IMPAIRMENT OF ENDOTHELIAL INWARDLY RECTIFYING K+ CHANNELS IN OBESITY

by

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ABSTRACT

Obesity-induced stress affects the vascular endothelium, leading to endothelial dysfunction (ED), a precursor to cardiovascular disease. Despite this, not all vasculature exhibits ED with obesity; visceral adipose arteries (VAA) are impaired, whereas subcutaneous adipose arteries (SAA) retain endothelial function. Our recent study identified that the impairment of inwardly rectifying K+ channel (Kir2.1) is responsible for ED in arteries within visceral adipose tissue (VAT) in cases of obesity while function of Kir2.1 within subcutaneous adipose tissue (SAT) endothelium is unaffected by obesity. However, the mechanisms underlying Kir2.1 impairment remain unclear. Kir2.1 is suppressed by increased levels of fatty acid derivatives within cells in obesity. Obesity is closely associated with elevated levels of circulating fatty acids and previous study has revealed that VAT in obesity is producing more long chain fatty acids (LCFA) and are lipolytically more active. This LCFA requires a transporter to get into the cells where CD36 comes to the play. CD36 is a fatty acid translocase that might have a role in transferring fatty acids from VAT into endothelial cells (EC) and impairing Kir2.1. Therefore, the purpose of the study was 1) to investigate the VAT being a mediator in endothelial Kir2.1 dysfunction in obesity 2) to examine if VAT mediating impairment in Kir2.1 is CD36-dependent. We hypothesized that 1) Endothelial cells exposed to VAT in obesity induces impairment in Kir2.1 and 2) VAT inhibits Kir2.1 current which is driven by CD36 resulting in ED.

We did an *in vitro* mice study to assess whether VAT and SAT from lean or diet-induced obese mice influenced the function of Kir2.1 channel. Our findings indicated that VAT from obese mice decreased Kir2.1 function with having no effect on channel expression, while adipose tissue (AT) from lean mice as well as SAT from obese mice did not impact Kir2.1 function compared to untreated control cells. Moreover, EC exposed to VAT from obese mice showed a remarkable rise in uptake of fatty acid driven by CD36. Our *ex vivo* pressure myography experiments on arteries provided further evidence. These studies showed that knocking down of CD36 restored the endothelium's ability to respond to flow by recovering Kir2.1 and eNOS function. Therefore, these results offer novel insights into the role of VAT and the function of CD36 in mediating the impairment of Kir2.1 and leading to ED.

Key words: endothelial dysfunction, inwardly rectifying K+ channel, long chain fatty acid, CD36.

Chapter 1

REVIEW OF LITERATURE

1.1 Obesity

Obesity is a major health concern in global population. It is a health condition characterized by the accumulation of excess body fat to a degree that can negatively impact one's health. It includes a complex set of factors that contribute to conditions like diabetes, cancer, cardiovascular problems and various metabolic diseases[1]. Per the World Health Organization (WHO) in 2022, 1 in 8 people were living with obesity in the world and the global count of overweight adults aged 18 and above exceeded 2.5 billion, with over 890 million falling into the category of obesity[2]. The WHO uses Body Mass Index (BMI) as a tool to assess overall body adiposity. The US Centers for Disease Control and Prevention (CDC) and the WHO designate a normal BMI range as 18.5 to 24.9 k kg/m^2. Overweight is categorized as a BMI ≥ 25 kg/m^2, and obesity is identified with a BMI ≥ 30 kg/m^2, while a BMI of ≥ 40 kg/m^2 is recognized as severe obesity[3]. National Health and Nutrition Examination Survey (NHANES) data in 2018 revealed that 31.10% Americans were overweight, 42.50% were obese and 9% were severely obese (**Figure 1.1**). Approximately 35.48% of the US population is affected by general obesity, while about 53.13% are affected by abdominal/visceral obesity[4].

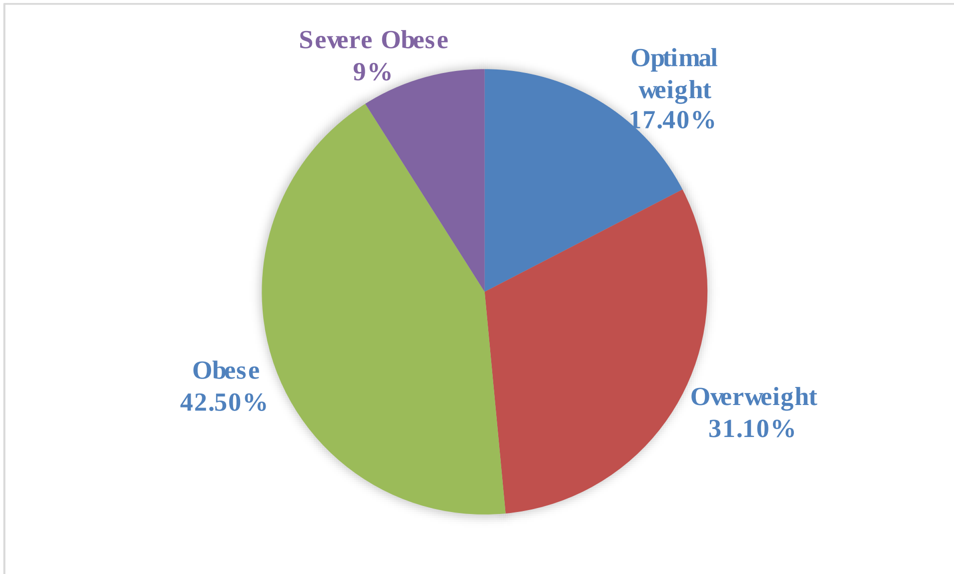


Figure 1.1: Pie chart of overweight and obesity, NHANES;2018

In the United States, cardiovascular disease (CVD) continues to be the primary cause of death, and the link between obesity and increased risk of CVD is extensively documented. However, various types of obesity present different risks for CVD: specifically, intra-abdominal or visceral obesity is strongly associated with the onset of CVD[5]. Visceral obesity exceed the general prevalence which is likely due to the use of BMI over waist-to-hip ratio to clinically assess obesity[6]. Using waist circumference (WC) as a measure of visceral obesity offers insight into body composition and provides valuable additional information when considered alongside BMI and is recommended to assess WC along with BMI in clinical evaluations[7,8]. It is evident that the global economic impact of obesity was $1.96

trillion in 2020 which will be surpassed by $4.32 trillion by 2035[9] and with the fact that US economic burden for obesity range $147 billion to $210 billion annually[10], it is an urgent to identify mechanisms governing the development of CVD in visceral obesity.

Obesity results due to imbalance between the intake and expenditure of energy acquired from food[11]. The hypertrophy of fat cells after storing extra energy is a pathological indicator of obesity. The clinical issues linked to obesity arise from enlarged fat cells, either due to the mass of excess fat or because of increased release of free fatty acids (FFA) from these enlarged cells[12]. Obesity serves as a risk factor for numerous diseases, primarily cardiovascular disease (CVD) and other metabolic diseases like dyslipidemia, diabetes, liver diseases, cancer, etc **(Figure 1.2).** While the precise mechanisms connecting obesity to CVD are not completely comprehended, it is thought to involve inflammation in adipose tissues (AT) and contributing metabolic responses triggered by increased body fat[13]. These changes in AT could be the reason affecting different vasculature in obesity. Mainly vascular complications like CVD lead to much morbidity and mortality in obesity[14]. The CVD progresses if the individuals with obesity induced endothelial dysfunction (ED), characterized by the loss of the endothelium's physiological properties, such as its ability to promote vasodilation and anti-coagulation. The link between obesity and CVD strengthens further when the distribution of fat is taken into account[15].

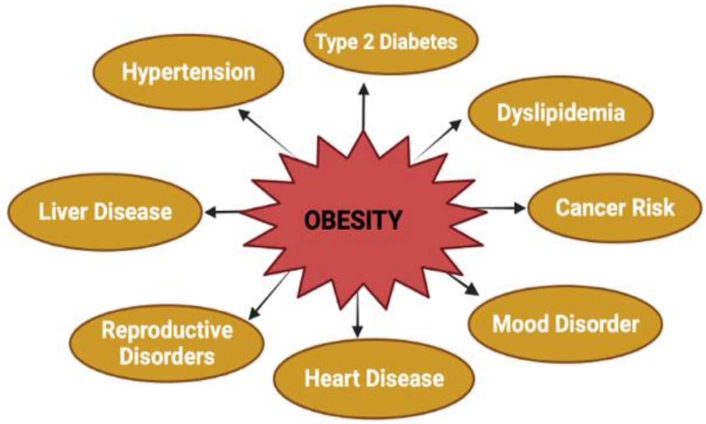


Figure 1.2: Diagram of metabolic complications of obesity. Created with BioRender.com

Previous studies have demonstrated that the endothelial function exhibits impairment in response to endothelium dependent vasodilators in obesity[16]. Various metabolic factors are implicated as a possible mechanism causing ED. It is important for endothelium to function properly to preserve homeostasis, maintain blood flow and vascular tone, and prevent genesis of vascular diseases[17]. The inner lining of the vessels is highly sensitive to both mechanical forces generated by the movement of blood, such as shear stress, and chemical signals that secrete vasoactive and trophic factors[18]. These elements play a pivotal role in controlling multiple features of vascular function, that includes vascular tension, adherence of cells, platelet activity, plasma clotting, the expansion of vascular smooth muscle cells, and inflammation within the vasculature wall[19]. ED, with marked lowering of dilating factors and an increase in constricting factors, serves as the initial stage leading to the deterioration of local blood circulation. This damages the tissues from vessels leading to conditions such as coronary circulatory failure[20]. A recent study has revealed that obesity-induced ED manifests uniquely in various vascular types. Specifically, subcutaneous adipose artery (SAA) from obese human subjects exhibits a robust reaction to endothelium-dependent agonists when subjected to flow and acetylcholine. However, visceral adipose artery (VAA) displays a diminished response, indicating a state of ED[21]. These ED are believed to be the result from compromised function of endothelial nitric oxide synthase (eNOS) or decreased bioavailability of nitric oxide (NO)[17] , but the underlying mechanism with the changes in SAA versus VAA is not well understood. Therefore, goal of my study was to identify the mechanism how ATs are affecting the arteries embedded on it and leading to ED.

