosclerotic plaques (Wu *et al.*, 2010; Lukasova *et al.*, 2011). More recently, Si *et al.* (2014) demonstrated, both *in vitro* and *in vivo*, that niacin exerted its anti-inflammatory effects via downregulated NF- κ B signaling pathways (Si *et al.*, 2014).

Niacin can also act as an antioxidant, protecting EC from oxidative stress. Treatment of coronary artery EC with niacin resulted in an upregulation of hemeoxygenase-1 (HO-1)

and resultant decrease in TNF α -mediated endothelial inflammation and oxidative stress (Wu *et al.*, 2012a). HO-1 has been consistently associated with clinical benefit in many pathological diseases, including CVD. Work by Ganji *et al.* (2009) has shown that niacin treatment increased endothelial NADPH and reduced glutathione which was associated with a reduction in ROS production and EC oxidative stress. Furthermore, in dyslipidemic patients, with low levels of HDL and hypercholesterolemia, treatment with niacin significantly decreased serum thiobarbituric acid and lipid peroxidases resulting in significantly reduced oxidative stress (Hamoud *et al.*, 2013).

Several studies exist to support the concept that niacin can directly improve EC function to provide a vascular benefit, independent of its lipid lowering effects. Niacin treatment improved angiogenesis following ischemic stroke, which was associated with increased expression of VEGF and increased PI3K activity (Chen *et al.*, 2009; Shehadah *et al.*, 2010). In diabetic mice, independent from changes in plasma lipids, niacin increased blood flow following peripheral ischemia, enhanced endothelial progenitor cell mobilization and improved revascularization in a NO-dependent manner (Huang *et al.*, 2012). In rabbits, niacin protected against endothelial dysfunction noted by increased vasodilation, decreased expression of VCAM-1 and MCP-1 and inhibition of vascular inflammation (Wu *et al.*, 2010). Recent work from our lab has demonstrated that niacin promotes HMVEC tube formation as well as enhances revascularization and functional recovery in diet-induced obese mice following ischemic injury (Hughes-Large *et al.*, 2014; Pang *et al.*, 2016).

Results from animal models suggesting that niacin directly improves EC function, independent of lipid lowering effects, are supported by human clinical studies. Niacin treatment significantly improved flow-mediated dilation (FMD) of the brachial artery in patients with T2DM (Hamilton *et al.*, 2010) and metabolic syndrome (Thoenes *et al.*, 2007), without changes in lipid profiles. More recently, in a small clinical trial, a seven day treatment with niacin was enough to improve FMD, without altering lipid levels (Nasser Figueiredo *et al.*, 2014). Furthermore, increased dietary niacin intake was associated with improved endothelial function as measured by FMD in the brachial artery and reduced oxidative stress (Kaplon *et al.*, 2014). A recent meta-analysis, found that niacin significantly improved endothelial dependent vasodilation without changes in lipid

parameters and the effect was more prominent in primary prevention of atherosclerotic CVD (Sahebkar, 2014). Finally, niacin treatment has been shown to significantly reduced coagulation factors in patients with PVD that may have been associated with the reduced CVD morbidity in these patients (Chesney *et al.*, 2000). Further understanding of the pleiotropic effects of niacin, including promotion of vascular function and regeneration, outline an important step in determining the clinical use of niacin for the treatment of PVD during obesity and metabolic syndrome.

1.5 Vitamin D and Vascular Function

Historically, vitamin D has largely been recognized for its significant role in bone and mineral homeostasis. Recently however, vitamin D supplementation has gained much attention as a possible therapeutic option for CVD as several preclinical and epidemiological studies have demonstrated an association between low levels of circulating vitamin D and increased risk of CVD (Forman *et al.*, 2007, 2008; Holick, 2007; Wang *et al.*, 2008a). Moreover, vitamin D deficiency has been identified as an independent risk factor for all-cause CVD morbidity and mortality (Brøndum-Jacobsen *et al.*, 2012; Kojima *et al.*, 2014). Consequently, widespread supplement use for the promotion of vascular health has increased, with the consumption of vitamin D supplements consistently rising (Pludowski *et al.*, 2018). However, it remains unclear whether widespread vitamin D supplementation for the promotion of vascular health is actually beneficial. Moreover, current dietary reference intakes and supplementation doses of vitamin D are based on the beneficial effects for the prevention of skeletal diseases such as rickets, osteomalacia, and fractures (Ross *et al.*, 2011; Cashman & Kiely, 2014) where evidence is lacking in regards to supplemental doses associated with improved cardiovascular health (Pilz *et al.*, 2016).

Vitamin D is a fat soluble vitamin and exists in two dominant forms, vitamin D₂ (ergocalciferol), synthesized by invertebrates and plants, and D₃ (cholecalciferol), that is naturally present in foods and endogenously photosynthesized in the skin in response to ultraviolet B radiation (Holick, 2003). Vitamin D itself is actually biologically inactive and requires two hydroxylation reactions in order to be biologically active (Bikle, 2014). The first hydroxylation reaction occurs in the liver where vitamin D is converted to 25-

hydroxyvitamin D [25(OH)D], which is then converted into the active form, 1 α ,25-dihydroxyvitamin D₃ [1 α ,25(OH)₂D₃], by 1 α -hydroxylation that occurs primarily in the kidney (Bikle, 2014). Although the kidney is the primary site of the final hydroxylation step, this process can occur at various extra-renal sites within the body including skin cells, endothelial cells, pancreatic islet cells and monocytes/macrophages (Norman, 2008). Finally, 1 α ,25(OH)₂D₃ can enter target cells and interact with the ligand-binding domain of the vitamin D receptor (VDR) in the cytoplasm (Norman, 2008). VDR is almost ubiquitously expressed and has been identified on various cardiovascular cells types including cardiomyocytes (Nibbelink *et al.*, 2007), endothelium (Merke *et al.*, 1989), vascular smooth muscle cells (VSMC) (Wu-Wong *et al.*, 2006) and myeloid cells (Wong *et al.*, 2014). Following activation, VDR translocates to the nucleus and heterodimerizes with the retinoid X receptor (RXR). The VDR-RXR heterodimer can then act as a transcription factor to regulate gene expression by binding directly to vitamin D response elements (VDRE) (Whitfield *et al.*, 1995). It has been estimated that VDR regulates approximately 3% of the genome (Carlberg, 2003), including processes important to cardiovascular physiology and pathology, such as proliferation, cellular differentiation, apoptosis and angiogenesis (Zittermann, 2014; Christakos *et al.*, 2016). In addition to genomic regulation, vitamin D can also interact with VDR localized in caveolae of the plasma membrane and elicit rapid, nongenomic, responses that include activation of second messengers, rapid opening of calcium channels and activation of signaling molecules (Norman, 2008). It has been suggested that both genomic and nongenomic responses of vitamin D are interrelated and may further modulate, or cross talk, between the two signaling pathways (Norman, 2008).

Several pathways and cell types that are relevant to cardiovascular physiology are influenced by vitamin D and may help to explain the role of vitamin D deficiency in the pathogenesis of CVD. Vitamin D has been proposed to reduce hypertension through the suppression of renin-angiotensin-aldosterone-system (RAAS) (Li *et al.*, 2002). *In vitro* studies have confirmed that 1 α ,25(OH)₂D₃ is a potent negative regulator of renin transcription by blocking the cAMP response element in the renin gene promoter (Yuan *et al.*, 2007). In addition, mice lacking VDR have increased levels of renin (Li *et al.*, 2004). Over activation of the RAAS pathway leads to blood vessels constriction,

contributing to hypertension and cardiac hypertrophy. Vitamin D may also protect against hypertension through its suppressive effects on parathyroid hormone (PTH) synthesis (Lee *et al.*, 2008; Wallis *et al.*, 2008; Lips, 2012), as increased serum concentrations of PTH are associated with increased risk for cardiovascular events including calcium overloading of cardiomyocytes (Watson *et al.*, 1997; Andersson *et al.*, 2004), increased oxidative stress, endothelial dysfunction (Gambardella *et al.*, 2018) and myocardial hypertrophy (Saleh *et al.*, 2003; Chen *et al.*, 2011). Experimental studies have also suggested that vitamin D may protect against atherosclerosis by inhibiting foam cell formation and increasing cholesterol efflux (Oh *et al.*, 2009; Yin *et al.*, 2015). Furthermore, VDR activation in VSMC results in upregulation of endothelin receptor type B and decreased oxytocin receptor expression to favour vessel relaxation (Bukoski *et al.*, 1989). Lastly, vitamin D may promote EC repair by inducing VEGF expression in VSMC (Cardús *et al.*, 2006; Wu-Wong *et al.*, 2006).

As mentioned above, several cell types have the vitamin D microendocrine system that allows for extra-renal synthesis of $1\alpha,25(\text{OH})_2\text{D}_3$, including EC (Merke *et al.*, 1989). A direct effect of VDR in the regulation of endothelial function was recently demonstrated by Ni *et al.* (2014) using endothelial-specific VDR knockout mice. Deletion of VDR resulted in impaired vessel relaxation accompanied by reduced eNOS expression and augmented blood pressure response to AngII (Ni *et al.*, 2014). Several studies have demonstrated beneficial effects of vitamin D supplementation on vascular endothelial NO production (Molinari *et al.*, 2011; Uberti *et al.*, 2014; Martínez-Miguel *et al.*, 2014). Activation of endothelial VDR has been shown to increase production of NO through p38/Akt-modulated eNOS activation (Molinari *et al.*, 2013). Additionally, experimental work performed in mice with functionally inactive VDR displayed lower NO bioavailability and decreased eNOS expression, which was associated with endothelial dysfunction and increased arterial stiffness (Andrukhova *et al.*, 2014). Treatment of HUVECs with vitamin D was able to improve NO production by reducing the deleterious effect of advanced glycation end products (Talmor *et al.*, 2008). Wong *et al.* (2010) confirmed that treatment with vitamin D was associated with improved EC functions noted by reduced blood pressure and EC vasoconstriction in the aorta of hypertensive rats.

Vitamin D may protect EC function through antioxidant pathways. In diabetic rats, treatment with the vitamin D analog, 22-oxacalcitriol, reduced EC NADPH oxidase expression, which was associated with reduced EC dysfunction in diabetic rats (Hirata *et al.*, 2013). Addition of $1\alpha,25(\text{OH})_2\text{D}_3$ to culture media was able to prevent hypoxia induced downregulation of CuZn-superoxide dismutase expression in EC (Zhong *et al.*, 2014). Furthermore, Polidoro *et al.* (2013) have shown that treatment of HUVECs with vitamin D induced MEK/ERK-Sirt-1 signalling pathway to decrease H_2O_2 oxidant injury. A more recent study found that treatment of HUVECs with $1\alpha,25(\text{OH})_2\text{D}_3$ led to decreased ROS production, increased expression of the known antioxidant, glutathione, and reversed the dysfunctional phenotype (Kanikarla-Marie & Jain, 2016). Vitamin D may also protect against EC oxidative stress by reducing apoptotic gene expression, while simultaneously enhancing autophagy by ERK 1/2 and Akt activation (Uberti *et al.*, 2014).

Vitamin D may improve EC function by downregulating inflammation, as treatment with $1\alpha,25(\text{OH})_2\text{D}_3$ has been associated with decreased expression of various inflammatory factors, such as $\text{TNF}\alpha$, IL-6, IL-10 and NF- κ B in EC (Martinesi *et al.*, 2006). Suzuki *et al.* (2009) showed that treatment of coronary EC with vitamin D inhibited $\text{TNF}\alpha$ -mediated NF- κ B activation and expression of E-selectin. Follow-up studies showed that vitamin D also inhibited $\text{TNF}\alpha$ -mediated expression of VCAM-1 and IL-8 in coronary EC (Kudo *et al.*, 2012). In HUVECs, treatment with vitamin D inhibited LPS-induced NF- κ B activation and expression of IL-6 and IL-8 (Equils *et al.*, 2006). Lastly, treatment of HUVECs with vitamin D has been shown to reduce monocyte adhesion to HUVECs, possibly through decreased expression of MCP-1 and ICAM-1 (Martinesi *et al.*, 2006; Kanikarla-Marie & Jain, 2016).

Taken together, there is compelling evidence linking vitamin D and EC function, raising the intriguing possibility that a causal relationship exists between vitamin D deficiency and CVD pathogenesis. However, establishing the clinical relevance of vitamin D supplementation and cardiovascular benefit in humans has been a challenge. In fact, several interventional studies examining the effect of vitamin D repletion on EC function in humans have shown conflicting results. Vitamin D supplementation improved EC function in T2DM patients with low vitamin D levels (Sugden *et al.*, 2008) and improved

EC function in obese adults as noted by increased FMD (Harris *et al.*, 2011). In stroke patients, short term vitamin D supplementation improved EC function, noted by increased FMD (Witham *et al.*, 2012). Additionally, a recent meta-analysis found that vitamin D supplementation was associated with improved EC function in a subgroup of patients with diabetes (Hussin *et al.*, 2017). Conversely, vitamin D supplementation had no significant effects on vascular EC function in T2DM patients following 12 weeks of oral supplementation (Yiu *et al.*, 2013). Vitamin D supplementation also had no effect on vascular function in various populations with low levels of vitamin D (Longenecker *et al.*, 2011; Witham *et al.*, 2013). Lastly, recent meta-analyses (Jenkins *et al.*, 2018) and primary end point analyses from the VITamin D and Omega-3 Trial (VITAL) (Manson *et al.*, 2019) indicate no general cardiovascular benefit associated with vitamin D supplementation. Discrepancy between results may be attributed to phenotypic characteristics of study participants (Hussin *et al.*, 2017; Bellan & Marzullo, 2018), outlining the importance of identifying the role of vitamin D in various patient populations to determine which cohort may have the most cardiovascular benefit, if at all, from vitamin D supplementation.

Few studies exist evaluating the effects of vitamin D supplementation on processes of revascularization for the prevention and/or intervention of PVD in humans. Neovascularization is a complex process requiring the coordinated proliferation and migration of EC, and their assembly into new, functional vasculature. Furthermore, the effects of vitamin D on this process appear to be context-dependent. During tumour progression, vitamin D is apparently anti-angiogenic (Saghiri *et al.*, 2017; Jamali *et al.*, 2018); while in the setting of vascular injury, it can promote vessel regeneration, and restoration of blood pressure control (Ni *et al.*, 2014; Wong *et al.*, 2014; Hussin *et al.*, 2017). However, in the setting of obesity and metabolic syndrome, the effects of vitamin D supplementation on peripheral ischemia, a characteristic of PVD, and the vascular regenerative mechanisms involved, are unclear.

1.6 Traditional, Complementary and Alternative Medicine

Several lines of evidence exist to show that people living with chronic conditions, especially those with obesity-related diseases, are often turning to use of Traditional,

Complementary and Alternative Medicines (TCAMs) (Falci *et al.*, 2016), including multivitamins (Yeh *et al.*, 2006; Saydah & Eberhardt, 2006; Bailey *et al.*, 2012). Moreover, studies show that individuals with two or more chronic conditions, a common scenario when considering metabolic disease, are more likely to use TCAM (Sirois, 2008; Jacobson *et al.*, 2009), with multivitamins being the most frequently used (Gahche *et al.*, 2011). Despite the popularity of multivitamins, it is not clear whether this approach is beneficial. Furthermore, the latest update from the United States Preventative Services Task Force (USPSTF) has concluded that, “The current evidence is insufficient to assess the balance of benefits and harms of single or paired nutrient supplements for the prevention of CVD” (Moyer & USPSTF, 2014). Understanding the effects of co-supplementation, such as combining vitamin D and niacin supplementation, for the promotion of vascular health will help to guide future recommendations for nutritional and/or TCAM strategies for the prevention of CVD.

Combining vitamin D supplementation with other vitamins, such as niacin, may offer additive or synergistic effects on endothelial cell function and vascular regeneration as they signal through separate endothelial cell receptors, GPR109A (Hughes-Large *et al.*, 2014) and VDR (Ni *et al.*, 2014), respectively. Both niacin and vitamin D appear to have overlapping pleotropic effects in promoting endothelial cell functions including increased NO signalling, antioxidant capacity and anti-inflammatory signalling. Combining vitamins may offer additive or synergist effects, as previous literature has suggest that combination of nutrients, such that would be found in whole foods offer additional benefit compared to single nutrients and isolated supplements (Liu, 2003; Jacobs *et al.*, 2009). The extent of the combined effects may depend on receptor expression levels as well as the receptor distribution on the different vascular cell types involved in vascular regeneration as well as their downstream signalling pathways and targets. Currently, the effect of combining vitamin D and niacin supplementation on vascular regeneration has not been tested. Moreover, understanding the interactions between these vitamins on this process is particularly relevant to the rise in popularity of TCAMs and widespread use of multivitamin supplements for the promotion of vascular health (Sesso *et al.*, 2012; Bailey *et al.*, 2013; Kantor *et al.*, 2016).