1.2 Visceral Obesity

Visceral obesity is an excessive deposition of fat that develops over time around the center of the body. Visceral adipose tissue (VAT) is situated in the abdominal cavity, surrounding internal organs, especially those related to digestion. It includes omental AT connected to the stomach, mesenteric adipose tissue attached to the intestine, and epiploic adipose tissue associated with the colon[22]. Obesity, particularly visceral obesity, is recognized as an independent cardiovascular risk factor. Findings from epidemiological investigations suggest that the presence of visceral obesity is linked to a higher prevalence or occurrence of CVD and overall mortality[23–25]. Visceral obesity is frequently linked to the up-regulation of adhesion molecules in the endothelium, increased hypercoagulability, reduced fibrinolytic activity, and elevated levels of von Willebrand factor and endothelin-1. Each of these elements contributes to the initiation and advancement of atherosclerosis[26–28]. A previous study conducted to explore the biological characteristics of both VAT and SAT (subcutaneous adipose artery) through gene-expression analysis revealed that higher portion of all genes in VAT encode secretory proteins known as adipocytokines, including leptin and adiponectin as compared to the SAT[29]. Adipocytes release adipocytokines to regulate the function of various organs and cells and these adipocytokines play a crucial role in regulating metabolism of fat and managing oxidative stress[30]. For instance, leptins have the capacity to trigger insulin resistance, while adiponectin enhances insulin sensitivity[30]. Hence it indicates that leptin might serve as a significant link between central (visceral) obesity, hypertension, and metabolic syndrome[30] . The mass of VAT is correlated with the progression of insulin resistance, unlike the overall or subcutaneous tissue mass, which does not exhibit the same relation[31,32] . These dysfunctional physiological changes within the VAT could be the reason contributing to the ED.

A previous study has confirmed that adipocytes in VAT exhibit higher lipolytic activity compared to adipocytes on SAT. Consequently, they play a more significant role in contributing to plasma free fatty acid levels[31,33]. This finding was specifically noted in individuals with diabetes and obesity, where there was a notable increase in leptin gene expression and a decrease in expression of adiponectin gene in mesenteric VAT[22]. Behavioral factors, such as excessive food intake or lack of physical activity, can lead to the buildup of visceral fat, resulting in adipocyte dysfunction. Excessive production of harmful adipokines, such as leptin, and insufficient secretion of protective adipokines, like adiponectin, are likely key mechanisms in behavior-related disorders, along with diabetes, hypertension, hyperlipidemia, ultimately contributing to CVD[24]. Another study also showed that in obesity, the process of breaking down triacylglycerol to glycerol and free fatty acid from VAT is correlated with various elements related to fat and glucose metabolism and increase in blood pressure[34]. Hence, with all these known changes within the AT, it is very important to explore impact of these AT on the adjacent arteries and their function.

1.3 Sex and age prevalence in obesity

The importance of sex on health have been increasingly recognized by clinicians, researchers and policymakers[35].The inclusion of sex considerations in science is crucial for filling in gaps in knowledge and producing information that is more precise and thorough. According to data from the WHO, obesity affects 650 million adults worldwide, with a prevalence of 11% in men and 15% in women. This emphasizes the importance of understanding and examining sex differences to develop targeted interventions and strategies and addressing obesity-related challenges including prevention and treatment of this disease[36]. The sex prevalence in obesity is associated with the stages of obesity, sociocultural, environmental, and psychological aspects. Sex differences extend to mental pressure and social behavior, contributing to variations in occurring obesity among different sexes. Perceived stress and its relation to eating habits have been found to be related with weight gain, being more prominent in women[37,38]. Understanding these sex-specific influences is important for developing targeted interventions and strategies to address obesity and its related factors. A study conducted in 2015 compared the prevalence of obesity over the past 35 years from 1980-2015. Throughout this period, the rates of getting obese have consistently been in the upper limit in females than in male. However, recent years have shown a pattern of diminishing sex differences in the overweight population, while sex differences in obesity have remained relatively constant. So, this statistical report reveals that prevalence of overweight and obese females is higher as compared to males over the time interval[39].

In the most recent decade between 2007-2008 and 2015-2016, a study revealed that the prevalence of youth obesity was 16.8% in 2007-2008 and increased to 18.5% in 2015-2016. The adjusted overall trends during this period were found to be non-significant, suggesting that there was no statistically significant change in youth obesity prevalence over this timeframe. Likewise, the adjusted overall weight gain in age group 2 -19 years showed an increment from 10.1% to 13.9% throughout the study period. However, between 2007-2008 and 2015-2016, the prevalence of adult obesity in the adjusted model revealed an overall significant trend with an increase from 33.7% to 39.6% and adult severe obesity from 5.7 to 7.7%. Therefore, over that study period, there was a persistent increase in the prevalence of obesity in adults with no significant increase observed in youth[40]. This highlights distinct patterns in the prevalence of obesity between these age groups over the specified time frame. Since past data revealed the variation in prevalence of obesity in different sexes and ages, I have included equal number of mice for both sex and age between 8 to 12 weeks in my study to examine the effect of diet-induced obesity in AT and vascular level.

1.4 Overview of blood regulation and vascular endothelium changes

The endothelium is a single-cell layer that lines the interior surface of blood vessels, serving as a boundary between the bloodstream and the vascular smooth muscle[41]. These cells regulate vessels tone by producing the elements that influence the contraction of the underlying smooth muscle[42].

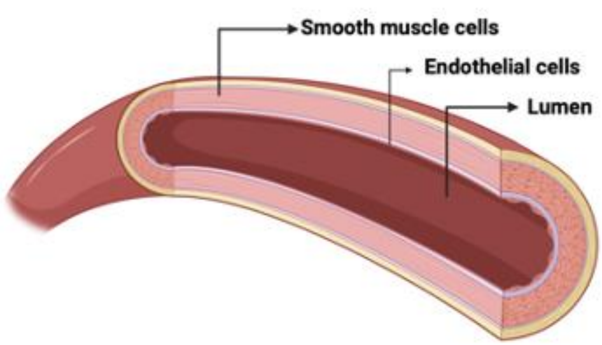


Figure 1.3: Diagram of normal endothelium with lumen in the interior surface and vascular smooth muscle outside. Created with BioRender.com

The function of the endothelium, including its regulation of vascular tone, can be influenced by mechanical forces such as flow-mediated shear stress. Fluid shear stress refers to the frictional force exerted on the walls of blood vessels by the flowing blood. This force plays a significant role in regulating vascular function. The mechanical stimulation from shear stress influences various endothelial functions by the production of endothelial factors and contributes to the maintenance of vessel tone and homeostasis[43]. Previous human studies have suggested that shear stress induces endothelium-dependent vasodilation in conductance vessels. This demonstrates the influence of shear stress on the ability of blood vessels to dilate through endothelial responses[44]. The inner lining of the vessels is highly sensitive to both mechanical forces generated by the movement of blood, such as shear stress, and chemical signals[19]. It maintains homeostasis and modulate vascular tone in vessels by producing different elements including NO, endothelin, endothelial growth factors, adhering molecules, and plasminogen inhibitors, endothelial-derived hyperpolarizing factors (EDHFs), and reactive oxygen species(ROS) in accordance to meet the changes in the body [45,46]. NO is a gas produced in endothelial cells by the nitric oxide synthase (NOS). NO has properties that allow to regulate and maintain vascular homeostasis. It regulates normal functioning of the endothelium by regulating local cell growth and modulating vascular dilator tone. The regulatory function of NO is responsible to maintain the health by proper vascular function. A reduction in the vasodilators, and an increase in contracting factors can lead to ED. This imbalance in vasoactive substances disrupts the normal regulatory functions of the endothelium, contributing to impaired vasodilation result in ED. Conditions related to atherosclerosis risk, like hypertension, hypercholesterolemia, DM, smoking, and heart failure, are linked to a reduced NO release into the arterial wall. This reduction can occur due to altered production or high oxidative deterioration. The low NO in this pathological condition contributes to significant issues in endothelial equilibrium. The impaired ability of the endothelium to release NO is a key factor for reduced vasodilation and progression of vascular dysfunction like development of atherosclerosis and leading to CVDs[41,45]. Dysfunctions of the small vessels initiate an elevation of peripheral resistance, contributing to the progression of cardiovascular issues. Due to the close connection between endothelial function and overall cardiovascular (CV) health which is a precursor to atherosclerosis and risk factor of CVD[41,47,48], the endothelium is a commonly studied aspect of CV health. We believe, among various mechanism that affects the normal functioning of ECs, local ATs might also have some role. Therefore, in my study I am investigating the changes in expression and function of proteins in endothelial cells of vessels after treating them with different AT.

1.4.1 Endothelial Dysfunction (ED)

ED refers to the detrimental alterations in endothelial physiology, leading to the process of developing atherosclerosis and CVD[49]. The alteration in balance in vasoactive substances contributes to impaired endothelium dependent vasodilation, a functional characteristic of ED that disrupt vascular homeostasis, contributing to pathophysiology of CVD. In addition, ED also favors atherogenesis that serve as a marker of CVD progression[49]. ED is considered a systemic disorder because it is not only confined to the coronary arteries; instead, it impacts peripheral vascular beds as well. This includes both conduit arteries, and resistance arteries. This makes the endothelium a useful marker of overall vascular health[49].

ED, marked by the loss of NO which is a major vasodilator and anti-inflammatory agent due to impaired eNOS, is a progression factor in CVD. The physiological properties of endothelial cells (EC), when defected, can shift the endothelium from vessels toward a pro-inflammatory, prothrombotic, and proatherogenic phenotype. This transformation results in leukocyte adhesion and platelets activation. Additionally, there is reduced NO production, decreased formation of EDHF, and an increase in vasoconstrictive factors, like angiotensin II (Ang II) and prostaglandin (PGH2). These alterations collectively contribute to the pathogenesis of various cardiovascular conditions progression in obesity[50,51]. Also, dysfunctional adipocytes secrete angiotensinogen, a component of the renin-angiotensin system (RAS). This will overexpress angiotensinogen in the RAS, promoting the production of ROS, and worsening the atherogenic and thromboembolic action of EC. Hence, inhibiting the inflammatory mechanisms and controlling obesity can alleviate the risks associated with cardiovascular complications[52].

Many of the risk factors associated with atherosclerosis and CVD morbidity and mortality, are also linked to ED. Risk factors such as hyperlipidemia, diabetes, hypertension, and smoking contribute to ED through mechanisms such as the excess production of ROS or elevated oxidative stress. This heightened oxidant stress is involved in the pathogenesis of ED and indeed, involves many CVD, including hypercholesterolemia, atherosclerosis, hypertension, and heart failure[53]. Previous studies in both animal and human have demonstrated that obesity results in reduction in vascular density within AT and is associated with metabolic dysfunction, which is more prominent in VAT as compared to other fat depot[54]. The distinct mechanism behind a specific depot being more dysfunctional is not well understood but this is leading to ED as consequence in arteries of VAT. It is widely believed that obesity causes diminished capillarization in adipose cells, resulting in reduced nutrient delivery. This, in turn, contributes to dysfunctional adipocytes and insulin resistance[54]. The study in mice has provided supportive evidence about a role of vascularization in obese AT is associated with metabolic dysfunction. Additionally, obesity induces endothelial cell activation in AT, accompanied by capillary rarefaction. AT of obese mice exhibit elevated levels of adhesion molecules in its EC[54].

In obesity, several fundamental actions combine and contribute to the vascular function impairment. These actions include elevated oxidative stress, increased vascular contractility, increased peroxynitrite, perivascular adipose tissue (PVAT) dysfunction, increased arginase activity and increased vascular inflammation. This increased oxidative stress can reduce NO bioavailability while elevated peroxynitrite may inactivate eNOS. Evidence indicates that high oxidative stress has a significant role in the ED. Generation of oxygen-derived free radicals in high amounts such as the superoxide anion is linked with impairment of endothelial vasomotor function in atherosclerosis models[55]. Also increased vascular contractility results in increased vascular inflammation and stimulated endothelial cells apoptosis can cooperate to create vascular dysfunction[56].

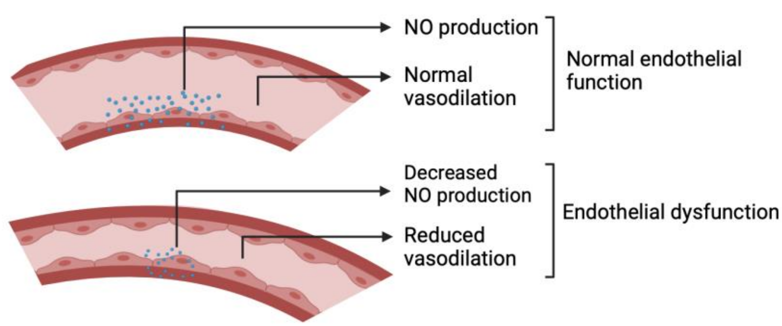


Figure 1.4: Diagram of endothelial dysfunction with reduced NO production. Created with BioRender.com

In central obesity, elevated plasma levels are associated with increased number of free fatty acids (FFA). Past study has demonstrated that pathophysiological concentrations of FFAs can affect the function of endothelium, as assessed through measurements of agonist-stimulated and flow-mediated vasodilatation[57]. This indicates that the increased presence of high FFAs, particularly in the context of central obesity, may contribute negatively to the function of EC. Conditions characterized by elevated plasma FFA concentrations have an impact on NO-mediated vasodilation. FFAs can bring significant alterations in crucial intracellular signal transduction pathways, including ion transport. This includes effects on key elements such as Na/K ATPase, sodium (Na) and potassium (K) channels, and calcium (Ca) currents[15]. In human studies, it has been observed that the distribution of fat, rather than obesity itself, has a negative impact on endothelial function. Specifically, individuals with visceral obesity, as opposed to subcutaneous obesity, exhibit impairment of endothelium-dependent vasodilatation mediated by flow in the brachial artery[58].

This impairment in flow-induced vasodilation (FIV) is indicative of compromised endothelial function and is a hallmark of vascular dysfunction associated with obesity indicating diminished ability of arteries to dilate in response to changes in blood flow[16]. FIV is affected by impaired NO production as it is a crucial vasodilator and anti-inflammatory agent[59]. Previous study showed that various other pathways involving prostacycline[60], EDHF leading to hyperpolarization and vasodilation [61–64], effect of sympathetic activation on shear-mediated NO release[65], flow-sensitive Kir (inwardly rectifying K+)[66,67] influence the FIV. The inhibition of the flow dependent response for vasodilation in mouse and human mesenteric arteries in obesity seems to be influenced by the depletion of the endothelial flow-sensitivity of the Kir (inwardly rectifying K+) channel. The previous study showed that this effect is attributed to obesity-induced thinning of the glycocalyx[68,69]. Hence, in this study I have used the arteries from VAT and SAT to examine the effect of different depot in vasodilation function.

1.4.2 Obesity and visceral adipose artery (VAA) endothelial dysfunction

Obesity being the multifactorial metabolic disease has a detrimental effect on vascular endothelial function. However, not all vascular beds get similar obesity-induced endothelial dysfunction. In obese mice, SAAs demonstrate a robust reaction to endothelium-dependent agonists like flow and acetylcholine. However, VAA show a comparatively diminished response, indicating ED[21]. The specific mechanisms underlying the difference in endothelial function between SAA and VAA in obesity are not well understood. However, it is believed that the mechanisms contributing to VAA ED involve a combination of impaired function of eNOS and/or reduced NO bioavailability[17,70] . A study showed that the diet induced obese mice which were in a high fat diet for eight weeks promotes ED with reduced flow induced vasodilation in VAA but not in SAAs[69]. With the fact that arteries were getting affected in 8 weeks of diet, I investigated the diet-induced mice model within that time frame to examine the effect of AT on vascular endothelial function.

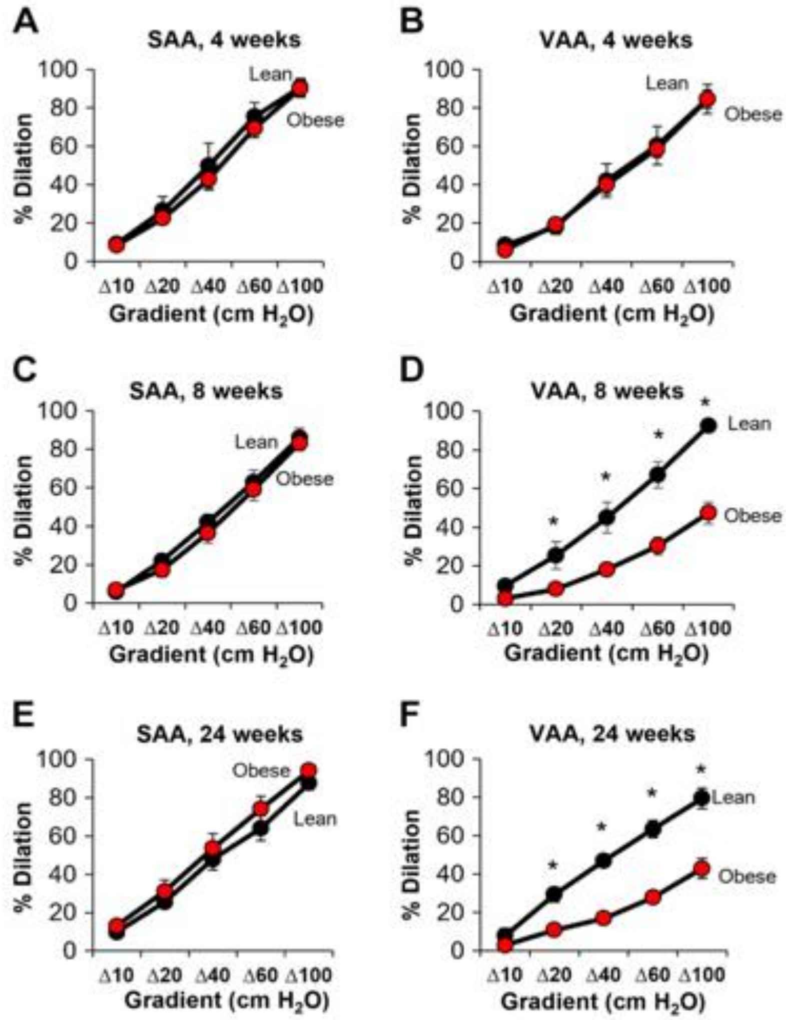


Figure 1.5: Diet-induced obesity contributes endothelial dysfunction in VAAs but preserves function in SAAs in a mouse model. Adapted from Sang Joon Ahn, et al 2022.

1.5 Inwardly Rectifying K^+ Channels and Flow Induced Vasodilation

Inwardly rectifying K^+ channels (Kir) are a significant type of K^+ channel that were first identified in skeletal muscle. These channels are expressed in many cell types like endothelial cells, cardiac myocytes, neurons, glial cells, osteoclasts, oocytes and epithelial cells[71]. In endothelial cells, inwardly rectifying K+ channels (Kir channels) are reactive to fluid shear stress, a significant element in the regulation of endothelial function[72–74]. Despite their sensitivity to shear stress, physiologically, the precise role of Kir channels in mediating flow-induced endothelial responses is not well understood. FIV is the hallmark showing the endothelial response to blood flow[75]. This physiological mechanism is crucial for the regulation of microcirculation blood flow. Endothelial K+ channels may generate the EDHF which is contributing to FIV[76]. There are two major classes of K+ channels which are expressed in vascular endothelial cells, first is inwardly rectifying Kir channels which are sensitive to fluid shear stress[72,77,78]and next is Ca^{2+}-activated K^+ (KCa) channels that are Ca^{2+} level sensitive [79].

Kir channels have seven subfamilies (Kir1–7) depending on their distinct biophysical properties[80] . Among them, Kir2 channels exhibit powerful inward rectification. Kir2.1 and Kir2.2 were shown in the human aortic ECs[77] whereas microvascular ECs primarily expressed Kir2.1[81]. The mesenteric ECs exhibits shear stress-sensitive Kir currents with Kir2.1 being the primary functional Kir channel. The distinctive biophysical properties of Kir channels, characterized by activation in response to membrane hyperpolarization and high extracellular K+, have led to the suggestion that these channels function as enhancers, amplifying the electrical activity produced by other K+ channels in vessels[66,82]. In our previous work, we showed that endothelial Kir channels play a role in the activation of eNOS in response to flow[74]. This signaling pathway is a major driver of various endothelial responses triggered by blood flow. In mesenteric circulation, Kir2.1 is a major contributor to FIV which is controlled by Akt/eNOS signaling and endothelial NO generation[68,74]. This suggests that the control of FIV by Kir2.1 channels primarily involves the NO-dependent pathway. Moreover, the study revealed that the activation of endothelial Kir2.1 during flow leads to the eNOS activation and subsequent NO production. Importantly, this activation is found to be not depending on calcium-sensitive potassium current. The study also confirmed that endothelial Kir2.1 supports flow-induced activation of Akt1, a kinase known for its role in activating eNOS[83]. These findings suggest that endothelial Kir2.1 serves as a primary upstream regulator which control flow-induced endothelial signaling. Impairment of this regulatory mechanism is key to ED associated with obesity.