In Canada, a population of interest when considering the use of TCAMs for the management of obesity and associated comorbidities is Canada's Indigenous community. There is a disproportionate burden of obesity-related diseases, including metabolic syndrome, diabetes and CVD, on Canada's Indigenous population with the prevalence of diabetes for on-reserve Indigenous people estimated at 17.2% compared to only 5% for non-Indigenous Canadians (Rice *et al.*, 2016). Current intervention strategies have shown limited success for Indigenous communities as strategies have failed to address culturally relevant interventions and account for Indigenous conceptualization of health and wellness (Rice *et al.*, 2016; Murdoch-Flowers *et al.*, 2017). Multiple research studies have shown that a person's beliefs towards medication and disease differ across cultures and can be influenced by multiple factors including historical factors, such as colonization, that may impact an individual's beliefs and/or attitude towards medication and their acceptance of treatment interventions (Simoni *et al.*, 2012; McQuaid & Landier, 2018). For these reasons, there have been multiple calls to action for culturally relevant intervention and prevention strategies for obesity and T2DM in Canada's Indigenous communities (Smylie *et al.*, 2009; McNamara *et al.*, 2011; Saini & Quinn, 2013).

Indigenous scholars have proposed to include a blending of traditional or culturally relevant healing, such as TCAM therapy, with Western medicine, effectively bridging the gap between Indigenous science and Western science (Massey & Kirk, 2015; Leung, 2016). This highlights the importance of studying the effects of natural, diet-derived compounds, such as niacin and vitamin D on vascular regeneration in the setting of obesity. Further understanding of the effects of these vitamins can help guide TCAM as well as nutritional recommendations for the prevention and/or intervention of PVD in obese and diabetic Indigenous people failing to respond or adhere to Westernized treatments.

1.7 Objectives and Hypothesis

Rationale

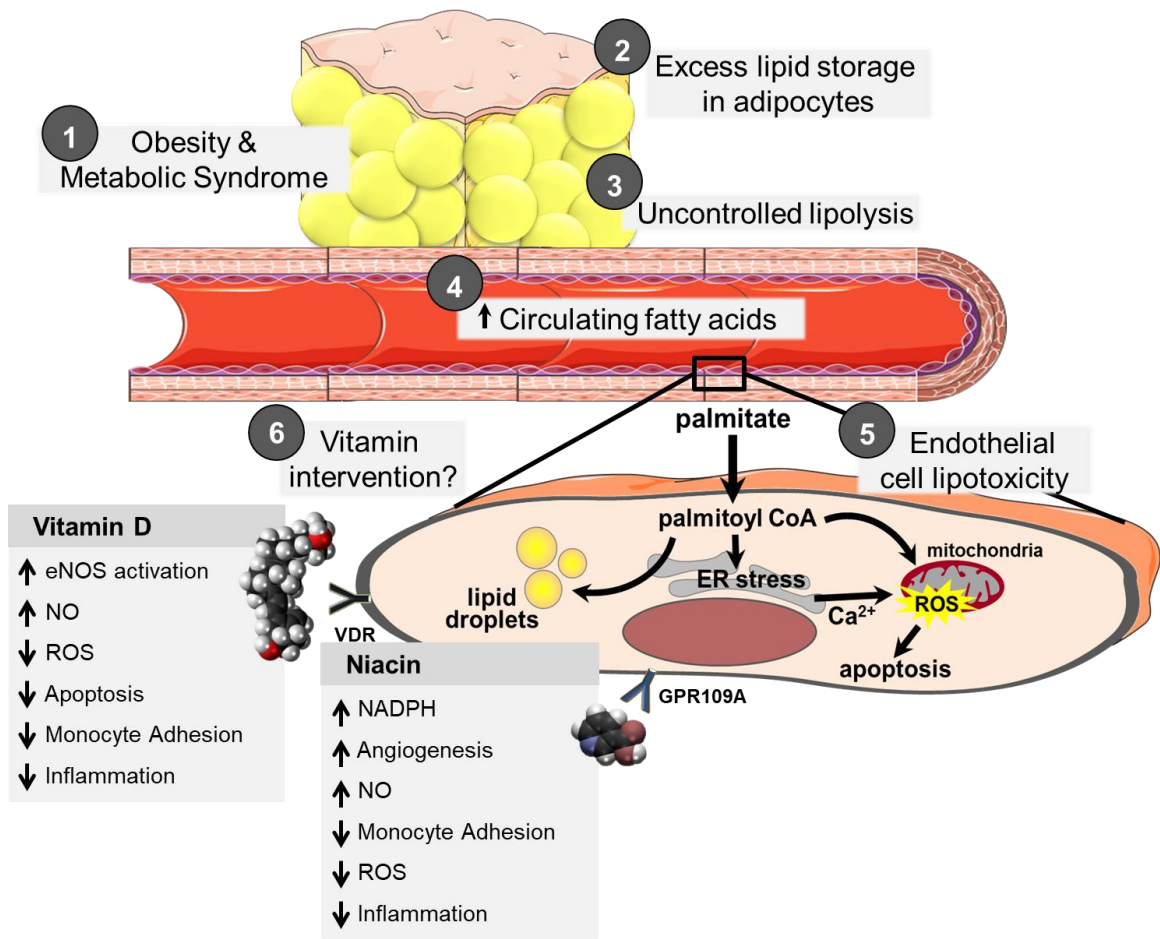
PVD is a common downstream complication associated with obesity and metabolic syndrome (Garg *et al.*, 2014). Current therapies for PVD are limited and fail to target endogenous revascularization of the ischemic tissue (Farber & Eberhardt, 2016).

Furthermore, there is a disproportionate burden of obesity-related diseases, including metabolic syndrome, CVD, and PVD on Canada's Indigenous population, with current intervention strategies showing limited success in this patient population (Rice *et al.*, 2016; Murdoch-Flowers *et al.*, 2017). Thus, studies are needed to identify compounds, including culturally relevant TCAMs such as natural diet-derived compounds, that support the formation of new blood vessels in ischemic tissues.

Endothelial dysfunction has been identified as a surrogate marker for the development of PVD (Campia *et al.*, 2012; Kim *et al.*, 2012) and highlights an important target for therapeutic intervention/prevention strategies. Recent studies have shown an association between low levels of vitamin D and increased risk for CVD (Forman *et al.*, 2007; Wang *et al.*, 2008b; Afshari *et al.*, 2015; Roy *et al.*, 2015), raising the intriguing notion that vitamin D supplementation may be beneficial for the promotion of vascular health. However, the effects of vitamin D on EC function and vascular regeneration have stirred much controversy and appear to be context-dependent. Whether vitamin D supplementation can improve EC function and promote vascular regeneration under lipotoxic conditions observed during obesity and metabolic syndrome is unclear. Furthermore, the effects of combining vitamin D with other TCAMs or vitamins, such as niacin, on endothelial function under lipotoxic conditions are unknown. Figure 1.2 summarizes the pathogenesis of endothelial dysfunction during obesity and metabolic syndrome and identifies potential mechanisms by which vitamin intervention may promote EC function.

Figure 1.2. Summary of endothelial dysfunction and potential therapeutic intervention strategies to protect endothelial function during obesity and metabolic syndrome.

During obesity and metabolic syndrome (1) there is accumulation of lipids in adipocytes (2). Once adipocyte capacity is exceeded uncontrolled, dysregulated lipolysis occurs (3), releasing excess fatty acids into circulation (4). Endothelial cells are continuously exposed to high levels of circulating lipids as they comprise the inner most layer of vasculature. Endothelial cells lack the metabolic programming to process excess fatty acids. Excess palmitate, an abundant dietary saturated fatty acid, can overwhelm the ability of a cell to store fatty acids in lipid droplets or to catabolize them through β -oxidation in mitochondria. This can result in production of reactive oxygen species (ROS) and ER stress, resulting in endothelial dysfunction and eventual death, through a process termed lipotoxicity. Palmitate can also readily be incorporated into the ER lipid membrane, resulting in impaired membrane structure and integrity. Oxidative stress and ER stress result in release of calcium from the ER further contributing to mitochondrial-mediated cell death (5). Niacin and vitamin D may be useful therapeutic compounds against endothelial dysfunction as they are known to promote normal endothelial function (6).



Hypothesis

Supplementation of vitamin D alone and in combination with niacin, will improve EC angiogenic function and promote vascular regeneration under lipotoxic conditions that occur during obesity and metabolic syndrome.

Objectives

The specific objectives of this thesis were to:

- 1) Determine whether vitamin D supplementation alone and in combination with niacin, improves *in vitro* HMVEC angiogenic function in the presence of high palmitate.
- 2) Determine global changes in HMVEC gene expression induced by supplementation of vitamin D or niacin during exposure to high palmitate in order to identify and compare genes and pathways that may underlie any angiogenic responses.
- 3) Determine whether vitamin D supplementation alone and in combination with niacin, improves functional recovery and neovascularization following femoral artery ligation and excision.

Relevance to Disease

Endothelial dysfunction has been identified as a surrogate marker for the development of PVD and highlights an important target for therapeutic intervention/prevention strategies (Nosova *et al.*, 2015; Farber & Eberhardt, 2016). Therefore, it is important to develop better understanding of therapeutic agents that may be useful interventions against endothelial dysfunction and promote EC maintenance under high fat settings. In light of the recent popularity of vitamin D supplementation for cardiovascular health, determining whether vitamin D can promote EC function and improve functional recovery, under a high fat setting, is important in determining future clinical applications of vitamin D. Furthermore, understanding the effects of co-supplementation of vitamin D with niacin is an important step in addressing the rise in popularity of TCAMs and widespread use of multivitamin supplements for the promotion of vascular health. The work described in this thesis will help to better understand the effect of vitamin D alone and in combination with niacin, on the vasculature during lipotoxic conditions and may help guide future therapeutic and nutritional recommendations for obese patients with PVD.

Chapter 2

2 Materials and Methods

2.1 Cell Culture and Treatments

For all cell culture experiments, primary human dermal microvascular endothelial cells (HMVEC) isolated from small vessels within dermal tissue were used as they are a close representation of cells likely to initiate vascular regeneration in the tibialis anterior muscle under low oxygen conditions during peripheral limb ischemia (Shireman, 2007). Cells from a single adult donor were obtained from Lonza (Cat.#CC-2811; Walkerville, MD USA) and maintained in Medium 199 (Life Technologies) supplemented with EGM-2-MV SingleQuots (Lonza). Cells were grown on 100 mm culture dishes, at 37°C and 5% CO₂ and growth medium was changed every 2-3 days. Cells were used between passage 4 and 10 and subcultured at 80% confluence using trypsin-EDTA solution (Lonza).

For high fat (lipotoxic) conditions, cells were exposed to medium supplemented with the saturated fatty acid palmitate at a concentration of 0.5 mM. A stock solution of 20 mM palmitate was prepared by saponification using 0.01 M NaOH and heated to 70°C for 30 minutes. Palmitate stock solutions were complexed to 30% bovine serum albumin (BSA) at a molar ratio of 2:1 prior to the addition of growth medium to reach a final fatty acid concentration of 0.5 mM (Hughes-Large *et al.* 2014). The concentration of palmitate used reflects a high physiological to pathophysiological concentration that would be observed during metabolic disease (Gordon, 1960; Soriguer *et al.*, 2009). Growth medium supplemented with fatty acid free 30% BSA alone was used as control.

To establish dose response, growth medium was supplemented with increasing concentrations of either vitamin D (1,25-dihydroxyvitamin D₃; Sigma Aldrich; 0 - 100 nM) solubilized in dimethyl sulfoxide (DMSO) and stored under N₂, niacin (Sigma Aldrich; 0 - 100 µM) solubilized in cell culture grade water at room temperature, a combination of vitamin D and niacin (0 - 100 nM and 0 - 100 µM, respectively), or vehicle (DMSO). Matrigel assays of endothelial tube formation and stability, described below, were used to evaluate dose response. For all subsequent vitamin treatments,

growth medium was supplemented with 10 μM niacin, and/or 10 nM vitamin D. Concentration ranges selected for niacin and vitamin D treatments are reflective of physiological plasma concentrations that can be achieved following oral administration of high dose niacin (2-3 g), which have been shown to be approximately 10 μM - 240 μM within 8 h of dosing (Menon *et al.*, 2007), or recommended supplemental dose vitamin D (1000 IU), which are 10 - 25nM (Lorvand Amiri *et al.*, 2017). Initially, the effects of vehicle treatments (water and DMSO) under both BSA and palmitate conditions were compared to confirm that there were no effects. For all subsequent experiments a conservative approach was taken and DMSO (2 $\mu\text{L}/\text{mL}$ of media) was used as the vehicle control.

2.2 Tube Formation and Stability Assays

Prior to cell harvesting, a pre-chilled 96-well plate was coated with 40 $\mu\text{L}/\text{well}$ of growth factor-replete Matrigel basement membrane Matrix (BD Biosciences). The plate was incubated for 30 minutes at 37°C before cells were plated to allow for solidification of Matrigel. HMVEC were harvested with EDTA-trypsin, counted using a hemocytometer, resuspended in growth medium and seeded onto matrigel coated plates at a density of 37,500 cells/cm². Cells were treated with corresponding treatments in order to establish a dose-response. Cells were incubated at 37°C and 5% CO₂ to allow endothelial tubes to form. Resulting tube networks were imaged by light microscopy at 18 hours for tube formation, and at 24 and 42 hours for tube stability. A tube was defined as an apparently three-dimensional, elongated structure stretching between branch points, with a width large enough along its entire length to permit the passage of an erythrocyte (Borradaile & Pickering, 2009a). For each condition, EC tube formation and stability were evaluated by measuring the total tube length for the entire well at all three time points using ImageJ software. Dose response was evaluated by comparing total EC tube lengths at 18, 24 and 42 h of incubation between the various concentrations of each treatment. Tube stability was evaluated by areas under the curve assessing total tube length over time from 18 hours to 42 hours, where a larger value indicates less tube degradation over the 24 hours following tube formation and thus improved tube stability.

2.3 HMVEC Cytotoxicity

Cytotoxicity was assessed by MTT [3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide] assays. HMVEC were seeded onto 96-well plates at a density of 31,250 cells/cm² and incubated for 5-6 hours to allow for adhesion to the plate. Cells were incubated with corresponding treatments in triplicate for 16 hours. Ten μ L of MTT (5 mg/mL of PBS) was added to each well and incubated for 3 hours, followed by addition of 100 μ L/well of MTT lysis buffer. Plates were covered in aluminum foil and incubated at room temperature overnight. Absorbance was quantified spectrophotometrically at a wavelength of 595 nm.

2.4 Cell Viability

HMVEC viability was assessed using trypan blue assays. HMVEC were seeded onto 6-well plates at a density of 2,500 cells/cm². Cells were incubated for 16 hours in corresponding treatments. Following incubation, cells were harvested using trypsin/EDTA. Fifty μ L of resuspended cells were mixed with 50 μ L of trypan blue. A hemocytometer was used to count cells. Dead cells were detected by blue staining and viable cells were detected by absence of blue staining. Percent viability was calculated using the equation:

$$\text{Viable cells per mL} / \text{total cells per mL} \times 100$$

2.5 Global Gene Expression

HMVEC were grown to 80-90% confluence in 100 mm dishes and treated for 16 hours with two or three dishes combined per treatment. Cells were harvest by direct lysis using 500 μ L of Buffer RLT added to every dish and lysed using QIAshredders (QIAGEN). An equal volume of 70% ethanol was added to the lysate, mixed by pipetting and transferred to an RNeasy spin column and centrifuged for 15 seconds at 8,000 x g. Flow through was discarded and 700 μ L Buffer RW 1 was added to the spin column and centrifuged for 15 seconds at 8,000 x g. Flow through was discarded and 500 μ L Buffer RPE was added to the spin column and centrifuged for 15 seconds at 8,000 x g. Flow through was discarded and another 500 μ L of Buffer RPE was added to the spin column and centrifuged for 2

minutes at 8,000 x g. The sample was then dried by centrifugation for an additional 1 minute at $\geq 8,000$ x g. RNA was extracted from the spin column by addition of 45 μ L of RNase-free water and centrifuged for 1 minute at 8,000 x g. All samples were stored at -80°C.

RNA concentration and purity were measured by NanoDrop (Thermo Scientific). Samples were diluted to a concentration of 200 ng/ μ L in nuclease-free water. All sample labeling and GeneChip processing were performed at the London Regional Genomics Centre (Robarts Research Institute).

RNA quality was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies) and the RNA 6000 Nano kit (Caliper Life Sciences). Following assessment, end labeled single stranded complementary DNA was prepared from 200 ng of total RNA and hybridized, for 16 hours at 45°C, to Human Gene 2.1 ST arrays, as described in the Affymetrix Technical Analysis Manual.

All liquid handling steps were performed by GeneChip Fluidics Station 450 and GeneChips were scanned with the GeneChip Scanner 3000 7G (Affymetrix) controlled by Command Console v3.2.4. Microarray probe data were imported into Partek Genomics Suite v6.6 using the RMA algorithm (Irizarry *et al.*, 2003).

Partek was used to determine gene level ANOVA p-values and fold changes. Filtered gene lists were generated for expression changes of greater than 2.0-fold and having a p-value of less than 0.05. Lists of genes exhibiting at least a 2.0-fold change in expression with a p-value less than 0.05 were used for gene ontology (GO) and KEGG pathway enrichment analyses. Over-represented transcripts were also imported into the open-source Reactome curated pathway data base (v58) (<http://www.reactome.org/>) for further analyses. Pathway overview schematics, indicating significantly represented ($p < 0.05$) nodes (pathways) and relationships between nodes were generated for each list of differentially expressed genes.

2.6 qRT-PCR

cDNA was synthesized from RNA samples using a High Capacity RNA-to-cDNA kit (Applied Biosystems) or TaqMan Advanced miRNA cDNA Synthesis kit (Applied Biosystems) following the manufacturer's protocol. For cDNA synthesis, RNA samples were mixed with 2X RT Buffer Mix, 20X Enzyme Mix and nuclease-free water in microfuge tubes. Reverse transcription of RNA samples was performed for 60 minutes at 37°C, followed by 5 minutes at 95°C, then cooling of the samples to 4°C. For miRNA cDNA synthesis, RNA samples underwent four different reaction steps. First, a poly(A) tailing reaction was performed. Poly(A) reaction components were mixed in a 1.5 mL microfuge tube. Ten ng of RNA sample was mixed with 3 µL poly(A) reaction mixture. For polyadenylation, samples were incubated at 37°C for 45 minutes, and then the reaction was stopped by incubating at 65°C for 10 minutes and cooled to 4°C. Immediately following, an adaptor ligation reaction was performed. Adaptor ligation components were mixed in a 1.5 mL microfuge tube and 10 µL of ligation reaction mix was added to the microfuge tube containing the poly(A) tailing reaction product. For ligation, samples were incubated at 16°C for 60 minutes and cooled to 4°C. Immediately following, reverse transcription reaction mixture was prepared and 15 µL of the reverse transcription reaction mixture was added to the microfuge tube containing the adaptor ligation reaction product. For reverse transcription, samples were incubated at 42°C for 15 minutes then the reaction was stopped by incubating at 85°C for 5 minutes and cooled to 4°C. Following, a miR-amplification reaction was performed. MiR-amplification reaction components were mixed in a 1.5 mL microfuge tube and 45 µL miR-amplification reaction mixture was mixed with 5 µL of the reverse transcription reaction product in a new microfuge tube. For miR-amplification, the samples were incubated at 95°C for 5 minutes to allow for enzyme activation and then run for 14 cycles with a 95°C denature step for 3 seconds, followed by 30 seconds of annealing at 60°C. The reaction was then stopped by incubating at 99°C for 10 minutes and cooled to 4°C.

Real-time qualitative polymerase chain reaction (qRT-PCR) was performed using reagents from Applied Biosystems. Taqman primers for *GAPDH* control, *CXCL8*, *DDIT3*, *IL1A*, *HMOX1*, *SMOX*, *BMX*, *MANF*, *APOLD1*, *BAMBI*, *CEBPB*, *CDK1*, *MCM5*, *CYP24A1*, pri/pre-miR126, miR126-5p and miR126-3p were obtained from

Applied Biosystems. Both cDNA and miRNA cDNA samples were diluted with nuclease-free water to 25 ng/ μ L. Reaction components were mixed according to the TaqMan Gene Expression Assays and TaqMan Advanced miRNA Assays Reference Cards. Samples (20 μ L) were plated in triplicate into a 384-well reaction plate. Plates were sealed, briefly centrifuged and loaded into the ViiA 7 qRT-PCR system (Applied Biosystems). The plate was run for 40 cycles with a 15 second denature at 95°C followed by a 1 minute anneal/extend step at 60°C. Cycle threshold (Ct) values were used to calculate $\Delta\Delta C_t$ values and expression fold changes ($2^{-\Delta\Delta C_t}$) for each sample. *GAPDH* was used as an endogenous control for all reactions.

2.7 Growth Rate

HMVEC were seeded onto 6-well plates at a density of 2,500 cells/cm². Cells were incubated with corresponding treatments for 16 hours, harvested using trypsin/EDTA and counted using a hemocytometer. Population doublings per day were calculated for each treatment using the equation:

$$\log_{10}(\text{number of cells harvested}) - \log_{10}(\text{number of cells seeded}) \times (\log_{10}2)^{-1}$$

2.8 Mouse Model

In order to induce obesity and characteristics of metabolic syndrome, 5 week old male 129S6/SvEv mice were fed a western diet containing 42% calories from fat, 15.2% calories from protein and 42.7% calories from carbohydrates (Envigo, Toronto, ON) *ad libitum* for a total of 17 weeks. Figure 2.1 outlines the mouse experimental procedure.

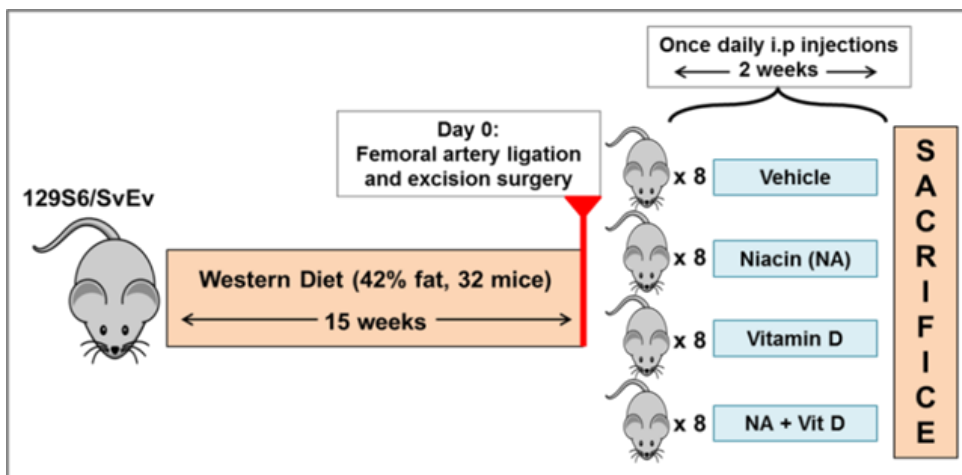


Figure 2.1. Outline of mouse experimental model and treatment.

After 15 weeks of feeding, mice underwent right hind limb femoral and saphenous artery ligation and excision surgery. Mice were anesthetized using a combination of intraperitoneal (i.p.) ketamine (150 mg/kg) and xylazine (5 mg/kg). A small incision was made in the right hind limb to expose the femoral artery. The proximal end of the femoral artery and the distal end of the saphenous artery were ligated using silk sutures. Mice were administered one i.p. dose of meloxicam (1 mg/kg), followed by a second dose 24 hours post-surgery. Following surgery, mice were randomized to one of four intervention treatments with vehicle, niacin (50 mg/kg), vitamin D (200 ng/kg), or niacin and vitamin D (50 mg/kg and 200 ng/kg, respectively) and received once daily i.p. injections for 2 weeks (14 days) following surgery. To ensure complete solubilization of each treatment, the vehicle for niacin was sterile water (Pang *et al.*, 2016), and the vehicle for vitamin D was sterile water:propylene glycol:ethanol (5:4:1, volumetric ratio) (Wong *et al.*, 2014). Each mouse received sequential, individual injections of each solution on opposing sides of the peritoneal cavity. The dose of niacin approximates a human dose of 250 mg (Reagan-Shaw *et al.*, 2008). The dose of vitamin D corresponds to the concentration found to improve HMVEC tube formation and stability (10 nM) from cell culture experiments performed in this study, and reflects increases in plasma concentrations achieved following oral administration of vitamin D (1000 IU) in obese humans with metabolic syndrome and fatty liver (Lorvand Amiri *et al.*, 2017). Two experiments consisting of 16 mice each were staggered by one month to accommodate surgeries on all mice. A total of eight mice were assigned to each treatment group.

Blood pressure and heart rate were taken pre- and post-surgery and measured with tail-cuff plethysmography (CODA, Kent Scientific Corp, Torrington, CT, USA).

Functional recovery of the hind limb was assessed on days 3, 9 and 15 post-surgery by gait analysis using a Catwalk system (Noldus) at the Neurobehavioural Core Facility at Robarts Research Institute. Mice were recorded as they traversed a glass walkway and the duration of contact for each paw was reordered to generate digital gait maps. Mean paw contact times were used to calculate hind limb use ratios of injured (right) to uninjured (left) limbs. A ratio of <1.0 indicates decreased use of the injured limb. Complete recovery of limb use is indicated by a hind limb use ratio of 1.0. Areas under the curve were calculated to assess functional recovery over the two-week treatment period.

On day 15 post-surgery, mice were sacrificed and blood, liver, pancreas, heart, and adipose tissues were harvested and weighed. The tibialis anterior, gastrocnemius, and adductor muscles were also isolated from each mouse. Plasma TG and cholesterol were determined by enzymatic, colorimetric assays (Roche Diagnostics). Blood glucose was determined using an Ascensia Elite glucometer (Bayer). Plasma insulin was measured using mouse ultrasensitive insulin enzyme-linked immunosorbent assay (ELISA, Alpco Diagnostic). Plasma liver enzymes (ALT and AST) were measured by the London Health Sciences Centre Core Laboratory. Liver cholesterol and triglycerides were determined by enzymatic, colorimetric assays (Roche Diagnostics/Wako Diagnostics). All plasma and tissue metabolic parameters, with the exception of AST/ALT measurements, were performed through the Metabolic Phenotyping Laboratory in Robarts Research Institute at Western University. Modified from Pang *et al.* (2016). Metabolic data was compared to previous observations of age-matched lean control 129S6 mice, fed a standard chow diet (Pang *et al.*, 2016).

2.9 Histology

At sacrifice, tibialis anterior, gastrocnemius, and adductor muscles were isolated and immersed in zinc fixation buffer (0.1 mol/L Tris-HCl buffer (pH 7.4) containing 0.5 g/L calcium acetate, 5.0 g/L zinc acetate, 5.0 g/L zinc chloride) followed by paraffin embedding. A microtome was used to cut 5 μm serial cross sections at three equally spaced locations spanning the length of the tibialis anterior muscle. Four images per section were captured for a total of twelve high powered fields of view per muscle (one muscle per mouse) using an Olympus BX51 light microscope. All tissue processing, cutting, and staining was performed at the Molecular Pathology Core Facility (Robarts Research Institute).

For determination of tibialis anterior muscle architecture, sections were stained with hematoxylin and eosin. Total myofiber and adipose tissue areas per section were calculated using a customized, automated ImageJ software protocol. Sections were manually analyzed, using ImageJ, to calculate areas of regenerating myofibers, identified by intense eosin staining and central nuclei, non-regenerating myofibers, identified by intense eosin staining and the presence of peripheral nuclei, and necrotic myofibers,

identified by pale eosin staining and absent nuclei (Pang *et al.*, 2016). Ratios of regenerating to non-regenerating myofibers per mouse were calculated. Adipose areas were normalized to total section areas and reported as percentages.

To determine vessel densities, sections were double immunostained for CD31 (BD Biosciences) and smooth muscle α -actin (α SMA) (Sigma Aldrich), sourced from rats and mouse clones 1A4, respectively. Hematoxylin was used as a counter stain (van der Veer *et al.*, 2005; Frontini *et al.*, 2011). Capillary and arteriole densities were determined by manually counting CD31⁺ vessels and α SMA⁺. Arteriole diameter frequencies were determined by automated analyses using a combination of ImageJ and MATLAB software that measured the diameter of every α SMA⁺ vessel across all images for each treatment group. Frequency distribution plots of vessel diameters for each treatment group were generated using GraphPad Prism 7.

Tibialis anterior muscle inflammation was determined by immunostaining for the pan-macrophage marker, F4/80 (Invitrogen, Thermo Fisher Scientific). Tibialis anterior muscle fibrosis was determined by Masson's Trichrome staining. Total macrophage and collagen areas were quantified using a customized, automated ImageJ protocol. Inflammation (F4/80⁺) and fibrosis (collagen) areas were normalized to total section areas and reported as percentages. A fibrosis location score was determined for each mouse, to provide relative quantification of myofiber-associated collagen. Each of the four images per section was assigned a value of 1 if myofiber-associated collagen was present, so that the maximum value per section would equal 4. Then the average value across sections for each mouse was calculated and multiplied by the percent collagen for that mouse. Thus, a higher score indicates greater presence of myofiber-associated collagen.

2.10 Statistics

All statistical analyses and generation of graphs were performed using GraphPad Prism 7 (GraphPad Software, La Jolla, CA, USA), except for microarray analyses. Data in Figure 3.1B,C were assessed by t-tests comparing PA vs. BSA within each treatment group. All remaining data were assessed using one-way ANOVA, followed by Tukey post-hoc tests. Differences in means were considered statistically significant at $p < 0.05$.

Chapter 3

3 Results

3.1 Niacin and vitamin D improve HMVEC tube formation and stability in high palmitate

Recently, vitamin D supplementation has gained much attention for the promotion of vascular health. However, effects of vitamin D on endothelial angiogenic processes appear to be disease-context dependent. During tumour progression, vitamin D is apparently anti-angiogenic (Saghiri *et al.*, 2017; Jamali *et al.*, 2018); while in the setting of vascular injury, several studies have demonstrated a beneficial effect of vitamin D supplementation on vascular endothelial cell functions (Hussin *et al.*, 2017; Wong *et al.*, 2014; Ni *et al.*, 2014). Combined supplementation with other vitamins, such as niacin, could also have additive or synergistic effects. Thus, we determined whether vitamin D supplementation in the absence or presence of niacin would improve HMVEC angiogenic processes under high palmitate conditions that would be observed during obesity and metabolic syndrome.

We previously found that supplementation of culture medium with low-dose niacin (10 μ M) preserves HMVEC tube formation during high palmitate under both normoxic (Hughes-Large *et al.* 2014) and hypoxic conditions (Pang *et al.*, 2016). We reproduced this earlier observation (Figure 3.1A) in order to compare the effects of vitamin D alone, and in combination with niacin, on tube formation during lipotoxic conditions.

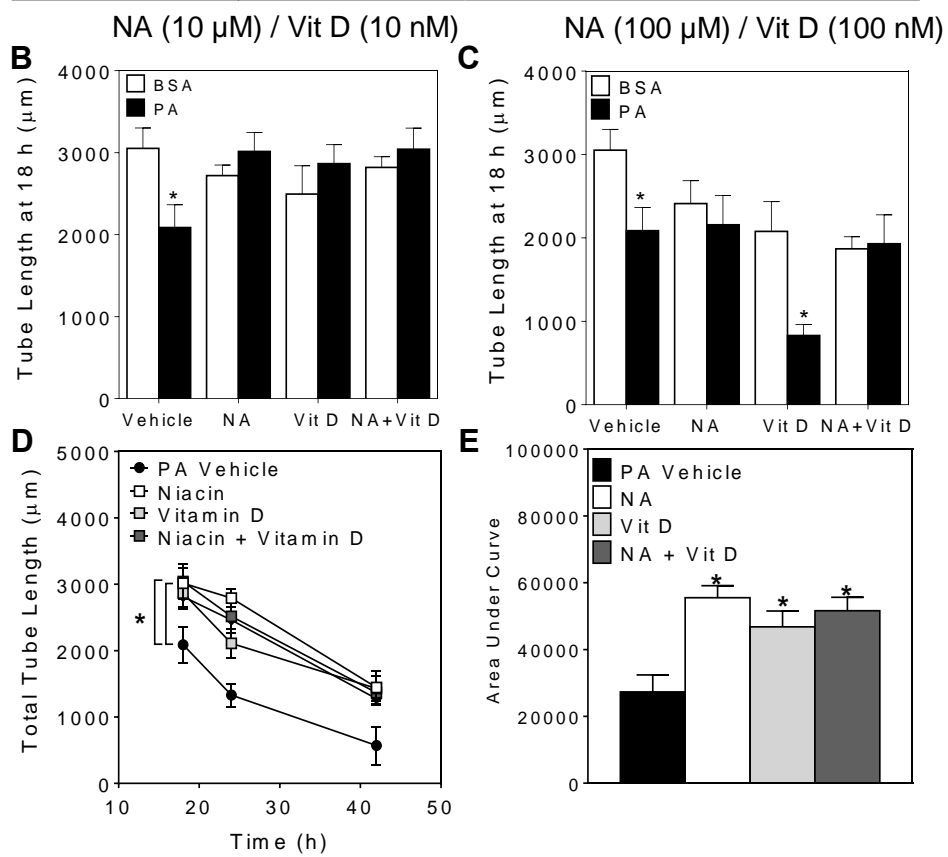
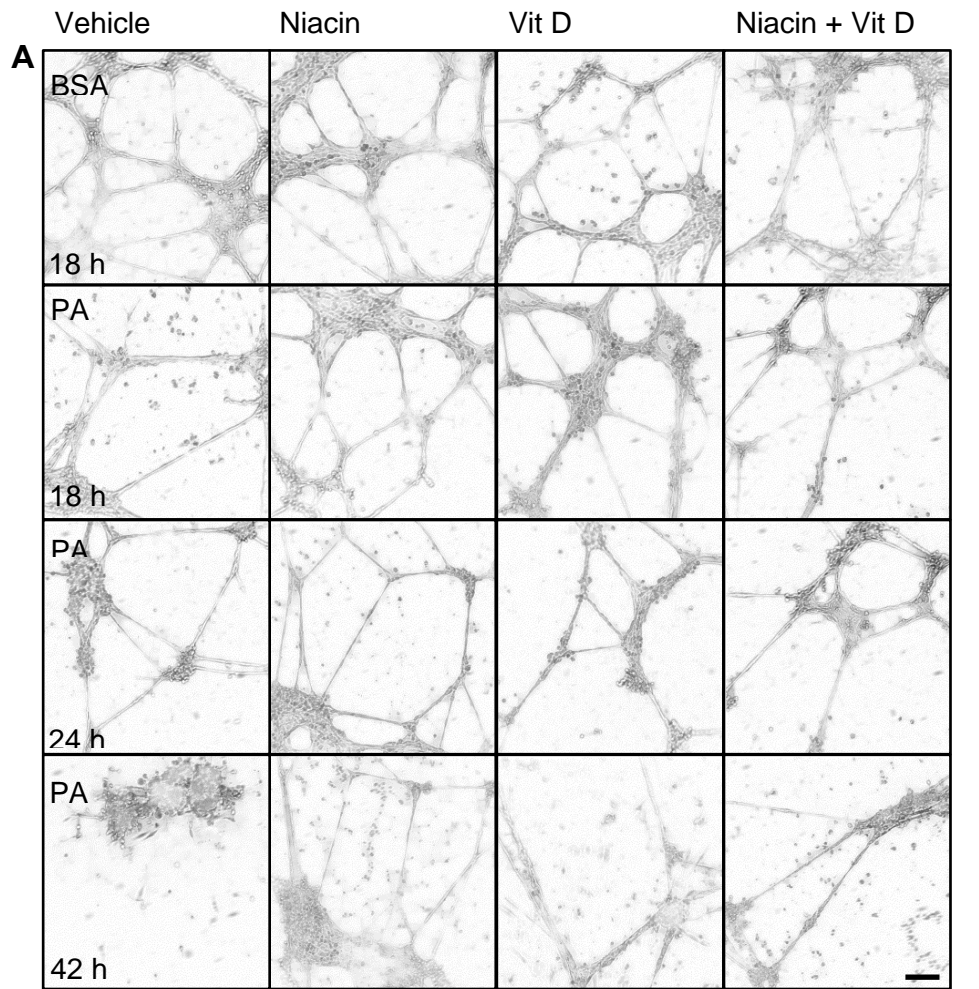
HMVEC tube formation on Matrigel was impaired after 18 hours of incubation in growth medium supplemented with the saturated fatty acid palmitate (Figure 3.1A-D). Media supplemented with low doses of niacin (10 μ M) alone, vitamin D (10 nM) alone, and the combination of niacin (10 μ M) and vitamin D (10 nM) significantly increased HMVEC tube formation at 18 hours of incubation in high palmitate (Figure 3.1A, B, D). It was found that vitamin D at a concentration of 10 nM and niacin at a concentration of 10 μ M were optimal concentrations for tube formation assays as higher concentrations offered no further benefit, and lower concentration were not as effective. In fact, Vitamin D at a