The study investigated the contribution of endothelial Kir2.1 in SAAs and VAAs in both lean and obese mice. The objective was to examine whether Kir2.1 plays a distinct and differential role in contributing to FIV in these two types of adipose arteries.

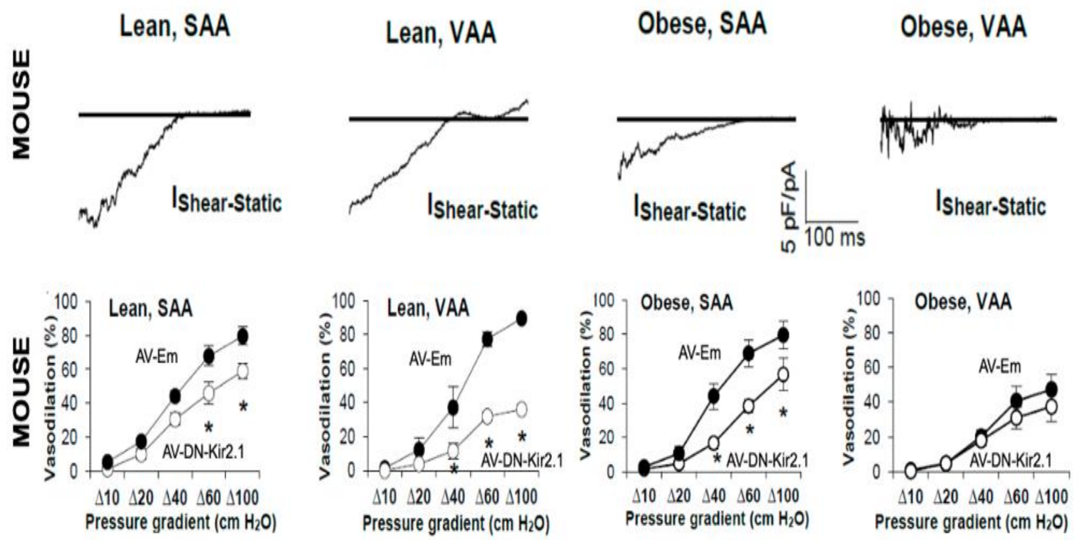


Figure 1.6: The involvement of endothelial Kir2.1 in FIV is maintained in SAAs, but not in VAAs of obese mice; Adapted from Sang Joon Ahn ,et al.

It demonstrated that Kir2.1 in endothelium supports FIV specifically in SAAs but not in VAAs from the mice with obesity. To assess FIV, arteries were pressurized and preconstricted and were treated with an endothelial membrane-targeted adeno-associated virus (Em-AV) or the endothelium-specific dominant-negative Kir2.1 adeno-associated virus (DN-Kir2.1-AV), after 8 weeks of a standard laboratory diet or a 42%kcal western diet. The impact of DN-Kir2.1-AV expression in the SAA and VAA on FIV show that VAAs have impaired functional endothelial Kir2.1 in obese mice. However, the SAAs and VAAs from lean mice and SAAs from obese mice preserve endothelial function[69].

1.6 Adipose tissue cellular changes in Obesity

AT is a multifaceted endocrine organ comprised of various cell types such as adipocytes, pre-adipocytes, mesenchymal stem cells, as well as immune cells like macrophages and lymphocytes[84]. Adipocytes within AT contribute to the increased pro-inflammatory condition observed in obesity which produces both inflammatory and anti-inflammatory proteins. The higher expression and synthesis of proinflammatory cytokines, including interleukin-6[21] , plasminogen activator inhibitor-1, and the production of regulated normal T cell expressed and secreted protein, are seen in VAT, that shows positive correlation with obesity[85–87]. An augmentation in VAT is proceeding towards the numerous metabolic disorders, including diabetes and cardiovascular issues[84]. In addition to adipocyte cells, the AT have some non-adipocyte cells such as progenitor cells, leukocytes, and other immune cells which shows it contains both endocrine and immune functions. In cases of obesity, there is a rise in fat accumulation within the VAT, typically accompanied by low grade and chronic inflammation, resulting in a range of metabolic diseases. A prior investigation demonstrated that the surgical extraction of VAT from rats enhances metabolic conditions and prolongs their survival[88]. Obesity induces stress on adipocytes, leading to the production of proinflammatory chemokines to attract immune cells. These immune cells infiltrate AT, contributing to its inflammatory state. While it is widely acknowledged that immune cells promote inflammation, there is still debate about the factors initiating the attraction of these cells. Given the vital role of cellular composition in regulating VAT function, it is essential to investigate specific cellular populations within VAT, identify any abnormalities in these populations, and assess their interactions with adipocytes, rather than studying VAT as a whole organ[84]. Studies have indicated that obesity is linked to changes in the types of macrophages present, resulting in a rise in proinflammatory macrophages M1. Specifically, in obese VAT, there is an elevation in M1 proinflammatory macrophages, particularly a notable increase in double negative (DN) macrophages that lack both M1 and M2 markers. Meanwhile, anti-inflammatory M2 macrophages tend to decrease in abundance[89,90]. These cellular changes in VAT could be one of the factors triggering the vasculature function by releasing chemicals or fatty acid to adjacent vessels. Hence, I targeted to examine if fatty acids were being released more from VAT into the arteries and causing ED.

1.7 Lipolysis and Fatty Acid

Lipolysis is the process of breaking down triglycerides (TGA) stored in adipocytes into one glycerol and three FFAs through the action of lipases, which include hormone-sensitive lipase and adipose tissue TGA lipase[91]. As obesity advances, the dysregulation of lipolysis indicates the emergence of comorbidities associated with obesity and is a significant factor contributing to the overall levels of circulating FFAs. There is documentation indicating that the release of FFAs escalates as the VAT mass increases[92]. The adipocytes of VAT are lipolytically more active, and contributes more to the plasma FFA levels[93]. This adipocyte lipolysis contributes to the rate of lipid oxidation. This implies that the link between higher levels of visceral fat and the metabolic issues related to obesity might result from an excess of FFAs originating from the lipolysis of VAT. Elevated FFAs, resulting from lipolysis from AT, are observed in central obesity, and increased levels of FFAs can lead to insulin resistance[94]. FAs are high-energy molecules and vital components of cell membranes, serving significant metabolic functions both at the cellular and systemic levels[95]. They are associated with the functions of channels and receptors and have hormone-like effects that impact the growth and differentiation of preadipocytes[96]. As explained, within AT, FAs play an important role in their primary function, being involved in the storage of TGA. The size of adipocyte cells and the regional expansion of AT are determined by the presence of unilocular lipid droplets. The composition of fatty acids significantly influences both the size and number of adipocytes[97,98].

Increased levels of FFAs originating from adipocyte cells contribute to ED. Human study has shown compromised responses to vasodilators dependent on endothelium in individuals subjected to lipid infusions[57,99]. Research indicated that the compromised microvascular function observed in obese individuals is influenced by elevated levels of FFAs, to some extent. Elevated FFAs in lean women led to a deterioration in microvascular function, comparable to that observed in obese women. Conversely, lowering FFA levels in obese women resulted in an enhancement of microvascular function, reaching a level similar to that seen in lean women. Hence, the increased levels of FFAs in obesity contribute to dysfunction in the microvasculature, potentially leading to the onset of insulin resistance, hypertension, and CVD associated with obesity[99]. Hence, my approaches aimed to examine if increased FFAs concentrations can promotes microvascular ED and causes CVD.

1.8 Fatty acid transporter/CD36 and LCFA

FFAs transport is a simple diffusion process for short and medium chain FFAs[100]. The transfer of circulating long-chain fatty acids (LCFAs) to parenchymal cells involves their movement across the EC barrier. The movement of LCFAs across the plasma has been understood to occur through a protein-mediated mechanism, which includes one or more LCFA-binding proteins. These proteins comprise a family known as fatty acid transport proteins and fatty acid-binding protein along with fatty acid translocase called cluster of differentiation 36 (FAT/CD36)[101]. CD36, also recognized as fatty acid translocase or FAT, was involved in importing fatty acids, specifically LCFA[102]. CD36 is an extensively glycosylated integral membrane protein with a molecular weight of 80 kDa, and it is broadly expressed. It is present in various cell types such as platelets, adipocytes, microvascular endothelial cells, immune cells, myocytes, retinal and mammary epithelial cells[103]. CD36 has an essential role in lipid homeostasis and functions in high-affinity tissue uptake of LCFAs into muscle and adipose tissues of rodents and humans[104,105].

In conditions of elevated fat availability, CD36 contributes to the accumulation of lipids and metabolic dysfunction. However, it remains unclear whether the uptake of fatty acids depends on CD36 in ECs, parenchymal cells, or a combination of both. Recent finding suggests that CD36 serves as a significant controller of the metabolic pathways associated with insulin resistance[43]. If the availability of FA surpasses the function of adipose tissue to store TGAs and/or the ability of muscles to oxidize FA, it can lead to the induction of insulin resistance[106]. The abundant glycosylation raises the apparent molecular weight of CD36 from 53 to 88 kDa. The protein is heavily modified post translationally as palmitoylation and glycosylation[107]. CD36 undergoes N-linked glycosylation, causing the protein to appear on 78–94 kDa on SDS-PAGE, despite the theoretical mass being 53 kDa[108,109].