concentration of 100 nM was found to significantly decrease HMVEC tube formation at 18 hours of incubation in high palmitate (Figure 3.1C), suggesting this concentration of vitamin D was becoming toxic to cells under lipotoxic conditions.

As expected, HMVEC tube stability was impaired when incubated in growth medium supplemented with palmitate over the 24 hours following optimal tube formation (Supplemental Figure 1). Supplementation with low dose niacin (10 μ M), vitamin D (10 nM), and the combination of niacin (10 μ M) and vitamin D (10 nM) significantly improved tube stability in high palmitate over the 24 h following tube formation (Figure 3.1E). No significant differences were observed between niacin, vitamin D, and combination treatments on HMVEC tube formation or stability.

Figure 3.1. Niacin and vitamin D improve HMVEC tube formation and stability in high palmitate.

(A) HMVEC were seeded onto growth factor replete Matrigel and incubated with media containing BSA (top row only) or 0.5 mM palmitate (PA) complexed to BSA (2:1 molar ratio). Resulting tube networks were visualized by light microscopy after 18, 24 and 42 h. Scale bar represents 100 μm . Cells were treated with vehicle (DMSO), niacin (NA), vitamin D (Vit D), or a combination of niacin plus vitamin D (NA + Vit D). For quantification, the entire well was assessed for total tube length using ImageJ software at each time point. (B) Cells were incubated with media containing BSA or PA media and treated with vehicle (DMSO), 10 μM niacin (NA), 10 nM Vitamin D (Vit D), a combination of 10 μM NA plus 10 nM Vit D for 18 hours. (C) Cells were incubated with media containing BSA or PA media and treated with vehicle (DMSO), 100 μM NA, 100 nM Vit D and a combination of 100 μM NA plus 100 nM Vit D for 18 hours. (D) Cells incubated with 0.5 mM PA and treated with vehicle (DMSO), 10 μM niacin (NA), 10 nM Vitamin D (Vit D), a combination of 10 μM NA plus 10 nM Vit D were assessed for total tube length at 18, 24 and 42 h. (E) Areas under the curve were calculated from data in (D). Data are means \pm SEM for n=4. (B, C) * p<0.05 versus BSA. (D, E) * p<0.05 versus vehicle.



3.2 Niacin and vitamin D have distinct effects on HMVEC gene expression in high palmitate

We took an unbiased approach of designing an Affymetrix GeneChip experiment to identify and compare genes and pathways that may underlie the improved endothelial cell formation observed in Matrigel assays. Using mRNA microarray analyses it was first confirmed that addition of palmitate to culture medium was representative of a lipotoxic environment by comparing the differential expression of genes between HMVEC treated with palmitate (0.5 mM) and BSA control following 16 hours of incubation. Unsurprisingly, palmitate induced expression of many genes involved in cell stress response, including ER stress (*DDIT3*, *CXCL8*), oxidative stress (*HMOX1*) and inflammation (*IL1A*), which is consistent with the known cellular responses to lipotoxic stress. Also consistent with lipotoxic conditions, the top 10 gene ontology (GO) categories and KEGG pathways for genes differentially expressed in response to high palmitate were related to cellular responses to stress (Table 3.1). Furthermore, treatment with palmitate did not alter the expression of niacin or vitamin D receptors, GPR109A and VDR, respectively.

To compare gene expression changes that were common or specific to treatments, gene lists were created for niacin plus palmitate and vitamin D plus palmitate both compared to BSA control and summarized using Venny 2.1 (Oliveros, 2018) (Figure 3.2). Differentially expressed genes of interest specific to niacin plus palmitate treatment included increased expression of *SMOX*, a suggested antioxidant, decreased expression of *BMX*, an inflammatory marker, and decreased expression of *MIR126*. Genes differentially expressed specific to vitamin D plus palmitate treatment displayed increased expression of proposed modulators of inflammation (*CEBPB* and *BAMBI*). Interestingly, however, changes specific to vitamin D plus palmitate were predominantly related to cell cycle regulation, including decreased expression of *CDK1* and *MCM5*. GO annotation and KEGG pathway analyses further confirmed the distinct effects of vitamin D on cell cycle-related genes and DNA repair signalling pathways (Table 3.2). Reactome pathway analyses were also consistent with pathway analyses from GO and KEGG. Reactome pathway analyses of differentially expressed genes in response to vitamin D

treatment identified robust overrepresentation of genes involved in pathways of cell cycle regulation, DNA replication, DNA repair, and signal transduction (Supplemental Figure 3).

To ensure that any observed changes in gene expression would not be due to the onset of cell death alone, the effects of vitamin D, niacin and palmitate on HMVEC cytotoxicity and viability were assessed before proceeding to RNA isolation. MTT assays showed that treatment of cells with BSA, palmitate, vitamin D or niacin or did not result in cellular cytotoxicity after 16 hours of incubation (Figure 3.3A). Percent viability calculated from trypan blue assays was not significantly different between treatment groups, with all treatments having viability greater than 80% (Figure 3.3B). These results suggest that vitamin supplementation or exposure to palmitate did not significantly affect HMVEC cytotoxicity or viability at 16 hours (Figure 3.3A,B). To further investigate results from the microarray bioinformatics suggesting that vitamin D was down regulating cell cycle gene expression, we performed a growth rate assay to determine the effect of niacin and vitamin D supplementation on HMVEC proliferation. Similar to our previous observations (Hughes-Large *et al.*, 2014; Pang *et al.*, 2016), niacin (10 μ M) did not have an effect on HMVEC proliferation compared to control (Figure 3.3C). However, supplementation with vitamin D (10 nM) plus palmitate resulted in a significant decrease in population doublings compared to BSA control (Figure 3.3C). These results strongly suggest vitamin D supplementation is downregulating EC cycle under lipotoxic conditions.

Transcript changes specific to niacin plus palmitate treatment revealed a highly significant and consistent 2-fold down regulation of *MIR126* (Figure 3.2). This gene may be of interest to the effect of niacin on HMVEC tube formation and stability during lipotoxicity as it is one of the most abundant miRs in EC and is known to play a role in the regulation of EC angiogenesis both under basal conditions and in response to stress (Boon & Dimmeler, 2014). However, the microarray gene chip (Human Gene 2.1 ST) used for this study was not specific for pri/pre-miR-126 versus mature miR-126. Furthermore, pre-miR-126 gives rise to two mature strands, miR-126-3p and miR-126-5p, which have been suggested to regulate EC angiogenesis under basal conditions and in response to stress, respectively (Boon & Dimmeler, 2014). Therefore, we examined if

there was a difference in expression of the three different forms of *MIR126* between treatment groups following 8, 16 and 24 hours of incubation by qRT-PCR (Figure 3.4A-C). In all three conditions, expression of pri/pre-miR-126 gradually decreased from 8 to 24 hours, which is consistent with miR processing. Treatment with palmitate (0.5 mM) induced transient increases in expression of both miR-126-3p and miR-126-5p at 16 hours, which returned to baseline expression by 24 hours (Figure 3.4A). In contrast, niacin treatment resulted in sustained 4- to 6-fold increases in both miR-126-3p and miR-126-5p from 16 to 24 hours (Figure 3.4B). In contrast, vitamin D treatment resulted in highly variable expression of miR-126-3p and miR-126-5p over time (Figure 3.4C).

Table 3.1. Top 10 GO categories and KEGG pathways for genes differentially expressed in response to high palmitate.

GO Category	KEGG Pathway
Response to stress	TNF signaling pathway
Response to chemical	IL-17 signaling pathway
Response to organic substance	Cytokine-cytokine receptor inter.
Cellular response to stimulus	Pathways in cancer
Response to stimulus	NOD-like receptor signal. pathway
Cellular response to stress	NF-kappa B signaling pathway
Response to O ₂ compound	AGE-RAGE signal. (Diabetes)
Response to ER stress	Protein processing in ER
ER unfolded protein response	Ala, asp, and glut metabolism
Cell response to chemical	ABC transporters

Cells were incubated for 16 hours in growth medium supplemented with BSA or 0.5 mM palmitate (n=3). RNA was extracted and Affymetrix GeneChip microarray analyses were performed. Gene ontology (GO) categories and KEGG pathways were ranked according to enrichment p-values from the lists of differentially expressed genes (both up- and down-regulated) at > 2-fold ($p < 0.05$).

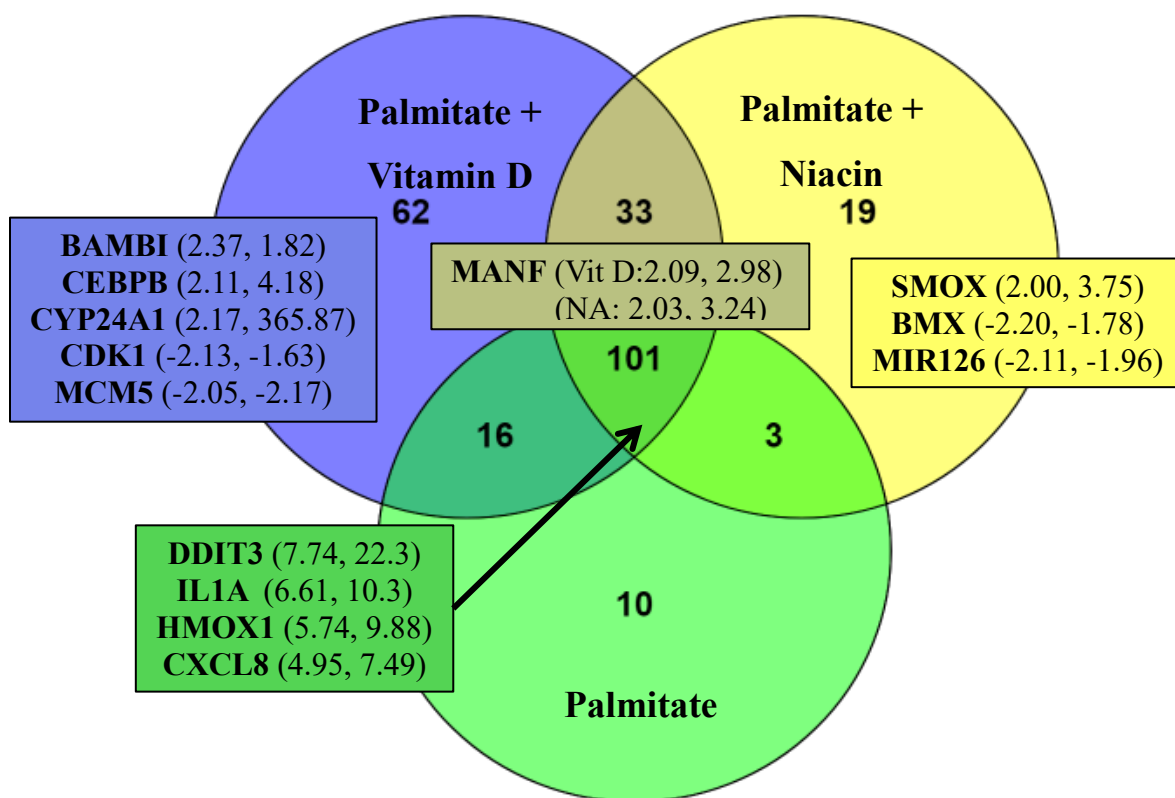


Figure 3.2. Comparison of gene expression changes in response to vitamin supplementation in the presence of high palmitate.

Cells were incubated for 16 hours in growth medium supplemented with BSA, 0.5 mM palmitate (PA), 10 μ M niacin, or 10 nM vitamin D (n=3). RNA was extracted and Affytrix GeneChip microarray analyses were performed followed by generation of lists of genes differentially expressed at >2-fold, $p < 0.05$, using Partek. Gene lists were summarized and compared to identify changes shared between treatments using Venny 2.1. Numbers within each Venn diagram compartment represent the number of genes specific to or shared between indicated treatment(s). Selected gene changes were confirmed by qRT-PCR as indicated. First number in parentheses indicated relative change in gene expression determined by microarray analysis, and the second number indicates relative change determined by qRT-PCR.

Table 3.2. GO categories and KEGG pathways corresponding to changes in gene expression highlighted in Figure 3.2.

GO Category	KEGG Pathway
Common to Palmitate, Palmitate + Niacin, and Palmitate + Vitamin D (101)	
Response to stress	TNF signaling pathway
Response to chemical	IL-17 signaling pathway
Response to organic substance	Cytokine-cytokine receptor interaction
Cellular response to stimulus	Pathways in Cancer
Response to stimulus	NOD-like receptor signaling pathway
Cellular response to stress	NK-kappa B signaling pathway
Response to O ₂ compound	AGE-RAGE signaling
Response to ER stress	Protein processing in ER
ER unfolded protein response	Ala, asp, and glut metabolism
Cell response to chemical	ABC transporters
Specific to Palmitate + Vitamin D (62)	
DNA metabolic process	Cell cycle
DNA replication	DNA replication
Chromosomal part	Homologous recombination
Cell cycle process	
DNA repair	
Chromosome organization	
DNA replication initiation	
Regulation of cell cycle	
Specific to Palmitate + Niacin and Palmitate + Vitamin D (33)	
Cell response to DNA damage	

Cells were incubated for 16 hours in growth medium supplemented with 0.5 mM palmitate plus vehicle, 10 μ M niacin, or 10 nM vitamin D. Gene ontology (GO) categories and KEGG pathways were ranked according to enrichment p-values from the comparative lists of differentially expressed genes (up- and down-regulated) at >2-fold, $p < 0.05$. Numbers in brackets indicate the number of differentially expressed genes identified through Venn analyses as shared between treatment groups.

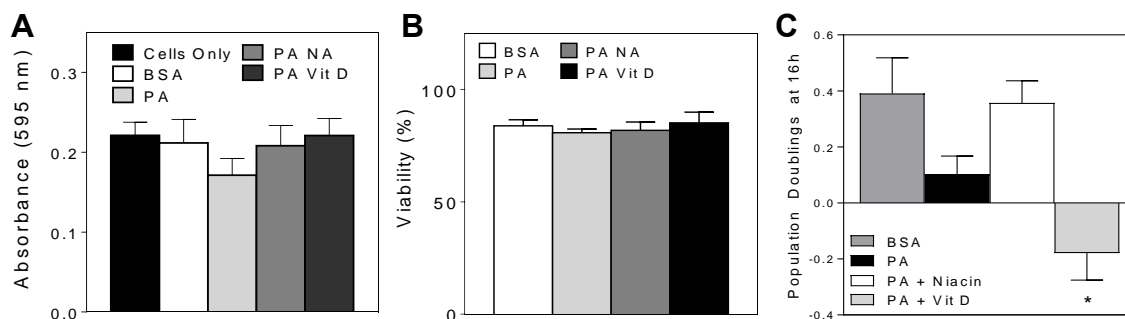


Figure 3.3. Vitamin D limits HMVEC population doubling without affecting cell viability in high palmitate.

(A,B) Cells were incubated in media containing media containing BSA or 0.5 mM palmitic acid (PA) and treated with vehicle (DMSO), 10 μ M niacin (NA), or 10 nM vitamin D (Vit D) for 16 h. (A) Absorbance was quantified spectrophotometrically at a wavelength of 595 nm from standard MTT assays. (B) HMVEC viability was calculated from trypan blue assays. (C) Cells were plated at known, sub-confluent density and incubated in medium containing BSA or 0.5 mM palmitate (PA) with vehicle (DMSO), 10 μ M niacin, or 10 nM vitamin D (Vit D) for 16 hours. Cells were counted, and population doublings calculated. Data are means \pm SEM for n=5, * p<0.05.

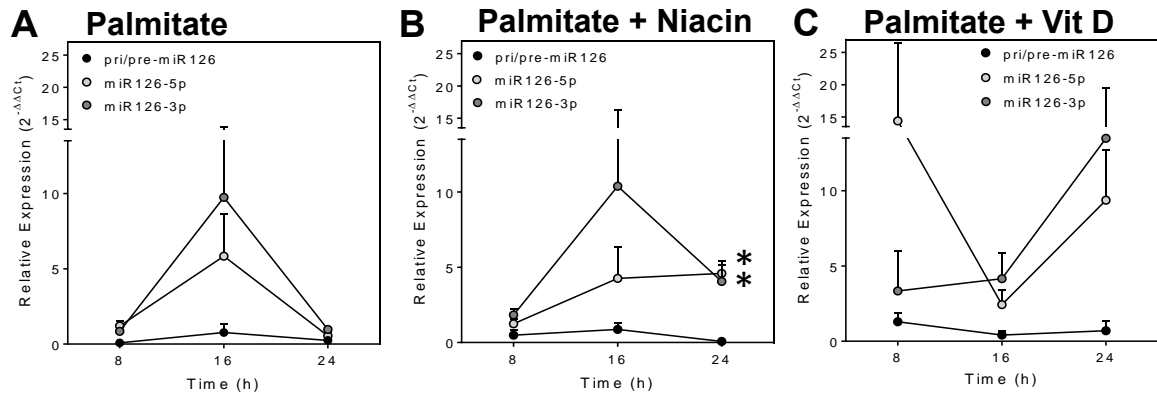


Figure 3.4. Niacin induces stable expression of miR126-5p and -3p in HMVEC exposed to high palmitate.

(A-C) Cells were incubated for 8, 16, or 24 hours in medium supplemented with 0.5 mM palmitate and treat with vehicle (A), 10 μ M niacin (B), or 10 nM Vit D (C). qRT-PCR was performed using primers for pri/pre-miR-126, miR-126-5p and miR-126-3p. Data are presented as fold changes normalized to baseline expression at 0 hours. Data are means \pm SEM for n=4, * p<0.05 for 24 hours compared to 8 hours.

3.3 Niacin, but not vitamin D, improves recovery of hind limb function following acute ischemic injury in obese mice with metabolic syndrome

Lipotoxicity-induced EC dysfunction limits vascular repair and regeneration *in vivo* (Kim *et al.*, 2012). In order to determine if the beneficial effects of niacin and vitamin D on angiogenic processes (Figure 3.1) could be translated to an *in vivo* model of obesity, hyperlipidemia, and PVD, we used western diet-fed 129S6 mice with surgically induced hind limb ischemia. Western diet feeding for a total of 17 weeks *ad libitum* induced characteristics of metabolic syndrome in all mice as indicated by increased adiposity, hyperlipidemia, and hepatic steatosis (Table 3.3) compared to observation of age-matched lean control 129S6 mice fed a standard show diet (epididymal fat: 0.37 ± 0.04 g; plasma cholesterol: 2.85 ± 0.12 mmol/L; liver triglycerides: 13.3 ± 2.0 mg/g) (Pang *et al.*, 2016). However, the extent of metabolic impairment induced by Western diet feeding in this thesis was not as dramatic as in our previous work, as indicated by near-normal fasting blood glucose concentrations in all groups (Table 3.3). Interestingly, both vitamin D treatment groups had significantly lower levels of liver cholesterol mirrored by increased levels of plasma TG and cholesterol, suggesting that vitamin D supplementation resulted in metabolic disturbances with impaired lipid homeostasis (Table 3.3). In order to determine if treatment with either vitamin had an effect on parameters of cardiovascular health, heart rate or blood pressure were measured pre- and post-surgery. Mean systolic blood pressure pre-surgery was 126 ± 3.8 mmHg with mean diastolic pressure of 84 ± 3.5 mmHg (Supplemental Figure 4A). Mean heart rate pre-surgery was 716.5 bpm (Supplemental Figure 4B). Baseline systolic blood pressure of 129S6 mice has previously been measured at 104 ± 3 mmHg using a tail-cuff computerized system (Salzler *et al.*, 2007). There was no statistical difference in blood pressure or heart rate between treatment groups. Furthermore no statistical significance was found between pre- and post-surgery (Supplemental Figure 4C).

On day 15 post-surgery, niacin-, but not vitamin D- or combination-treated mice had significant increases in mean hind limb contact time ratios (Figure 3.4A, B). Furthermore, over the two week treatment period only niacin-treated mice had a significant increase in

mean hind limb contact time ratios compared to vehicle-treated mice (Figure 3.4C). Similar to our previous observations in obese mice with hind limb ischemic injury (Pang *et al.*, 2016), there were no significant differences found in mean hind limb contact intensity ratios between treatment groups (Figure 3.4D, E).

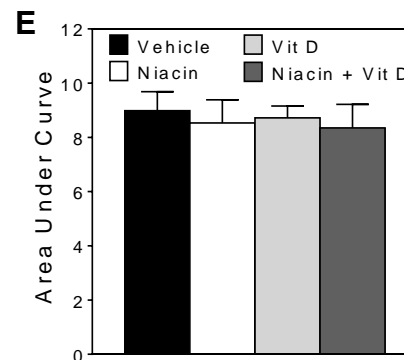
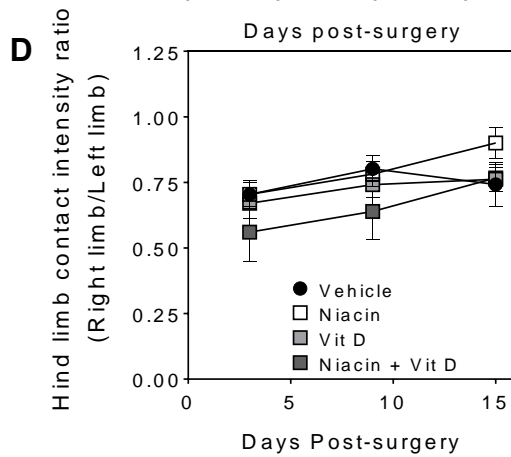
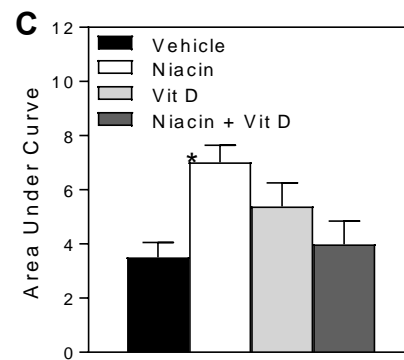
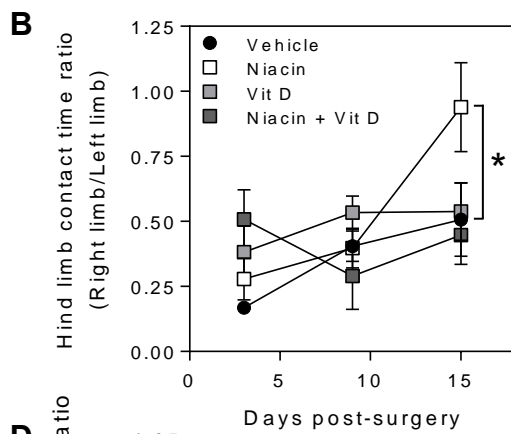
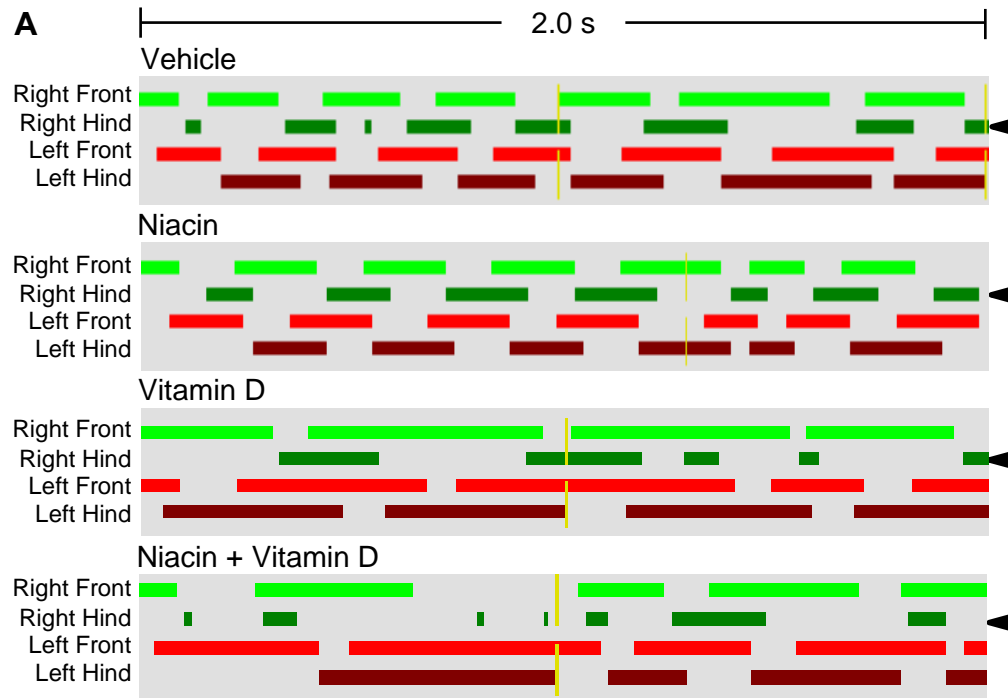
Table 3.3. Metabolic parameters of 129S6/SvEv mice.

Treatment	Vehicle	Niacin	Vitamin D	Niacin + Vitamin D
Initial body weight (g)	20.26 ± 1.04	21.04 ± 0.41	21.04 ± 0.56	20.40 ± 0.41
Body weight at week 15 (g)	33.16 ± 2.24	34.60 ± 1.25	34.50 ± 1.65	33.88 ± 0.86
Body weight at sacrifice (g)	28.95 ± 1.90	29.5 ± 0.74	27.96 ± 1.05	27.21 ± 1.61
Food Intake (kcal/day)	9.02 ± 0.50	9.26 ± 0.45	8.58 ± 0.49	7.93 ± 1.01
Epididymal fat weight (g)	1.36 ± 0.19	1.18 ± 0.06	1.18 ± 0.13	1.10 ± 0.13
Liver weight (g)	1.04 ± 0.07	1.02 ± 0.05	1.01 ± 0.06	1.02 ± 0.05
Liver triglycerides (mg/g)	73.34 ± 9.24	69.04 ± 5.58	50.79 ± 9.32	33.84 ± 7.00*
Liver cholesterol ester (mg/g)	8.11 ± 0.76	8.30 ± 1.12	4.56 ± 1.12*	2.47 ± 0.32*
Liver free cholesterol (mg/g)	3.00 ± 0.09	2.88 ± 0.04	2.83 ± 0.05	2.76 ± 0.08
Plasma ALT (U/L)	20.14 ± 2.13	20.00 ± 2.13	21.83 ± 2.92	30.33 ± 10.99
Plasma AST (U/L)	92.86 ± 25.61	80.83 ± 11.33	73.50 ± 15.88	130.5 ± 18.58
Plasma triglycerides (mmol/L)	0.63 ± 0.04	0.66 ± 0.03	0.95 ± 0.05*	0.78 ± 0.07
Plasma free fatty acids (mmol/L)	0.43 ± 0.05	0.57 ± 0.04	0.59 ± 0.06	0.40 ± 0.03
Plasma cholesterol (mmol/L)	4.28 ± 0.10	3.86 ± 0.16	5.04 ± 0.19*	3.69 ± 0.25
Blood glucose (mmol/L)	5.24 ± 0.32	4.24 ± 0.31	5.19 ± 0.39	4.77 ± 0.39
Insulin (ng/ml)	0.35 ± 0.05	0.40 ± 0.07	0.36 ± 0.04	0.22 ± 0.02

Five week old male 129S6 mice were maintained on a western diet (42% calories from fat) for 17 weeks. Following 15 weeks of feeding, mice underwent unilateral femoral artery ligation and excision surgery. Following surgery, mice received once daily i.p. injections of vehicle (DMSO), niacin (50 mg/kg), vitamin D (200 ng/kg), or niacin and vitamin D (50 mg/kg and 200 ng/kg, respectively) for 14 days. Food intake was measured post-surgery. Body weight and blood glucose measurements were performed immediately prior to sacrifice (day 15). All remaining parameters were determined as indicated, or post-mortem. Data are means±SEM. * p<0.05 versus vehicle, n=7-8.

Figure 3.5. Niacin improves recovery of hind limb function after hind limb ischemic injury in obese mice with metabolic syndrome.

Five week old male mice were maintained on a western diet for 15 weeks, followed by unilateral femoral artery ligation and excision surgery. Following surgery, mice received once daily i.p injections of vehicle, niacin (50 mg/kg), vitamin D (200 ng/kg), or niacin plus vitamin D (50 mg/kg and 200 ng/kg, respectively) for 14 days. **(A)** On days 3, 9 and 15 post-surgery, gait analyses were performed using a Catwalk system. Representative contact duration maps are shown for day 15. Black arrows indicate the injured limb. **(B)** Mean hind limb contact times and **(D)** mean hind limb contact intensities were used calculate hind limb use ratios for each day. A ratio of <1.0 indicates decreased use of the injured limb. **(C, E)** Areas under the curve for paw contact times and intensities over the course of treatment were calculated from data in (B, D, respectively). Data are means±SEM for n = 8. * p<0.05 versus vehicle.



3.4 Vitamin D does not promote vascular or myofiber regeneration of tibialis anterior muscles in diet-induced obese mice with metabolic syndrome

The tibialis anterior muscle undergoes significant necrosis and inflammation with subsequent vascular and muscular regeneration in response to femoral artery ligation and excision surgery (Arpino *et al.*, 2017). To determine if recovery of hind limb function was associated with corresponding tissue revascularization and myofiber regeneration, vessel densities, arteriole diameters and muscle architecture were quantified. There were no significant differences between treatment groups for capillary and arteriole densities per myofiber area across the length of the tibialis anterior muscle, as determined by staining for CD31+ and α SMA+, respectively (Figure 3.6A-C). Furthermore, there were no significant differences in arteriole diameter observed between treatment groups (Figure 3.6D). These results are consistent with previous findings from our lab, published by Pang *et al.* (2016), which showed similar results in arteriole and capillary density data with niacin treatment compared to control.

Quantification of tibialis anterior muscle architecture revealed no significant differences in percentages of regenerating (central nuclei), non-regenerating (peripheral nuclei), and necrotic (no visible nuclei) myofibers (Figure 3.7A, B). However, the proportions of regenerating to non-regenerating myofibers were markedly lower in mice treated with vitamin D, either alone or in combination with niacin (Figure 3.7C). These results may suggest that proportions of regenerating to non-regenerating myofibers may partially account for functional differences observed between treatment groups. Since accumulation of interstitial adipose tissue is associated with persistent muscle damage and can impair muscle function (Sciorati *et al.*, 2015), adipose tissue areas were measured in hematoxylin and eosin stained sections. There were no significant differences in adipose tissue area between treatment groups (Figure 3.7D).