CD36 facilitates the cellular uptake of LCFA[110,111] and involved in the process of lipolysis[112]. However, the direct involvement of CD36 in the process of lipolysis has not been clearly established, and the molecular mechanisms governing the mobilization of LCFA from adipocytes are not thoroughly understood. It is evident that CD36 is expressed in both ECs and adipocytes. Experiments involving mice lacking the CD36 system either systemically[110] or specifically in ECs[113] have shown that it is essential for the typical uptake of LCFAs in multiple organs. The activity of CD36 in both the endothelium and adipocytes governs the systemic distribution of LCFA and CD36 facilitates the mobilization of fatty acids from adipose tissue[114]. Previous research examined the function of CD36 in AT and found that it exacerbates both inflammation and cell death in mice adipocytes exposed to a high-fat diet[115]. Additionally, the study demonstrated that the expression of CD36 in cultured primary adipocytes leads to increased expression of inflammatory cytokines, suggesting that CD36 plays a role in promoting inflammatory responses in macrophages and in adipocytes. Moreover, the inflammatory reactions in these two cell types are coordinated and are CD36 dependent. Additionally, mice lacking CD36 (CD36 KO) exhibit resistance to diet-induced obesity caused by a high-fat diet compared to wild-type (WT)[115]. The findings additionally verified that mice with an absence of CD36 showed significant protection against obesity induced by a high-fat diet, which was linked to elevated plasma insulin and glucose levels and diminished glucose tolerance[116–118]. Since CD36 is promoting more lipolysis, inflammation and facilitating the intracellular FFA mobilization, I have focused on investigating its role in VAT with the hypothesis that CD36 mediates ED by translocating LCFA from VAT into the ECs.

1.9 Conclusion

It is evident that obesity is the primary risk factor for the CVD along with various other metabolic diseases. The dysfunctional changes in AT of different types could serve as a major indicator leading to the ED in vasculature. With the evidence suggesting VAT being more inflamed and undergo more alteration in obesity, visceral obesity is more likely to affect endothelial function adversely within the vessels. Despite the alteration in endothelial function, it has been shown that only

VAAs are getting affected by obesity while SAAs maintaining normal function. It is evident that the obese VAA has impaired Kir2.1 that mediated ED whereas SAA retain endothelial function. However, the mechanism governing Kir2.1impairment is unknown and needs to be investigated. It is well known that Kir2.1 current is inherently sensitive and is inhibited by lipids. Since CD36 has been proved to translocate LCFA in various cell types including EC and adipocytes; and VAT being more lipolytically active and inflamed, there is need of additional studies to investigate the role of VAT and CD36 in impairing Kir2.1 current and causing ED. An *in vitro* mice study was conducted to explore the role of VAT in inducing impairment of vascular Kir2.1 currents, driven by CD36. Subsequently, an *ex vivo* study in mice can be employed to further investigate and confirm the involvement of CD36 in the impairment of Kir2.1 and vascular function.

Chapter 2

VISCERAL ADIPOSE TISSUE INHIBITS ENDOTHELIAL KIR2.1 IN OBESITY

2.1 Introduction

Obesity is a significant global public health challenge characterized by a multiple pathophysiology that contributes to a range of health issues, including diabetes, cancer, and cardiovascular complications[1]. It is linked to the accumulation of visceral fat which is now considered an independent risk factor for onset and progression of cardiovascular disease (CVD), being a leading cause of mortality in the United States[119]. Research has revealed that 35.48% of the United States population is affected by general obesity, while a notable 53.13% experience visceral obesity[4]. Being a significant precursor to CVD, endothelial dysfunction (ED) is distinguished by the reduction of nitric oxide (NO) production in obesity[48]. The decline in NO levels leads to a diminished dilatory response to endothelium-dependent stimuli, such as flow/shear stress and acetylcholine[21]. Previous studies have consistently demonstrated the influence of visceral obesity on endothelial function. Specifically, arteries associated with visceral adipose tissues (VAT) consistently exhibit pronounced ED, while arteries connected to subcutaneous adipose tissues (SAT) maintain endothelial function and NO production in both obese humans and rodent models of diet-induced obesity[21].

The perivascular adipose tissues (PVAT) which is situated outside of the blood vessel are clinically categorized as either SAT or VAT. The PVAT surrounding large arteries such as the aorta or mesenteric arteries is typically classified with VAT and is strongly associated with cardiovascular risk[120]. PVAT plays a significant role in regulating vascular tone, intravascular thermoregulation, and the proliferation of vascular smooth muscle cells (VSMCs)[121].

The consequence of obesity is dysfunctional changes within the adipose tissue (AT). With obesity, AT expands to accommodate the excessive caloric intake and leads to the changes in structure and cellular composition. This changes is associated with generation of pro-inflammatory state[54]. AT produces and releases a variety of proinflammatory and anti-inflammatory substances, such as adipokines, cytokines, and chemokines, which are typically elevated in obesity[122]. The alteration of different AT might play a role in ED of the vessels where these arteries are embedded. Previous studies showed that VAT has been characterized as a pro-inflammatory tissue and is more associated with a higher risk for type 2 diabetes, hypertension and dyslipidemia than SAT[123]. Previous research has demonstrated a direct link between VAT accumulation and impaired endothelial function. While the vascular function physiology in various adipose depots remains incompletely understood in humans, several studies have indicated that the mechanisms of dilation to flow in VAT during CVD are similar to those observed in the coronarycirculation[124,125]. The study revealed that VAT exhibits a more pro-atherogenic profile and demonstrates decreased vasodilator reactivity to acetylcholine (ACh) compared to SAT. Additionally, it was observed that the vasodilator reactivity of VAA to ACh was diminished in obese individuals compared to lean ones[21]. The importance of VAT is also focused by the study that showed the surgical removal of VAT from rats improves metabolic status and extends survival[88] . It has been shown that adipocytes of VAT are lipolytically more active, and it contributes more to the plasma free fatty acid (FFA) levels[93]. The composition of fatty acids significantly influences both the size and number of adipocytes[97,98]. Increased FFAs from AT lipolysis results in ED that leads to the onset of insulin resistance, hypertension, and CVD associated with obesity[99].

Previous study showed that inwardly rectifying potassium channel (Kir2.1) channels which are shear sensitive are crucial to flow induced vasodilation (FIV), and is a key indicator of endothelial function[75], They are also an upstream of endothelial nitric oxide synthase (eNOS) and NO production[74]. Endothelial Kir2.1 from human and mice study supports FIV specifically in SAAs but not in VAAs from obese mice[69]. Kir2.1 is inherently lipid sensitive and is inhibited by several lipid species like fatty acid derivatives[126]. Hence, VAT which are lipolytically very active possibly mediating Kir2.1 dysfunction in a CD36-dependent fashion. CD36 is a known scavenger receptor which activate a variety of downstream signaling cascades[127,128]**.** It has many ligands including oxidized low-density lipoprotein, apoptotic cells, and thrombospondin and is well-established in promoting the formation of atherosclerotic lesions[127]. Previous research has identified CD36 as a fatty acid translocase (FAT) that plays a significant role in maintaining lipid balance and facilitating the transport and uptake of long chain fatty acids (LCFAs) into the muscle and AT of both rodents and humans[104,129]. It also contribute in increasing the uptake of FFA into VAA endothelium[130]. But we still need to examine if CD36 is facilitating more fatty acid uptake locally from VAT into endothelial cells (EC).

Therefore, purpose of this study was to investigate if the VAT serve as a mediator in endothelial Kir2.1 dysfunction in obesity. We used an *in vitro* study where ECs were exposed to VAT and SAT from lean and diet-induced obese mice and investigated their impact. We also focused on determining the role of CD36 in obesity-induced ED in VAT ECs. We hypothesized that VAT inhibiting Kir2.1 current was driven by CD36 and that is resulting in ED **(Figure 2.1).**

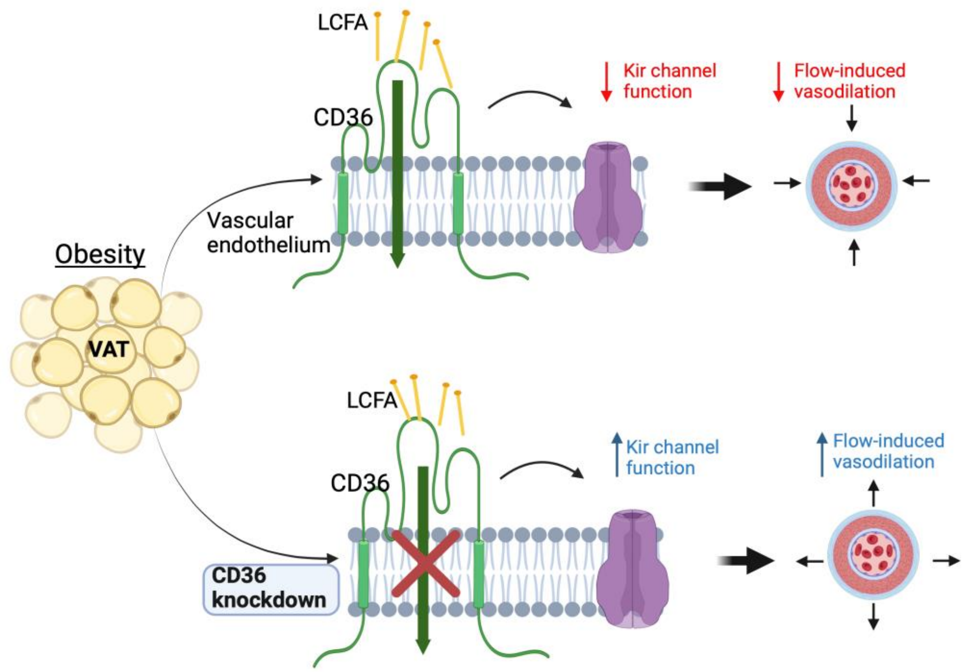


Figure 2.1: Working hypothesis of VAT impairing Kir2.1 driven by CD36.

2.2 Method

This study was designed by preparing the animal model which is approved by the Institutional Animal Care and Use Committee at the University of Delaware. *In vitro* studies were performed by maintaining human adipose microvascular endothelial cells (HAMECs) in the standard cell culture and using AT from the mice. This is followed by the examination of expression and function of Kir2.1 current in the HAMECs.

2.2.1 Animal preparation

Ten-week-old C57BL/6J WT mice from The Jackson Laboratory were randomly assigned to two diet groups with lean as a control group fed a standard laboratory diet, and the other group of mice receiving a high-fat western diet containing 42% of calories fat. These diets were maintained for 8 weeks since the past study has shown that ED is observed in WT mice after this time point of diet[68,69]. Male and female were classified accordingly and separated. Mice were euthanized by cervical dislocation followed by thoracotomy. Abdominal SAT and mesenteric VAT were isolated with the dissection and cleaning of vessels and parenchymal tissues in a HEPES buffer. These isolated AT, 5mg/ml of each type were incubated en bloc with the HAMECs prior to downstream analyses. Both male and female mice were used, and the data was combined.