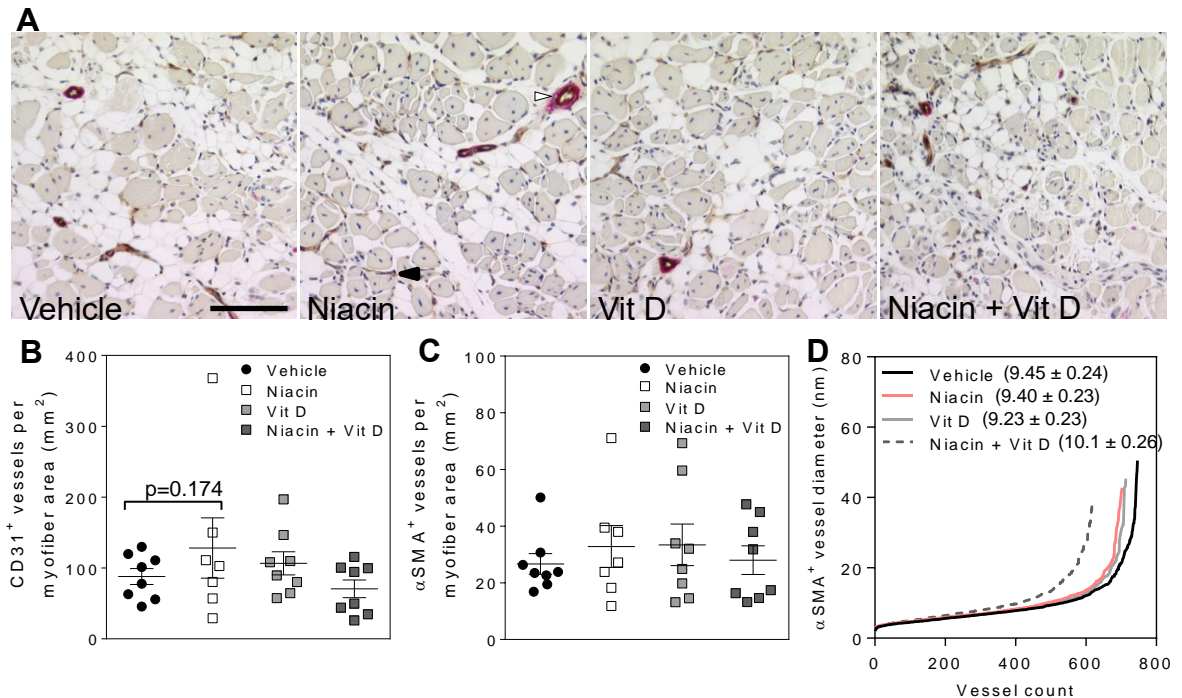


Figure 3.6. Effect of niacin and vitamin D on vascular regeneration in diet-induced obese mice with hind limb ischemia.

(A) Sections were immunostained for CD31 (brown, black arrowhead), to identify endothelial cells, and smooth muscle α -actin (α SMA) (red, white arrowhead), to identify vascular smooth muscle cells. Scale bar = 100 μ m. (B, C) Capillary and arteriole densities were determined by counting CD31⁺ vessels and α SMA⁺ vessels, respectively. Vessel densities were normalized to myofiber areas per section. Data are means \pm SEM for n = 7-8. (D) To generate arteriole diameter frequency plots for each treatment, arteriole diameters for all vessels in all muscle sections were determined. Values in parentheses indicate mean vessel diameters \pm SEM for each treatment group.

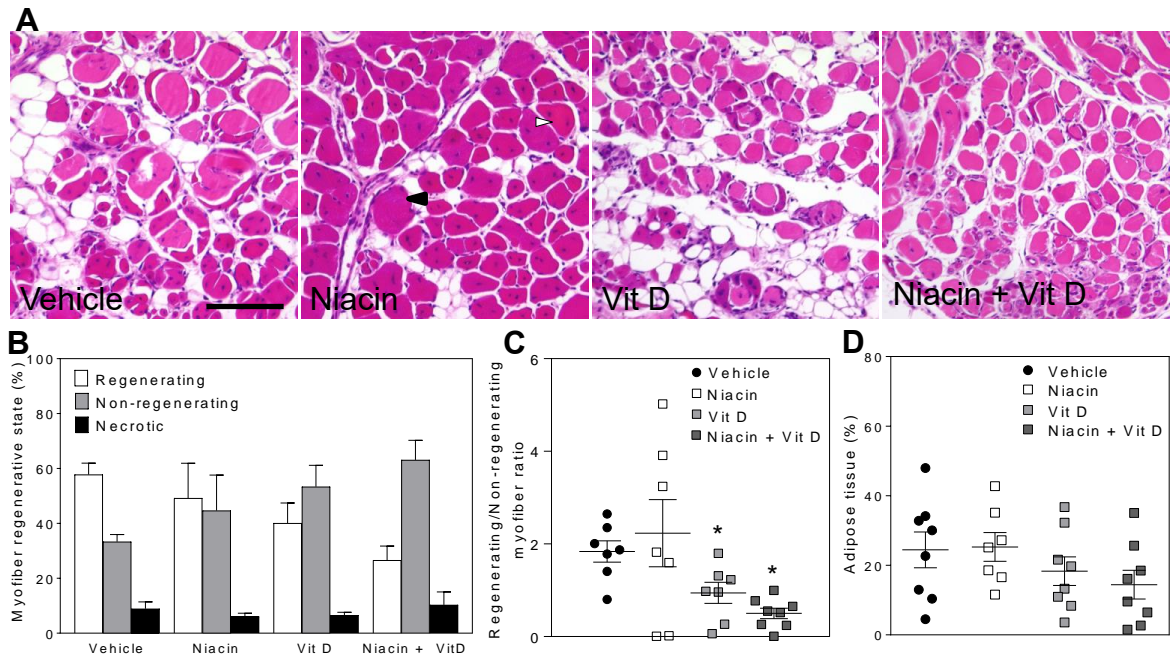


Figure 3.7. Vitamin D does not promote myofiber regeneration in tibialis anterior muscles following acute ischemic injury in diet-induced obese mice

(A) Sections were stained with hematoxylin and eosin and regenerating myofibers were identified by intense eosin staining and central nuclei (white arrowhead), non-regenerating myofibers were identified by intense eosin staining and the presence of peripheral nuclei (black arrowhead), and necrotic myofibers were identified by pale eosin staining and absent nuclei. Scale bar = 100 μ m. (B) Myofiber areas were determined, and percentages of each myofiber type (regenerating, non-regenerating, or necrotic) were calculated for each section. (C) Ratio of regenerating to non-regenerating myofibers were calculated for each section. (D) Adipose tissue areas were determined and calculated as percentages of total section area. Data are means \pm SEM for n=7-8. * $p < 0.05$ versus vehicle.

3.5 Vitamin D does not decrease inflammation or fibrosis of tibialis anterior muscles in diet-induced obese mice with metabolic syndrome

Sustained inflammation and increased interstitial fibrosis are associated with impaired myofiber regeneration (Sciorati *et al.*, 2015). Therefore, macrophage content and collagen deposition were quantified. Furthermore, a fibrosis location score was quantified for each mouse, where a higher score indicates increased presence of myofiber-associated collagen. Collagen associated with myofibers has a greater negative impact on muscle function than adipose-associated collagen. Treatment with vitamin D, either alone or in combination with niacin did not decrease inflammation or fibrosis (Figure 3.8A-C) and did not significantly impact the location of collagen deposition (fibrosis location score) (Figure 3.8E). Interestingly, mice treated with niacin alone exhibited consistent, though not statistically significant, decreases in inflammation and fibrosis (Figure 3.8C-E).

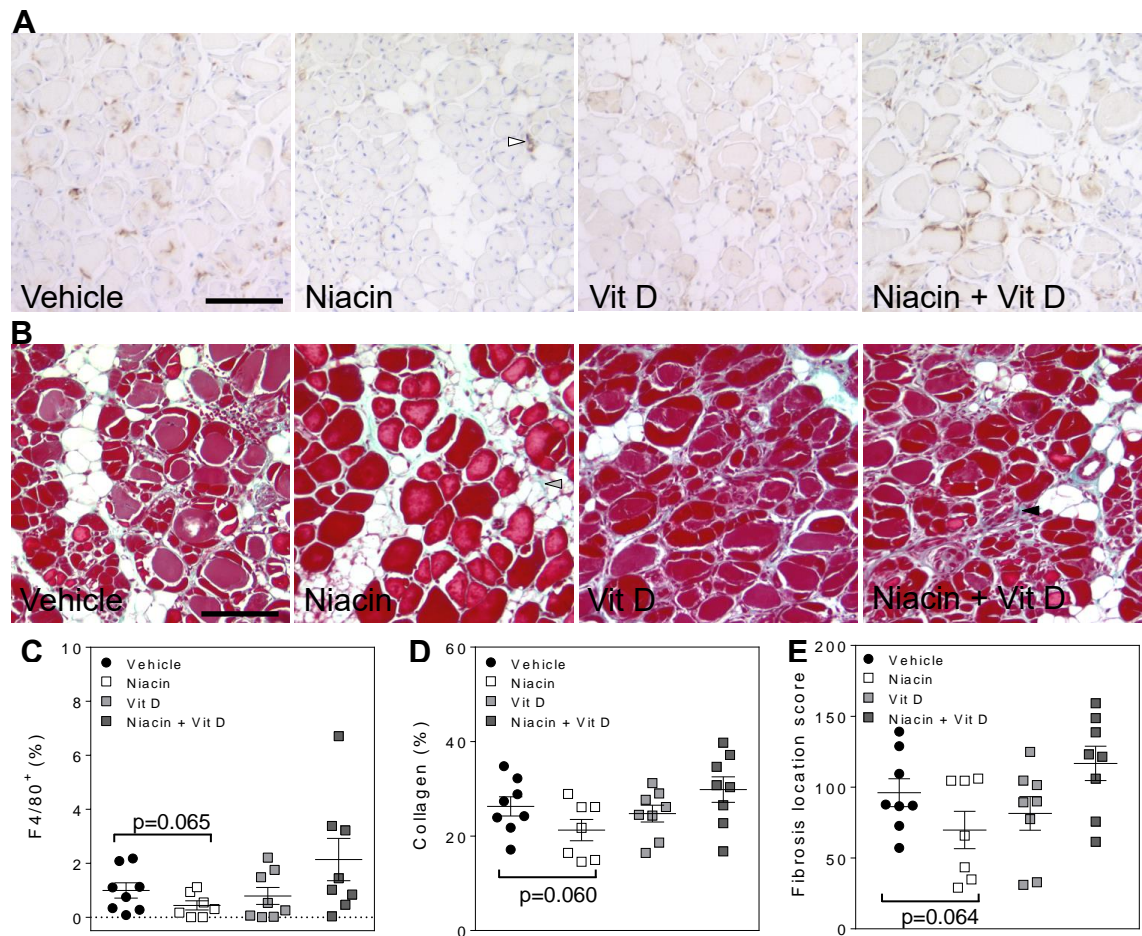


Figure 3.8. Vitamin D does not improve inflammation or fibrosis in tibialis anterior muscles following acute ischemic injury in diet-induced obese mice.

(A) Sections were immunostained for F4/80 (brown, white arrowhead) to identify macrophages. (B) Sections were stained with Masson's trichrome to identify collagen fibrosis (light blue, black and grey arrowheads). Scale bars = 100 μ m. (C, D) Macrophage and collagen areas were quantified and expressed as percentages of total section areas. (E) Fibrosis location score was determined for each mouse. A higher score indicates greater presence of myofiber-associated collagen (black arrowhead). Data are means \pm SEM for n=7-8.

Chapter 4

4 Discussion

4.1 Summary of Results

Endothelial dysfunction, in the setting of lipotoxicity during obesity and metabolic syndrome, has been identified as a surrogate marker for the development of vascular complications, including the commonly associated, but often underdiagnosed condition of PVD (Campia *et al.*, 2012). Current therapies for PVD are limited and fail to target endogenous revascularization of the ischemic tissue. Furthermore, current intervention strategies have shown limited success in Canada's Indigenous population that carries a disproportionate burden of obesity-related diseases. Therefore, it is important to develop better understanding of therapeutic agents that may be useful interventions against endothelial dysfunction during obesity and metabolic syndrome. Observational studies have suggested an association between low circulating levels of vitamin D and increased PVD risk in human populations (Gouveri *et al.*, 2012; Nsengiyumva *et al.*, 2015), offering the notion that increased vitamin D supplementation either through pharmacological doses, or increased dietary intake, may provide vascular benefit. Current knowledge of the effects of vitamin D supplementation for prevention of CVD are conflicting with no data yet available from large randomized, placebo-controlled vitamin D primary prevention or intervention trials. Furthermore, the effects of vitamin D on peripheral ischemia and vascular regeneration in the setting of obesity and metabolic syndrome are lacking and may be context dependent. Additionally, combined supplementation with other vitamins associated with improved endothelial cell function, such as niacin, may offer additive or synergist effects. Recent work from our lab has shown that low dose niacin promotes HMVEC tube formation as well as enhances vessel smooth muscle investment and improves functional recovery and tissue architecture of tibialis anterior muscles in diet-induced obese mice following ischemic injury (Hughes-Large *et al.*, 2014; Pang *et al.*, 2016). Therefore, it was hypothesized that supplementation of vitamin D alone and in combination with niacin, would improve EC angiogenic function and promote vascular regeneration under lipotoxic conditions that occur during obesity and metabolic syndrome.

Figure 4.1 summarizes the major findings of this thesis, which are as follows: 1) Both niacin and vitamin D improved HMVEC tube formation and stability in high palmitate. 2) Niacin and vitamin D increased stress response and anti-inflammatory gene expression in response to high palmitate. However, vitamin D decreased cell cycle gene expression, which was associated with reduced HMVEC population doubling, while niacin induced stable expression of miR126-3p and -5p. 3) Niacin, but not vitamin D or the combination, improved recovery of hind-limb function following ischemic injury in obese mice with metabolic syndrome. 4) Recovery of hind limb function was achieved despite no improvements in revascularization, regeneration, inflammation and fibrosis of the tibialis anterior muscle of niacin-treated mice. However, treatment with vitamin D, either alone or in combination with niacin, significantly impaired myofiber regeneration.

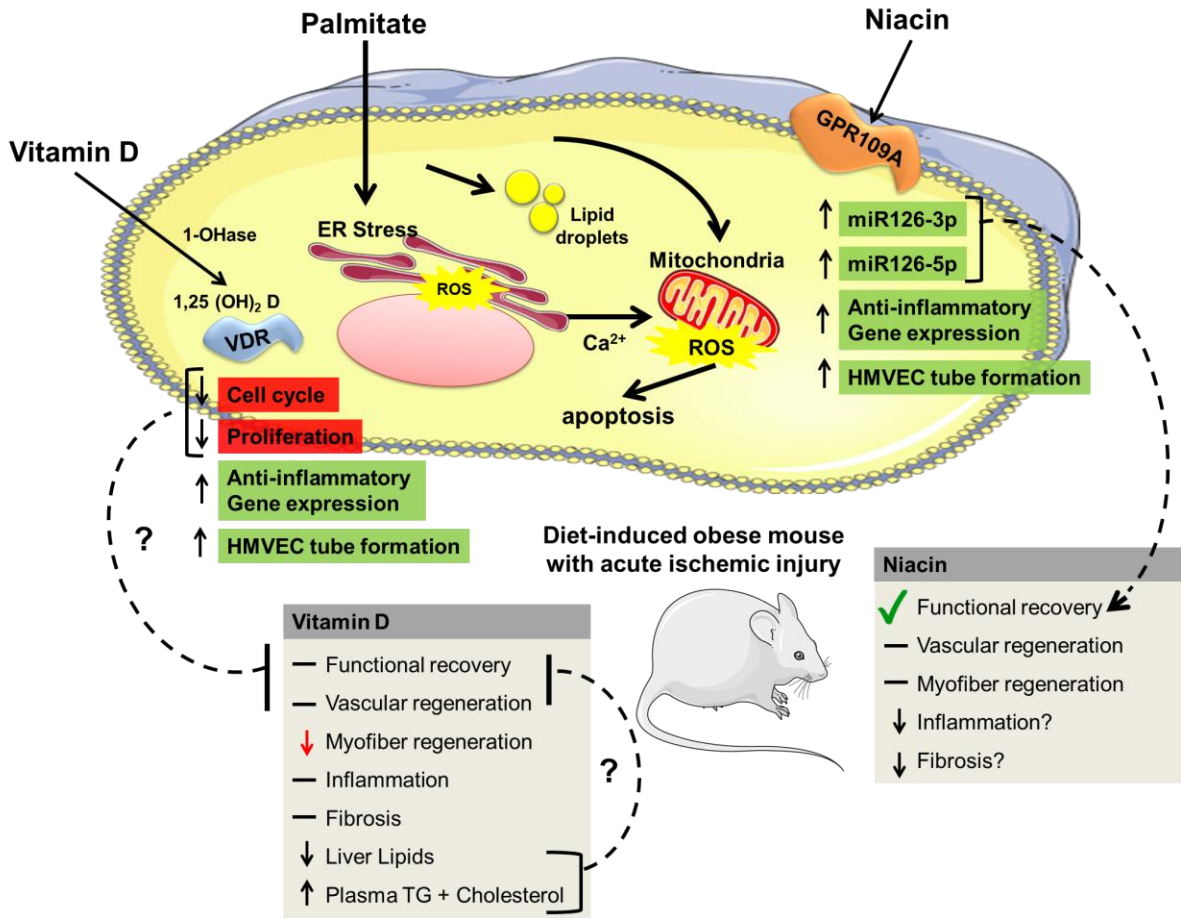
4.2 Niacin and Vitamin D Improves Endothelial Cell Angiogenic Function in High Palmitate

There is compelling evidence linking vitamin D and cardiovascular function, raising the intriguing possibility that a causal relationship exists between vitamin D deficiency and CVD progression. In support of this concept, Wong *et al.* (2014) showed improved vascular regeneration in lean, diabetic rodent models of vascular injury. Furthermore, several clinical studies have demonstrated improvements in endothelial function following vitamin D supplementation, in patients with T2DM (Sugden *et al.*, 2008; Hussin *et al.*, 2017), in healthy patients with vitamin D deficiency (Tarcin *et al.*, 2009) and in patients at risk for CVD development (Reynolds *et al.*, 2016). However, the effects of vitamin D on EC function and vascular regeneration under lipotoxic conditions are lacking. Thus, it was determined whether vitamin D would have similar benefits on endothelial function in a high fat setting that would be typical during obesity and metabolic syndrome. Furthermore, the effects of combined supplementation with niacin, were determined to identify any potential additive or synergistic effects.