2.2.2. Cell Culture

Under standard culture conditions, HAMECs were maintained using endothelial cell medium and supplements. Experiments were conducted by seeding cells at approximately 80% confluency. Cells were allowed to become a monolayer before being treated with AT obtained from either lean or obese mice at a concentration of 5 mg/ml. Cells were incubated with AT for 48 hours and AT was discarded and washed away prior to the examination of expression and function of Kir2.1. Cells without adipose treatment were used as control. To determine the effects of CD36 downregulation on Kir2.1 function *in vitro*, transfection was performed prior to AT treatments. At 50% confluency, HAMECs were transfected with siRNA to CD36 or scrambled siRNA control using lipofectamine. This was followed by AT treatment from both lean and obese mice and downstream examination.

2.2.3 Patch clamp electrophysiology

After 48 hours of AT treatments, AT was discarded, and cells were washed with RT PBS. The HAMECs were then dissociated using Versene (commercially available EDTA), resuspended, and stored on ice. The cells were subjected to whole-cell voltage clamp using thick-walled borosilicate glass pipettes with resistances ranging from 3-5 MΩ. The pipettes were fire-polished before use. Inwardly rectifying K+ currents were recorded in a high 60 mM K+ bath using an EPC10 amplifier and corresponding Pulse and PulseFit acquisition and analysis software. HAMECs were held at −30 mV, and a voltage ramp from −120 to +40 mV was applied over 400 ms. Recordings were low-pass filtered at 2 kHz and digitized at 10 kHz. Before the data collection for analysis, it was ensured that inwardly rectifying K+ currents had stabilized. To be considered for offline analysis, the patch had to maintain a consistent membrane resistance, and the series resistance had to be ≤10 MΩ throughout the voltage ramp protocol, which included at least 20 stable recordings in a static bath. Currents were normalized to cell capacitance to obtain current densities (pA/pF) for analysis. For group analysis, offline leak subtraction was conducted to ensure the accuracy of data points at −100 mV, if needed.

2.2.4 Protein isolation and Western immunoblotting

After 48 hours of AT treatment, AT was discarded and the HAMECs were washed with ice cold PBS. Cells were then homogenized in ice cold radioimmunoprecipitation assay buffer containing protease inhibitors. Lysed samples were centrifuged at 16,000 x g, at 4°C for 15 minutes to remove membrane lipids. Supernatants were collected via tube transfer and bicinchoninic acid (BCA) protein assays were performed to ensure equal loading of protein concentration(15ug). Through boiling at 70°C, the samples underwent reduction and denaturation for 10 minutes with SDS sample buffer. Subsequently, they were separated on 10% SDS gels and transferred onto nitrocellulose membranes. The membranes were then blocked using 5% BSA in TBST and undergo an overnight incubation with primary antibodies specific to Kir2.1, CD36 or the loading control β-actin at 4°C. Next day, the membranes were washed in TBST with Tween for 30 minutes. Following this, appropriate secondary antibodies were applied and allowed to incubate for 2 hours at room temperature (RT). The membranes were washed again and exposed to enhanced chemiluminescence before being imaged using the iBright FL1500 imaging system. Densitometry was performed in Image J[131] and were normalized to β actin for both Kir2.1 and CD36 values.

2.2.5 Fatty acid uptake

To investigate the role of CD36 in cells treated with different AT in driving ED *in vitro*, its function was evaluated with fatty acid uptake. The assessment of fatty acid uptake was conducted using a QBT fatty acid uptake kit from Molecular Devices. This kit employs a BODIPY-fluorescent dodecanoic acid analog, which has been verified as an appropriate substrate for fatty acid transporters, mimicking naturally occurring fatty acids and specific quenching substance[132]. After AT treatments for 48 hours, the AT was removed and HAMECs were washed with PBS. Subsequently, the cells were then deprived of serum for one hour and was exposed to fluorescent fatty acid for 90 minutes at 37°C. Following this, the cells were used for imaging using an EVOS fluorescent microscope. Imaging was conducted at a 20x magnification, capturing at least three fields of view per sample for subsequent analysis. The mean fluorescent intensity (MFI) of the entire field of view was quantified using Image J by subtracting background and normalizing the cell number in each field of view. An average normalized MFI was calculated for each sample for analysis.

2.2.6 Statistical Analysis

A Kruskal-Wallis one-way analysis of variance ($p < 0.05$) followed by Dunn-Sidak post hoc analysis (*) was used to determine significant differences in expression of adipose treated cells. A Mann Whitney test was used to detect significant differences (*) in both types of transfected cells treated with individual AT group from obese and lean mice. A similar number of male and female mice were included ($n =$ 8/group/sex) and data were combined after testing for significant sex differences.

2.3 Results

2.3.1 Endothelial Kir2.1 function is diminished by VAT in mice exhibiting obesity.

In obesity, endothelial function of VAA embedded in VAT is robustly impaired whereas SAA embedded in SAT retain endothelial function[21,42,69,133]. Our previous study showed that endothelial Kir2.1 channels regulates the endothelial vasodilation via NO production[74]. Kir2.1 is impaired in VAT endothelium of both obese mice and humans but not in SAT or lean mice[69]. These findings led us to determine the role of VAT as a mediator in impairing endothelial Kir2.1 leading to endothelial dysfunction in obesity. To determine the role of VAT from obese mice to induce endothelial Kir2.1 impairment, VAT and SAT were isolated from both groups and incubated with HAMECs as described above. Following this, whole cell patch clamp showed Kir current recordings from control cells without AT, and cells exposed to SAT from lean mice, VAT from lean mice, SAT from obese mice, or VAT from obese mice. Kir2.1 were recorded using a protocol of voltage ramp of -140 to +40 mV over 400 ms in a 60 mM K + bath. The figures demonstrated that the Kir current density is similar in the HAMECs exposed to SAT and VAT from lean mice whereas there is reduction in Kir currents in HAMECs exposed to VAT from obese mice as compared to control **(Figure 2.2A)**. When all groups were normalized and compared to control Kir currents, only the cells that were exposed to VAT from obese mice showed significant reduction in Kir currents. This indicates that VAT impairs endothelial Kir2.1 in obesity (**Figure 2.2A**, **group data**). These findings suggest that obesity-induced alterations in VAT may results in endothelial Kir2.1 dysfunction in the mesenteric arteries. An equal number of male and female mice (n = 8/group/sex) were used for this study. After analysis, all data were combined due to the evidence of having no sex differences.

In addition, result from immunoblotting **(Figure 2.2B)** where the expression of Kir2.1 in HAMECs following exposure to AT from lean and obese mice were determined, showed that despite the VAT from obese mice impairs Kir2.1 current densities, it has no effect on endothelial Kir2.1 expression. This finding supports the past study revealing that obesity does not influence endothelial Kir2.1 expression[134]. Together this result suggest that VAT may play a vital role in promoting functional impairment of Kir2.1 channels in obesity without affecting its expression. These findings support our previous studies suggesting that VAT may promote a functional impairment of Kir2.1 channels in obesity.

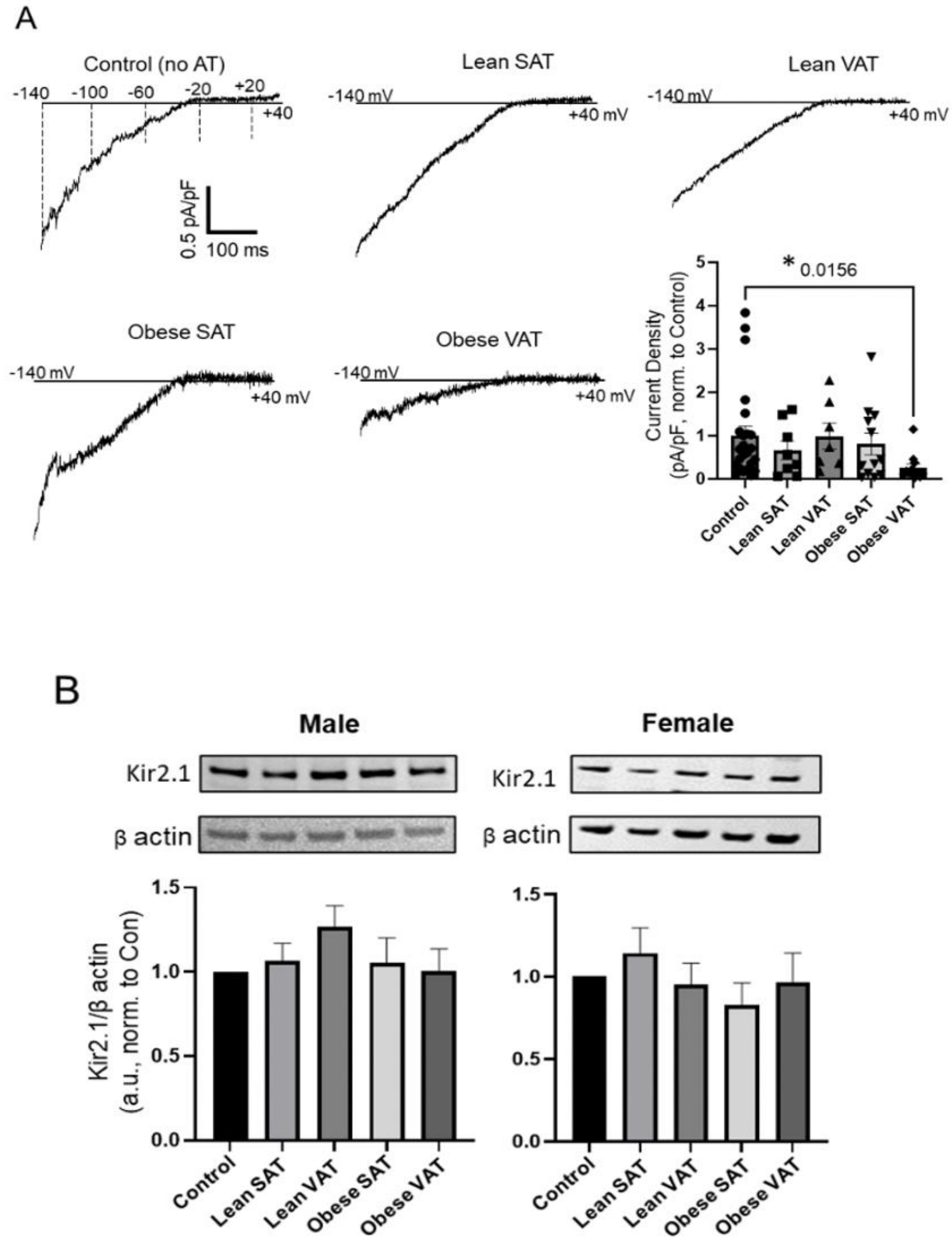


Figure 2.2. In Vitro study showing VAT from obese mice decreases endothelial Kir2.1 channel function. A) Patch clamp of HAMECS in control without AT treatment and with AT treatment from lean and obese mice showing

significant decrease in Kir2.1 in HAMECs with obese VAT (n=7-14cells/AT group). Group data with normalized current densities (pA/pF) that were analyzed and compared at -100 mV (n = 7-14 cells/AT group from 4 mice/diet group) indicate significant reduction in Kir2.1 in HAMECs with obese VAT. A Kruskal-Wallis test followed by Dunn's post hoc tests were used to determine significant differences relative to control (*). B) Western blots and group data for Kir2.1expressions in HAMECs after AT treatment from female and male mice show no difference (n=8/group/sex). Untreated cells were used as controls. Kir2.1 expression densities were normalized to that of β-actin prior to analysis.