In vitro data showed that low dose supplementation of vitamin D alone and in combination with niacin, improved EC tube formation in high palmitate. These data are consistent with an earlier study showing increased tube formation with vitamin D during

Figure 4.1. Summary of vitamin D and niacin supplementation on EC function and vascular regeneration during lipotoxicity.

Both niacin and vitamin D improved endothelial tube formation and stability in high palmitate. However, only niacin improved functional recovery in obese mice following ischemic injury. Treatment with vitamin D, either alone or in combination with niacin, significantly impaired myofiber regeneration with no improvements observed in hind-limb function, revascularization, inflammation or fibrosis. Both niacin and vitamin D increased stress response and anti-inflammatory gene expression in EC exposed to high palmitate. However, vitamin D decreased cell cycle gene expression, which was associated with suppression of EC proliferation, which may have limited vascular regeneration and recovery of limb function *in vivo*. Furthermore, vitamin D-treated mice exhibited metabolic disturbances and impaired lipid homeostasis, which may affect processes of vascular regeneration. Niacin-treated mice exhibited consistent, though not statistically significant, decreases in inflammation and fibrosis of the tibialis anterior muscle. Niacin induced relatively stable expression of miR-126-3p and miR-126-5p, which may have contributed to improved EC function *in vitro*.



exposure to preeclampsia-related inflammatory factors (Brodowski *et al.*, 2014). Furthermore, consistent with our previous results (Hughes-Large *et al.*, 2014; Pang *et al.*, 2016), low dose niacin supplementation improved HMVEC tube formation upon exposure to palmitate. In addition to improved EC tube formation, low dose supplementation of vitamin D alone and in combination with niacin, also promoted HMVEC tube network stabilization in high palmitate. This observation is consistent with earlier studies, showing that niacin plays a key role in increasing the longevity of endothelial tube networks in an NAD⁺-dependent manner, under the stress inducing condition of high glucose (Borradaile & Pickering, 2009b). Vitamin D has also been shown to promote EC longevity, by protecting EC from oxidative stress and promotion of cell survival pathways (Uberti *et al.*, 2014). Promotion of EC survival and longevity are important considerations for revascularization following vascular injury. Functional revascularization requires newly formed microvasculature networks to survive long enough to recruit smooth muscle cells and arteriogenesis initiation (van der Veer *et al.*, 2005; Borradaile & Pickering, 2009b). Interestingly, no additional benefit on tube formation or stability was observed with the combination treatment, suggesting no additive or synergistic effect of using the combination treatment on HMVEC function *in vitro*.

4.3 Modulation of Endothelial Cell Gene Expression by Niacin and Vitamin D

High levels of circulating fatty acids during obesity and metabolic syndrome are well known risk factors for the development of CVD (Mathew *et al.*, 2010). Saturated fatty acids, in particular, are known to play an important role in mediating endothelial dysfunction (Kim *et al.*, 2005; Liu *et al.*, 2012). Palmitate, the most abundant dietary saturated fatty acid, has been shown to induce oxidative stress, ER stress, and inflammatory pathways in endothelial cells (Battson *et al.*, 2017; Ghosh *et al.*, 2017). The global gene expression data provided in this thesis clearly support this earlier work (Table 3.1). Treatment with vitamin D in high palmitate resulted in decreased expression of CEBPB and BMABI, which are proposed modulators of inflammation; whereas treatment with niacin resulted in increased expression of *SMOX*, a suggested antioxidant,

and decreased expression of *BMX*, an inflammatory marker. These results are consistent with previous findings that niacin and vitamin D are both able to suppress EC mediators of inflammation and oxidative stress in response to inflammatory stimuli (Digby *et al.*, 2012b; Dalan *et al.*, 2014). However, neither vitamin was able to completely suppress stress response and inflammatory pathways during high palmitate *in vitro* (Figure 3.2). This *in vitro* model only tested the effect of niacin and vitamin D on one vascular cell type, which may account for the lack of complete stress response and inflammatory suppression as indicated by global gene expression. Both niacin and vitamin D treatment have been shown to have anti-inflammatory effects on various cell types including adipocytes (Digby *et al.*, 2010; Cimini *et al.*, 2017), as well as macrophages and neutrophils (Lipszyc *et al.*, 2013; Digby *et al.*, 2012a; Bellan *et al.*, 2018), to limit the contribution of systemic inflammation to perivascular inflammation and stress (Berg & Scherer, 2005; Libby *et al.*, 2009).

Although vitamin D was able to improve EC angiogenic function as measured by tube formation and stability in high palmitate, microarray data revealed that vitamin D decreased the expression of several cell cycle genes, which was associated with suppression of cell proliferation. Vitamin D exerts its effects predominantly through activation of its receptor, VDR, and has been reported to have effects on cell cycling, proliferation, and apoptosis in a wide variety of cell types (Holick, 2007). Consistent with our findings, vitamin D has been shown to elicit anti-proliferative effect on EC in the setting of tumor progression and in malignant cell types (Majewski *et al.*, 1993; Saghiri *et al.*, 2017; Jamali *et al.*, 2018). Furthermore, it has been shown that vitamin D can suppress proliferation of other vascular cell types including cardiomyocytes and VSMC by promoting cell cycle arrest in G1/G0 phase (Wu-Wong *et al.*, 2006; Artaza *et al.*, 2010). Despite downregulation of cell cycle genes and suppressed proliferation, this did not impact *in vitro* angiogenic function as measured by Matrigel assays. This assay relies predominantly on endothelial cell migration rather than proliferation, and thus the beneficial effects of vitamin D on anti-inflammatory gene expression, were likely able to contribute to improved tube formation and stability *in vitro*.

MicroRNAs (miRs) are small noncoding RNAs that modulate gene expression post-transcriptionally (Bartel, 2004). Several miRNAs have been shown play important roles

in the regulation and control of vascular function, contributing to both vascular homeostasis and disease (Latronico *et al.*, 2007; Feinberg & Moore, 2016). Microarray analyses identified miR126 to be differentially expressed in response to niacin treatment in high palmitate. Further investigation of miR126 regulation through qRT-PCR analyses revealed that niacin may induce relatively stable expression of both miR126-3p and miR126-5p. MiR-126 has been identified as a regulator of angiogenesis and plays a critical role in vascular development (Wang *et al.*, 2008a; Chistiakov *et al.*, 2016). The precursor, pre-miR-126, gives rise to two mature strands, miR-126-3p and miR126-5p, both of which are biologically active (Fish *et al.*, 2009; Weber *et al.*, 2014). Similar to our findings, one study has shown that treatment with niacin upregulated miR-126 in retinal EC under the stress inducing condition of high glucose (Wang & Yan, 2016). miR126-3p and miR126-5p have been implicated in the promotion of an anti-inflammatory state, and of proliferation in response to EC stress, respectively (Boon & Dimmeler, 2014; Feinberg & Moore, 2016). Schober *et al.* (2014) found that expression of miR-126-5p was essential to EC proliferation during hyperlipidemia and resulted in reduced atherosclerotic lesions. These findings suggest that therapeutic agents promoting miR-126-5p expression in EC could have atheroprotective potential (Schober *et al.*, 2014). Since miR-126 is known to play a key role in the regulation of EC angiogenic function, it is possible that niacin-mediated expression of miR-126 may be one mechanism by which niacin improved EC tube formation and stability under lipotoxic conditions in Matrigel assays.

4.4 Vitamin D does not Improve Functional Recovery in Obese Mice with Acute Ischemic Injury

Although observational studies have suggested an inverse relationship between vitamin D intake and PVD risk in human populations, there are no data yet available from large randomized, placebo-controlled vitamin D primary prevention or intervention trials. There is convincing evidence to support the relationship between vitamin D supplementation and improved vascular function *in vitro*; however, the translation of this relationship *in vivo* is often conflicting or inconclusive (Bellan & Marzullo, 2018). Data presented in this thesis show that although vitamin D supplementation was able to

improve *in vitro* angiogenic function of EC under lipotoxic conditions, intervention did not improve recovery of hind limb function in obese mice following acute hind limb ischemic injury. This finding raises questions as to whether vitamin D supplementation may be beneficial under established lipotoxic environments *in vivo*.

Recent reports indicate that vitamin D supplementation can promote vascular regeneration in lean, diabetic rodent models of vascular injury (Wong *et al.*, 2014; Leisegang *et al.*, 2016). However, equally compelling work clearly showed that treatment with vitamin D limited neovascularization and promoted anti-angiogenic effects on tumor derived EC (Majewski *et al.*, 1993; Mantell *et al.*, 2000), and on retinal EC (Albert *et al.*, 2007). It is possible that the timing of vitamin D administration determines its therapeutic effect on vascular regeneration. For studies of vascular regeneration, vitamin D was administered prior to vascular injury; while for studies of tumour progression, vitamin D administration was either concurrent with or after the initial establishment of tumours. The latter intervention protocol is most similar to the protocol we used, is relevant to the human treatment scenario, and could partly account for the lack of benefit of vitamin D that we observed. This general concept is consistent with negative data from the EVITA trial of vitamin D supplementation in heart failure patients (Zittermann *et al.*, 2017), and with preliminary analyses from VITAL showing fewer advanced cancer diagnoses among patients receiving vitamin D (Manson *et al.*, 2019).

Multiple observations have been reported that suggest a relationship between abnormal serum vitamin D levels and dysregulation of vitamin D metabolism during obesity (Earthman *et al.*, 2012; Ding *et al.*, 2012; Clemente-Postigo *et al.*, 2015). The lack of effect on hind limb revascularization and recovery of limb function could be related to increased adiposity of the diet-induced mouse model. Evidence suggests that increased adiposity in an obese individual acts as a reservoir for vitamin D, reducing serum vitamin D concentration, which may account for conflicting results from trials evaluating vitamin D supplementation on endothelial function (Drincic *et al.*, 2012; Carrelli *et al.*, 2017). For example, Carrara *et al.*, (2014) found that vitamin D improved endothelial function, as measured by FMD following treatment with vitamin D. However, a recent finding from Borgi *et al.*, (2017) that used similar dosing and endpoint evaluations, did not find a benefit of vitamin D on endothelial function. The latter trial included overweight and

obese subjects, raising the intriguing possibility that in the setting of obesity, vitamin D may not be a useful intervention to protect against endothelial dysfunction. In further support of this concept, Lee *et al.* (2018) showed that vitamin D supplementation was able to modulate innate immunity in lean mice, but this effect was absent in diet-induced obese mice.

Not only can obesity and increased adiposity disrupt the effectiveness of vitamin D, but emerging evidence suggests that vitamin D may play a role in regulating energy metabolism and lipid levels. However, the effect of vitamin D supplementation on lipid and lipoprotein metabolism is highly controversial and unclear (Challoumas, 2014; Schwetz *et al.*, 2018). Results from this thesis suggested that Vitamin D may have disrupted lipid homeostasis, as observed by decreased liver lipids and corresponding increases in plasma TG and cholesterol (Table 3.3). A recent trial found that vitamin D supplementation resulted in unfavorable effects on lipid and lipoprotein metabolism, noted by increased serum lipids including total cholesterol and serum TG (Schwetz *et al.*, 2018). These results are consistent with previous randomized control trials noting an unfavourable effect of vitamin D on lipid levels (Maki *et al.*, 2011; Pilz *et al.*, 2015). Conversely, observational studies have shown that patients with higher serum vitamin D concentrations displayed favourable lipid profiles compared to vitamin D deficient patients (Ponda *et al.*, 2012; Chaudhuri *et al.*, 2013; Kelishadi *et al.*, 2014). In addition, several intervention studies have also shown neutral effects of vitamin D supplementation on lipid metabolism (Nagpal *et al.*, 2009; Jorde *et al.*, 2010; Witham *et al.*, 2013). Consistent with our results, several studies using diet-induced obese mice have shown an increase in hepatic fatty acid oxidation with corresponding decreases in hepatic lipid accumulation following vitamin D supplementation (Yin *et al.*, 2012; Marcotorchino *et al.*, 2014; El-Sherbiny *et al.*, 2018). Unfavorable lipid profiles are known contributors to EC lipotoxicity, disrupted EC function and limited vascular regeneration (Campia *et al.*, 2012; Kim *et al.*, 2012). Despite controversial evidence for the effect of vitamin D on serum lipids, it is possible that the changes in lipid metabolism associated with vitamin D intervention in our model, may have limited vascular regeneration, repair and functional recovery *in vivo*.

The lack of effect on hind limb revascularization and recovery of limb function could also be related to the inhibition of cell cycle gene expression observed in cultured EC exposed to vitamin D in the presence of high palmitate. Although vitamin D supplementation resulted in improved *in vitro* angiogenic function, it is entirely possible that impaired endothelial cell proliferation, in the setting of pre-existing tissue damage induced by femoral artery ligation and excision, limited vascular regeneration *in vivo*. This is consistent with the anti-angiogenic effects of vitamin D previously observed in various experimental models. Albert *et al.* (2007) showed that vitamin D inhibited retinal neovascularization via suppression of EC proliferation. Numerous studies have outlined the anti-angiogenic effect of vitamin D on tumor vascularization (Mantell *et al.*, 2000; Maj *et al.*, 2016; Jamali *et al.*, 2018). Lastly, vitamin D has also been shown to inhibit angiogenesis in a rodent model of endometriosis by reducing angiogenic factors and inhibiting ECM degradation (Yildirim *et al.*, 2014).

Vascular regeneration is a complex process, not only requiring proliferation and migration of EC, but also the co-ordination and recruitment of several other vascular cells types, and their assembly into new, functional vessels (Shirali *et al.*, 2018). The angiogenic effects of vitamin D may depend on vascular cell type. Although vitamin D has been shown to elicit pro-angiogenic effects on vascular EC in lean rodent models of vascular injury, it has been shown to inhibit VSMC proliferation and migration (Raymond *et al.*, 2005). VSMC are required for vascular integrity and function and are a major cellular component involved in vascular regeneration (Greenberg *et al.*, 2008). It is possible that vascular regeneration and functional recovery were limited *in vivo* due to different effects of vitamin D on the various vascular cell types involved in the vascular regenerative process.

4.5 Combined Vitamin Supplementation and Functional Recovery in Obese Mice with Acute Ischemic Injury

Previous work from our lab has shown that treatment with niacin improved recovery of hind limb function in the same mouse model (Pang *et al.*, 2016). Data presented in this thesis confirmed the effect of niacin on functional recovery, which corresponded with consistent, although not statistically significant, beneficial effect on tibialis anterior

macrophage content (-56%, $p=0.065$) and fibrosis (-24%, $p=0.060$). These findings support previous work showing that niacin, through modulation of immune cell activity, promoted vascular function and repair (Ganji *et al.*, 2009; Lukasova *et al.*, 2011; Digby *et al.*, 2012a; Wu *et al.*, 2012). Interestingly, however, improvement in functional recovery and the associated modest effect at the tissue level were not evident in mice treated with vitamin D alone or in combination with niacin, providing evidence that intervention with vitamin D was sufficient to abrogate the beneficial effects of niacin.

Both niacin and vitamin promote normal EC function through similar mechanisms as shown in Figure 1.2, including increased NO production via increased eNOS activation, decreased ROS production and decreased production of inflammatory molecules (Digby *et al.*, 2012b; Dalan *et al.*, 2014). The lack of beneficial effect with the combination treatment *in vivo* and absence of additive or synergistic effects with the combination *in vitro* could partly be related to saturation of these pathways. The enzymatic activity of NOS is tightly regulated and largely dependent on cofactor availability as well as arginine and oxygen availability. Changes in concentration or availability to any of these major substrates can result in profound influences on NOS activity and thus NO signalling (Thomas *et al.*, 2008). It is possible that combining niacin and vitamin D offered no additional increase in NOS activity and NO-mediated signalling in EC if their downstream targets converged on the similar pathways. Furthermore, it is possible that the combination of niacin and vitamin D did not offer an additive or synergistic interaction with respect to total antioxidant capacity as it is possible that signalling pathways related to increased antioxidant potential may have been saturated by either vitamin alone.

In addition, the absence of beneficial effects of niacin in the combination treatment could be partly related to the distinct effects of these vitamins on gene expression in EC. In contrast to vitamin D, niacin did not decrease expression of cell cycle regulators (Table 3.2) and did not inhibit EC population doublings (Figure 3.3C). Furthermore, niacin induced relatively stable expression of miR126-3p and -5p, which have been implicated in regulation of EC functions; it is possible that these distinct and opposing effects on EC function may account for the lack of functional recovery and revascularization *in vivo* with the combination treatment.

These observations of co-supplementation of only two vitamins in a tightly controlled experimental system raise questions as to the effectiveness of multivitamin supplements for CVD benefit. It is possible that constituents of multi-vitamins may have opposing effects on gene expression related to vascular process in the humans and may account for the lack of CVD benefit observed in recent meta-analyses (Jenkins *et al.*, 2018). Furthermore, this outlines the importance of studying the potential risk of interactions between vitamins and micronutrients to elucidate the advantages and disadvantages of different combination for the promotion of health.

4.6 Limitations and Future Directions

Our *in vitro* model of EC tube formation and stability only tested the effect of niacin and vitamin D on one vascular cell type. Vascular regeneration is a complex process, not only requiring proliferation and migration of EC, but also the co-ordination and recruitment of several other vascular cells types, such as VSCM, which are required for vascular integrity and function and are a major cellular component involved in vascular regeneration (Shirali *et al.*, 2018). Vitamin D has been show to inhibit VSMC proliferation and migration (Raymond *et al.*, 2005). It would be interesting to expand the findings from this thesis to other vascular cell types, especially since vitamin D has been show to inhibit VSMC proliferation and migration (Raymond *et al.*, 2005). One focus would be to co-culture EC with VSMC in Matrigel assays of tube formation and stability. Co-culturing with VSMC, or other cell types such as pericytes, may provide insight into differences between findings *in vitro* of improved EC tube formation and stability with vitamin D supplementation, and findings *in vivo* of impaired myofiber regeneration and lack of function recovery following vitamin D intervention.

We previously showed that niacin enhanced recovery of hind limb function in the same mouse model (Pang *et al.*, 2016). Here, we confirmed the effect of niacin on recovery of limb function, and found that this was associated with consistent, though statistically non-significant, reductions in tibialis anterior macrophage content (-56%, $p=0.065$) and fibrosis (-24%, $p=0.060$). These beneficial effects of niacin were less dramatic than observed as in our earlier work (Pang *et al.*, 2016), most likely because induction of metabolic disease was less pronounced in the current study, as indicated by near normal

fasting blood glucose concentrations in all groups (Table 3.3). This resulted in greater recovery of limb function in the vehicle-treated group in the study presented in this thesis. Nonetheless, these data generally support previous work in lean rodent models showing that niacin can promote vascular function and repair by modulating the activity of immune cells (Ganji *et al.*, 2009; Lukasova *et al.*, 2011b; Tavintharan *et al.*, 2011; Digby *et al.*, 2012a; Wu *et al.*, 2012).

The unexpected negative effects of vitamin D on vascular regeneration in an obese mouse model presented in this thesis highlights the need for further research to identify potential TCAM therapies that may be useful intervention and/or prevention strategies for CVD risk. Currently available pharmacotherapies for PVD are limited and generally focus on symptom relief for thrombosis and claudication pain management rather than promoting endogenous revascularization of the ischemic tissues (Farber & Eberhardt, 2016). It would be interesting to expand this project to investigate other naturally occurring, diet-derived compounds that may be useful TCAM interventions for endothelial dysfunction resulting from lipotoxic environments seen in patients with obesity, metabolic syndrome and T2DM. The naturally occurring modified amino acid, ergothioneine is a suggested powerful antioxidant. Ergothioneine is mainly acquired through fungi dietary sources, and thus like niacin and vitamin D, is enriched in commonly consumed edible mushrooms. Ergothioneine is absorbed through the specific transporter organic cation transporter novel, type 1 (OCTN1) expressed in all human tissues (Gründemann *et al.*, 2005). Recent studies indicate that ergothioneine can accumulate in injured tissues, and it has been hypothesized that it acts through a protective or adaptive mechanism of action to minimize oxidative damage (Halliwell *et al.*, 2018). However, there is limited knowledge of the effects of ergothioneine on EC angiogenic function under lipotoxic conditions. Future studies could be performed to determine the effects of ergothioneine supplementation, possibly with and without co-supplementation with niacin, on EC function and vascular regeneration under lipotoxic conditions to determine its potential use as a therapeutic agent for PVD.

Beyond assessing MIR126 expression, the *in vitro* and *in vivo* findings in this thesis did not thoroughly investigate signaling pathways that may be involved in improved EC function and improvement in hind limb functional recovery observed with niacin

treatment. Previous findings from our lab have shown that the pro-angiogenic effect of niacin on EC is mediated by GPR109A (Hughes-Large *et al.*, 2014; Pang *et al.*, 2016). One downstream signaling pathway of niacin-mediated GPR109A activation that may be particularly relevant to vascular regeneration is prostaglandin receptor activation, specifically prostaglandin D₂ receptor (DP1) activation. Production of PGD₂ is known to be mediated by GPR109A activation and is responsible for the adverse, yet relatively harmless, skin flushing experienced with niacin supplementation (Kamanna *et al.*, 2009). GPR109A/PGD₂/DP1 axis may be relevant to vascular regenerative properties of niacin supplementation as prostaglandins are known to have vasodilatory and cytoprotective effects (Suzuki *et al.*, 2016). PGD₂ has been shown to improve the barrier function of EC (Murata *et al.*, 2008; Kobayashi *et al.*, 2013), and a recent study demonstrated that DP1 activation appears to be required for the myocardial protective effects of niacin (Kong *et al.*, 2017). Future *in vitro* and *in vivo* studies using selective activators and/or inhibitors of the GPR109A/PGD₂/DP1 axis will provide insight into whether the effects of niacin are mediated through this axis. It would also be useful to determine if niacin promotes EC functions through GPR109A activation independent of PGD₂ activation of DP1. In order to determine if either receptor alone or in combination is required for the effects of niacin, cells could be transfected with either control siRNA or silencer select siRNA against either receptor.

4.7 Relevance and Conclusions

Maintenance of EC function can help reverse or attenuate vascular disease. Therefore, it is important to develop better understanding of therapeutic agents that may be useful interventions against endothelial dysfunction. Future PVD intervention and prevention strategies could target endogenous revascularization to improve CVD disease morbidity. Niacin and vitamin D are considered promising natural, therapeutic compounds as they are known to promote normal endothelial function. Results presented here raise questions as to the effectiveness of vitamin D supplementation to promote vascular regeneration under lipotoxic conditions that would be seen during obesity and metabolic syndrome.

Another important finding of this thesis was that the beneficial effects of niacin were absent when co-supplemented with vitamin D. This underscores the importance of

understanding interactions between different therapeutic agents, especially TCAMs, and their effects on disease states. Ultimately, better understanding of the effects and mechanisms of action of TCAM therapies on the vasculature will help to guide therapeutic as well as nutritional recommendations for obese patients with PVD.

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Appendices

Appendix A: Animal Use Protocol



AUP Number: 2011-044

PI Name: Borradaile, Nica M

AUP Title: Vascular Regeneration In Diet-induced Metabolic Syndrome

Approval Date: 08/03/2016

Official Notice of Animal Use Subcommittee (AUS) Approval: Your new Animal Use Protocol (AUP) entitled "Vascular Regeneration In Diet-induced Metabolic Syndrome" has been APPROVED by the Animal Use Subcommittee of the University Council on Animal Care. This approval, although valid for four years, and is subject to annual Protocol Renewal.2011-044::5

1. This AUP number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this AUP number.
3. Purchases of animals other than through this system must be cleared through the ACVS office. Health certificates will be required.

The holder of this Animal Use Protocol is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.

Submitted by: Copeman, Laura
on behalf of the Animal Use Subcommittee
University Council on Animal Care

The University of Western Ontario
Animal Use Subcommittee / University Council on Animal Care
Health Sciences Centre, • London, Ontario • CANADA – N6A 5C1

Appendix B: Permission for use of Article in Master's Thesis

6/24/2019

RE: Using my Article as part of my Master's Thesis

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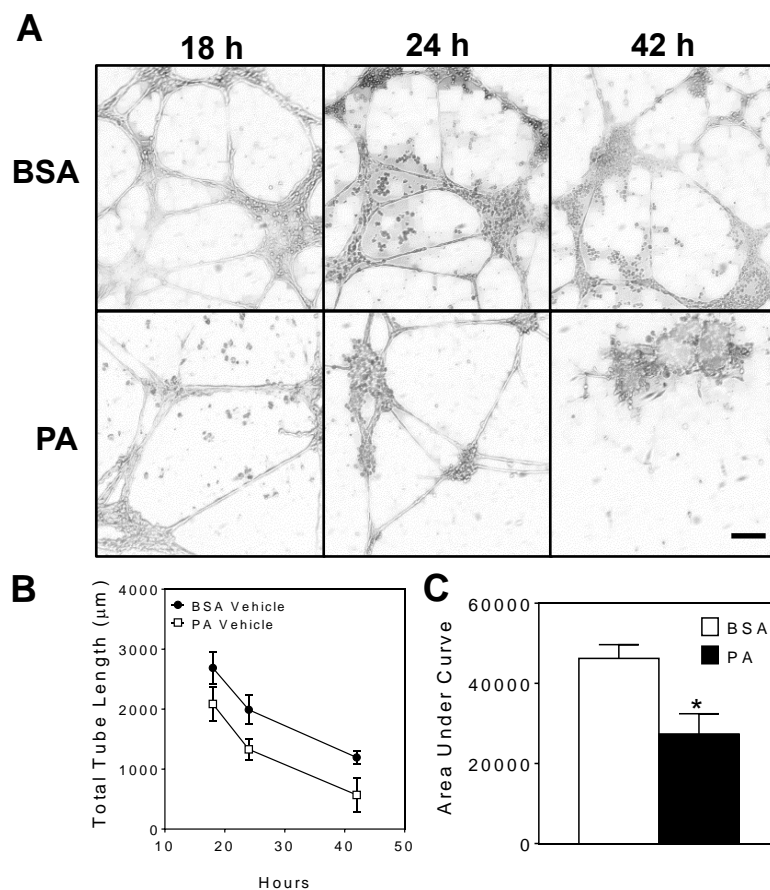
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Appendix C: Supplemental Information

**Supplemental Figure 1. HMVEC tube stability was impaired in high palmitate.**

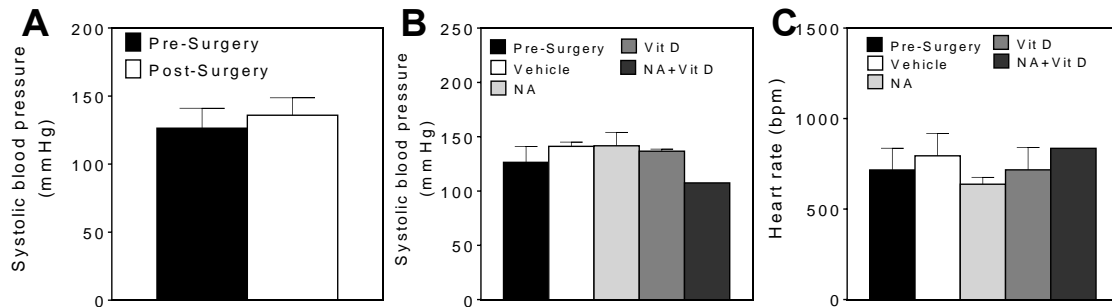
(A) Cells were seeded onto growth factor replete Matrigel and incubated with media containing BSA or 0.5 mM palmitate (PA) complexed to BSA. Cells were treated with vehicle (DMSO). Resulting tube networks were imaged at 18, 24 and 42 hours by light microscopy. (B) The entire well was assessed for total tube length using ImageJ software. (C) Areas under the curve were calculated from tube length data in (B). Data are means±SEM for n=4. *p < 0.5.

Supplemental Figure 2. Reactome pathways corresponding to changes in gene expression during palmitate treatment.

Pathways overrepresented in the list of differentially expressed genes in human microvascular endothelial cells treated with 0.5 mM palmitate plus vehicle (**A**), 0.5 mM palmitate plus 10 μ M niacin (**B**), or 0.5 mM palmitate plus 10 nM vitamin D (**C**) compared to BSA control. Top level pathways are represented by central nodes, with nodes in the outer rings representing sub-pathways. Relationships between nodes are represented by arcs (edges). Significantly ($p < 0.05$) overrepresented (enriched) pathways and relationships are indicated by increasing yellow signal. Black boxes

highlight comparisons in significantly overrepresented top-level pathways and associated sub-pathways between treatment groups.





Supplemental Figure 3. Blood pressure and heart rate are unaffected by vitamin supplementation.

Five week old male 129S6 mice were fed a western diet for 15 weeks, followed by right hind limb femoral artery ligation and excision surgery. Mice received once daily i.p injections for 14 days following surgery of either vehicle, niacin (NA) (50 mg/kg), vitamin D (Vit D) (200 ng/kg), or a combination of NA and Vit D (50 mg/kg and 200 ng/kg, respectively). Three days before the surgery (Pre-surgery), and on day 14 following surgery (Post-surgery), blood pressure and heart rate were measured using tail-cuff plethysmography. **(A)** Mean systolic blood pressure pre- and post-surgery. Data are means \pm SEM for n=15 for pre-surgery and n=8 for post-surgery. **(B)** Mean systolic blood pressure (mmHg) and **(C)** mean heart rate (bpm). Data are means \pm SEM for n=15 for pre-surgery and n=8 for post-surgery, n=3 for vehicle, n=2 for NA, n=2 for Vit D and n=1 for NA and Vit D.

Curriculum Vitae

Kia Mae Peters

EDUCATION

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- September 2012-
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ACADEMIC AWARDS

- May 2019 Wayne State Lipid Symposium Poster Award – 3rd place
- 2019-Present Ontario Graduate Scholarship – Indigenous to Canada
- June 2018 Canadian Lipoprotein Conference Poster Award
- April 2018 Interdisciplinary Development Initiative in Applied
Indigenous Scholarship
- 2018 Graduate Student Innovation Scholar
- October 2017 Canadian Lipoprotein Conference Poster Award
- 2017-2018 Queen Elizabeth II Graduate Scholarship in Science and
Technology
- November 2016 Physiology & Pharmacology Research Day Poster Award

PUBLICATIONS

Kia M. Peters, Richard Zhang, Chanho Park, Zengxuan Nong, Hao Yin, Rachel B. Wilson, Brian G. Sutherland, Cynthia G. Sawyez, Geoffrey J. Pickering, Nica M. Borradaile (2019). Vitamin D intervention does not improve vascular regeneration in diet-induced obese male mice with peripheral ischemia. *J Nutr Biochem* 70:65-74.

Kia M. Peters*, Rachel B. Wilson*, Nica M. Borradaile (2018). Non-parenchymal hepatic cell lipotoxicity and the coordinated progression of non-alcoholic fatty liver disease and atherosclerosis. *Curr Opin in Lipidol.* 29(5): 417-422.

*Authors contributed equally

Sarah J Lehnert, Ian A.E. Butts, Erin W. Flannery, **Kia M. Peters**, Daniel D Heath, and Trevor E. Pitcher (2017). Effects of ovarian fluid and genetic differences on sperm performance and fertilization success of alternative reproductive tactics in Chinook salmon. *J Evol Biol.* 30(6): 1236-1245.

PRESENTATION & CONFERENCES

Peters K, Nong Z, Park C, Wilson R, Zhang R, Sutherland B, Sawyez C, J. Pickering JG, Borradaile N Effects of low dose niacin and vitamin D on vascular regeneration under lipotoxic conditions. International Symposium on Atherosclerosis, Toronto, Ontario. June, 2018.

[Poster]

Peters K, Nong Z, Park C, Wilson R, Zhang R, Sutherland B, Sawyez C, J. Pickering JG, Borradaile N. Effects of low dose niacin and vitamin D on vascular regeneration under lipotoxic conditions. Canadian Lipoprotein Conference, Toronto, Ontario. June, 2018.

[Poster]

Peters K, Nong Z, Park C, Wilson R, Zhang R, Sutherland B, Sawyez C, J. Pickering JG, Borradaile N. Effects of low dose niacin and vitamin D on endothelial cell angiogenic function and vascular regeneration under lipotoxic conditions. London Health Research Day, London, Ontario. May, 2018.

[Poster]

Peters K, Wilson R, Sawyez C, Sutherland B, Borradaile N. Effects of low dose niacin and vitamin D on endothelial cell angiogenic function and vascular regeneration under lipotoxic conditions. Physiology & Pharmacology Research Day, Western University, London, Ontario. November, 2017.

[Poster]

Peters K, Wilson R, Sawyez C, Sutherland B, Borradaile N. Effects of low dose niacin and vitamin D on endothelial cell angiogenic function and vascular regeneration under lipotoxic conditions. Canadian Lipoprotein Conference, Ottawa, Ontario. October, 2017.

[Poster]

Kia M. Peters. Why are we, as Indigenous People, unhealthy?. Caldwell First Family Services Expo and Retreat, Niagara Falls, Ontario. March 25, 2017.

[Oral Presentation]

Peters K. Sawyez C, Borradaile N. Effect of vitamin supplementation on endothelial cell angiogenic function during lipotoxicity. London Health Research Day, London, Ontario. March 28, 2017.

[Poster]

Kia M. Peters. Did you take your vitamins today?. Caldwell First Nation Annual General Meeting, Leamington, Ontario. February 2017.

[Oral Presentation]

Peters K. & Borradaile N. Vitamin Supplementation for vascular regeneration during metabolic syndrome. Physiology & Pharmacology Research Day, Western University, London, Ontario. November 2016.
[Poster]

TEACHING EXPERIENCE

January 2018- April 2018	Teaching Assistant, Western University, London, ON Department of Physiology and Pharmacology <i>Physiology 4100B - Digestion, Metabolism & Metabolic Disease</i>
May 2017- August 2017	Teaching Assistant, Western University, London, ON Department of Physiology and Pharmacology <i>Pharmacology 2060 Summer Session - Introduction to Pharmacology and Therapeutics</i>
January 2017- April 2017	Teaching Assistant, Western University, London, ON Department of Physiology and Pharmacology <i>Pharmacology 2060B - Introduction to Pharmacology and Therapeutics</i>