2.3.2 Downregulation of CD36 prevents VAT-induced dysfunction in endothelial Kir2.1.

It has been shown that Kir2.1 is inherently lipid sensitive and is inhibited by several lipid species like fatty acid derivatives[126]. Hence, we hypothesized that VAT may be mediating Kir2.1 dysfunction in a CD36-dependent fashion. Even though role of CD36 in mediating endothelial dysfunction is not well understood but previous studies showed that tissues need CD36 for utilization of fatty acids[113] being a major endothelial fatty acid translocase.

After our finding showing VAT from obese mice impairs endothelial Kir2.1 in obesity, we further focus on determining whether knocking down of CD36 from endothelium could reverse or prevent the impairment of Kir2.1. First, we downregulated CD36 expression in HAMECs using siRNA **(Figure 2.3A)** and exposed HAMECs to VAT from obese mice and this was followed by assessing endothelial Kir 2.1 currents via whole cell patch clamp. Patch clamp showed CD36 knockdown (KD) HAMECs treated with VAT had significant increase in Kir2.1 current density (**Figure 2.3B**) when normalized to scramble control. However, CD36

KD cells without any AT treatment showed no effect on Kir2.1 current density (**Figure 2.3C).** These figures revealed that CD36 KD from cells reverses the effect of VAT from obese mice inducing endothelial Kir2.1 channel impairment and bring back significant current density which is required for normal functioning of endothelium. Together, this suggests that endothelial CD36 is a mediator of VAT-induced Kir2.1 dysfunction *in vitro*.

After the result showing endothelial CD36 is driving VAT-induced Kir2.1 dysfunction in vitro, we examined the expression of CD36 in HAMECs via western blotting. We tested HAMECs with exposure to AT from lean and obese mice to determine if VAT from obese mice increases CD36 expression and contribute to Kir2.1 dysfunction leading to ED. It revealed that there was no difference in CD36 expression in HAMECs treated to AT from male and female mice of both lean and obese group as compared to control **(Figure 2.3D).** These results indicate that VAT in obesity may induce increase in function of CD36 by translocating the fatty acid and impair the Kir2.1 channel leading to ED as its expression remain unchanged.

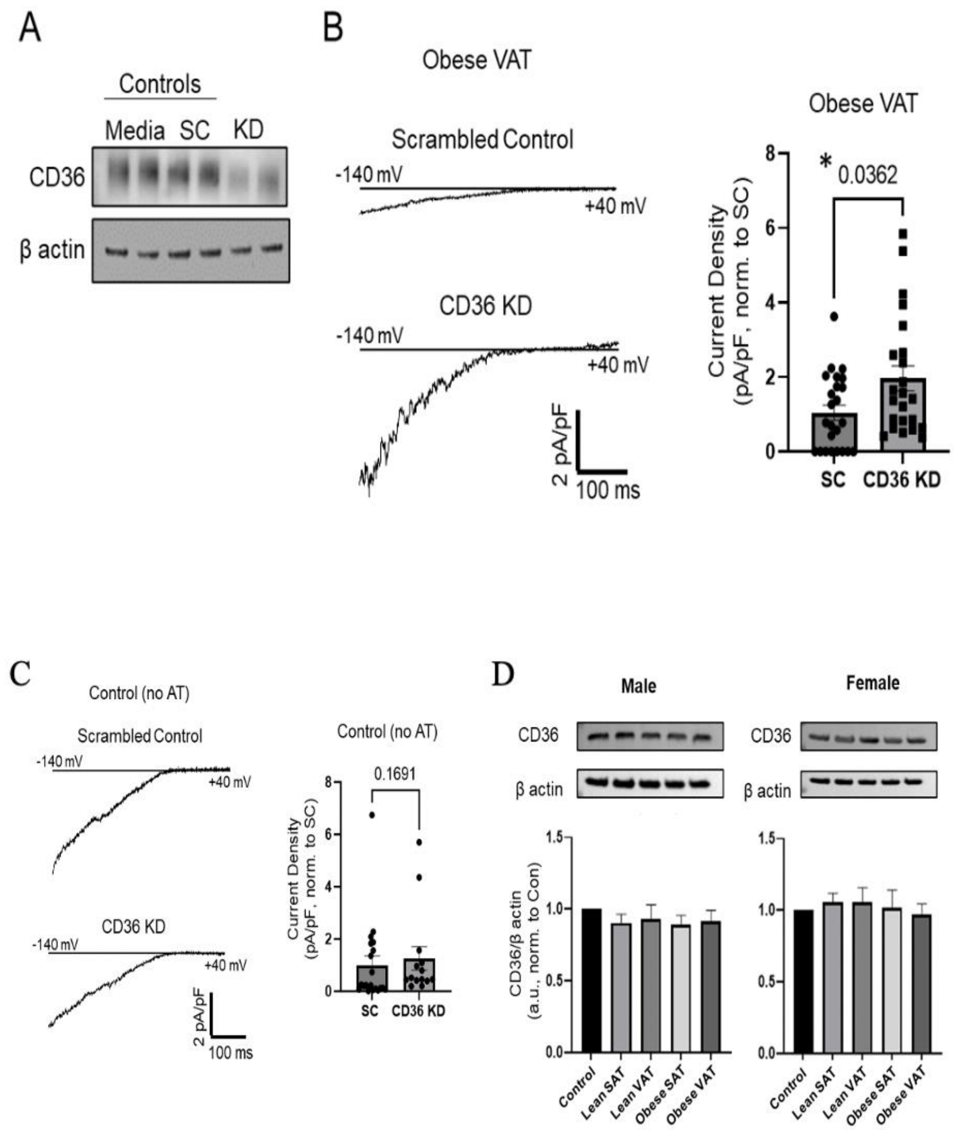


Figure 2.3. Knockdown of CD36 restore endothelial Kir2.1 function in HAMECs exposed to VAT from obese mice. A) Western blot showing reduced CD36 in HAMECs after CD36 siRNA as compared to the scramble siRNA treatment and untreated cells (Media Control). B) Whole patch clamp in HAMECs after CD36 siRNA and obese VAT treatment showing reduced current density. Group data reveal normalized current densities (pA/pF) that were analyzed and compared at -100 mV showing knocking down CD36 significantly rescuing Kir2.1 function (n = 23-24 cells/group from 4 obese mice). C) CD36 KD cells without any AT treatment showed no effect on Kir2.1 current density (n = 14

cells). D) Western blots and group data showing no changes in CD36 expression in HAMECs with AT treatment from male and female mice and control without AT treatment and were normalized to that of β actin(n=8).

2.3.3 Fatty acid uptake by ECs exposed to VAT from obese mice relies heavily on CD36.

To investigate whether VAT from obese mice triggers enhanced activity of endothelial CD36, HAMECs were treated with a fluorescent fatty acid analogue after being exposed to AT from both lean and obese mice. Following the incubation, HAMECs were then observed under fluorescent microscopy to visualize the uptake of internalized fatty acids. The images demonstrated the uptake of fatty acids in HAMECs treated with either scrambled control or CD36 siRNA prior to exposure to AT (**Figure 2.4A**). It showed that ECs treated with the scrambled control and exposed to AT from either lean or obese mice did not display a notable difference in fatty acid uptake compared to the control group without AT treatment. Cells that were exposed to VAT from obese mice demonstrated a significant uptake of fatty acid in comparison to cells exposed to SAT from obese mice and VAT from lean mice and (**Figure2.4B**). This observation suggested a distinctive role for VAT in obesity in promoting endothelial uptake of fatty acids.

Our *in vitro* result revealed that ECs that were exposed to VAT from obese mice showed a strong dependence on CD36 showing knocking down CD36 significantly blunting the fatty acid uptake as compared to the scrambled control treated cells (**Figure 2.4C**). However, CD36 knockdown did not result in a significant reduction in fatty acid uptake in untreated control cells. Also, this lack of effect of CD36 knockdown was observed in cells exposed to AT taken from lean mice and SAT taken from obese mice, where CD36 knockdown showed no impact on fatty acid uptake.

These findings indicate that HAMECs likely utilize alternative mechanisms for fatty acid uptake besides CD36. However, VAT from obese mice appears to facilitate a shift towards CD36-mediated fatty acid uptake, leading to increased uptake of fatty acids into endothelial cells *in vitro*. Since the observation from past study demonstrated endothelial Kir2.1 function can be rescued by knocking down CD36 to restore endothelial function in obesity[134], we next used a diet-induced obese mouse model to investigate the role of CD36 in obesity-induced ED in arteries located in VAT.

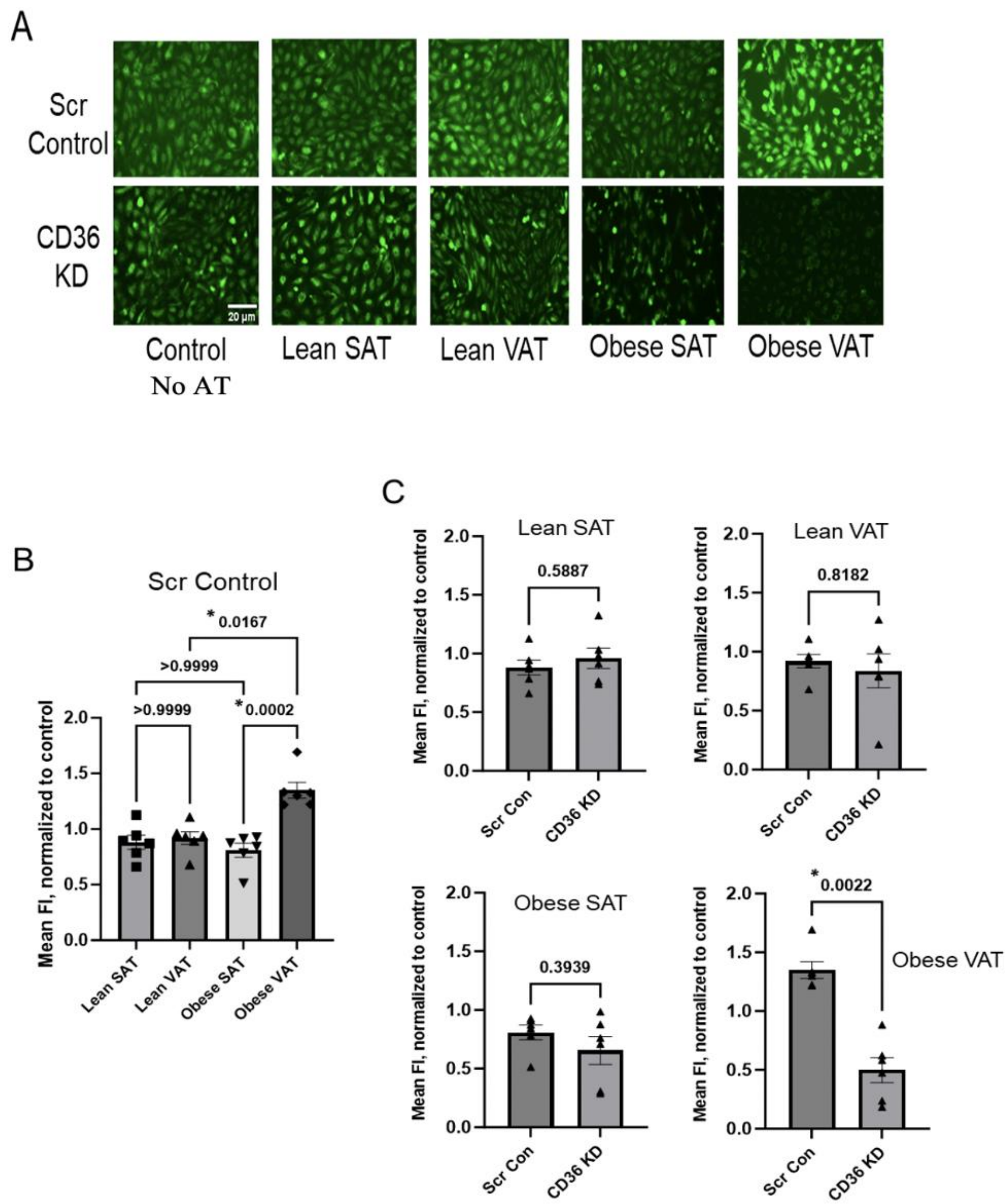


Figure 2.4. HAMECs exposed to VAT from obese mice heavily depend on CD36 for fatty acid uptake. A) Representative fluorescent microscopy images (20x) showing fluorescent fatty acid analogue in HAMECs after cell transfection with the scrambled (Scr) control or CD36 siRNA and AT treatment. The

scale bar is 20 μm. B) Group data showing the effects of AT on the mean fluorescence intensity (FI) in Scr-treated cells normalized to that of control (n = 6 independent experiments using 6 mice/group). A Kruskal-Wallis test followed by Dunn's post hoc tests were used to identify significant differences (*) between AT groups. C) The effect of CD36 KD on fatty acid uptake in each AT treatment group showing obese VAT treated cells has significant decrease in siRNA group compared to Scr. The mean FI of cells that received either Scr or siRNA were normalized to the respective control group to assess the role of AT on CD36-mediated fatty acid uptake. A Mann Whitney test was used to detect significant differences (*) in cells treated with different AT from lean and obese mice (n=6 experiments using 6 mice/group).

2.4 Discussion

Obesity-induced ED is a major precursor to the development of severe CVD characterized by a loss of NO production[47,48]. AT as a paracrine tissue, may affects the vasculature which lies adjacent to it. Previous study showed that, in obesity, VAT depots exhibit an elevated inflammatory state as compared to SAT, indicating the alterations in VAT depot[13,135,136]**.** This alteration in AT might have a deleterious impact on the local vasculature since several studies revealed that VAT arteries exhibit ED whereas arteries embedded in SAT retain endothelial function[21,42,69,133]**.** However, this mechanism is not yet clearly understood.

To elucidate the mechanism, in our present study, we used an *in vitro* model. In this model, HAMECs in culture were incubated with SAT and VAT from lean or obese mice to determine the effects of AT on endothelial Kir2.1 channel function. Endothelial Kir2.1 is a critical component of endothelium-dependent NO production and vasodilation, and this is disrupted in obesity[137,138]**.** In our previous study we showed that endothelial Kir2.1 channel were impaired in endothelium of VAT arteries isolated from obese mice and human[69,134]**.** Our findings in this study suggested that VAT from obese mice robustly decreases endothelial Kir2.1 currents with no changes in its expression.

Obesity is closely linked to elevated levels of circulating free fatty acids. These fatty acids, when converted into cellular derivatives, are known to act as inhibitors of Kir2.1[126,139]. Therefore, we examined if increase in endothelial CD36 function has a role in VAT-mediated impairment of Kir2.1 with the hypothesis that VAT-mediated impairment of Kir2.1 is dependent on an increase in endothelial CD36 function. Our *in vitro* study revealed that knocking down of CD36 recovered endothelial Kir2.1 currents in HAMECs subjected to VAT isolated from obese mice. However, there was no effect of CD36 KD on Kir2.1 currents in cells that were not exposed to AT. This suggests that VAT is inducing upregulation of CD36 function that might be the underlining mechanism for the impairment of endothelial Kir2.1 in obesity. Furthermore, our data revealed that ECs subjected to VAT of obese mice showed a remarkable rise in CD36-dependent uptake of fatty acid as compared to cells exposed to SAT from obese mice and AT from lean mice. This finding additionally supports that a VAT is mediating increase in CD36 function that may leads to the impairment of endothelial Kir2.1. Our study demonstrated that VAT from obese mice has the tendency to reduce Kir2.1 currents, which is not the same case in cells treated with AT from lean mice or SAT from obese mice. However, our data also revealed that AT from lean and obese does not affect endothelial Kir2.1 expression. This evidence supports existing of a modification of channel activity and/or trafficking to the membrane. This suggested that VAT is altered by obesity and may also influence the local endothelium. Together, these results indicated that the specific AT in obesity alters the regulation of the vascular K^+ ion channels. Hence, we proposed that the VAT act as an upstream mediator that drives Kir2.1 and affects endothelial function in obesity.

A variety of mechanisms are involved in driving ED and several groups have worked on identifying that mechanism. The effect of obese VAT on vascular function which was investigated by some researcher previously were more focused on identifying PVAT-derived reactive oxygen species (ROS) as a causative factor in ED[140]. However we did not investigate the role of AT-derived ROS in our study but the mechanisms like increased fatty acid accumulation in endothelium are known to promote ROS production elevation[137]. The accumulation of fatty acid in endothelium indicates impairment of Kir2.1 may be mediated by CD36. Previous study from our lab showed that endothelial Kir2.1 is regulated by the glycocalyx and was disrupted in mesenteric arteries from VAT of obese mice[43]. Another study in mice also showed that aortic PVAT induces ED[141]. Hence, this indicates that with obese PVAT and impairment of K^+ channels, there might be the involvement of ROS and/or glycocalyx disruption that is leading to ED.

2.4.1 Conclusion

Our *in vitro* study findings reveal that the abdominal SAT and mesenteric VAT, differentially affect endothelial Kir2.1 in obesity. Mesenteric VAT is described to be more dysfunctional, and our findings support this notion as we observed the inhibition of endothelial Kir2.1 in obesity. Furthermore, this study provides support in the involvement of endothelial CD36 in mediating the impairment of Kir2.1 in VAA, potentially contributing to ED. Ultimately, our findings suggest that targeting CD36 may be beneficial to prevent the progress of CVD and rescue NO production mediated by Kir2.1.

2.4.2 Perspectives and Future Directions

There may be the various distinct mechanism playing role in altering ED. One of the mechanisms could be an increase in endothelial fatty acid accumulation that promote elevated ROS production which may indirectly induce Kir2.1 dysfunction by degrading the glycocalyx. Next mechanism may include direct inhibition of Kir2.1 by CD36 which convert intracellular fatty acids into metabolite derivatives including long chain fatty acyl CoA-esters and ceramides. It may inhibit the channel directly or indirectly through cell signaling events[126,139]. Therefore, future studies to identify potential source of ROS and fatty acid derivative formation in VAT-mediated impairment of Kir2.1 are necessary. In addition, distinct mechanisms by which VAT regulates or triggers modification of CD36 in endothelium and how CD36 is promoting Kir2.1 impairment in obesity-induced ED needs to be addressed. Next, we are going to examine the increase in LCFAs by investigating uptake of fatty acid in endothelium after VAT treatment by using fatty acid uptake kit that will direct if VAT is releasing more LCFA and allowing to accumulate in endothelium and affecting its function. Similarly, we will test the modification of CD36 by evaluating the s-acylation of CD36 in the endothelium after VAT treatment. Increased s-acylation in ECs triggered by AT suggests increase in LCFA uptake in ECs and de-acylation in AT indicates increase in release in LCFA from AT which, in together, result in ED in the arteries.

Chapter 3

IMPAIREMENT OF ENDOTHELIAL KIR2.1 IN VISCERAL ARTERY IS DRIVEN BY CD36 IN OBESITY

3.1 Introduction

CD36, a glycosylated integral membrane protein ,broadly expressed in various cell types such as platelets, adipocytes, microvascular endothelial cells, immune cells, myocytes, retinal and mammary epithelial cells[103]. CD36 is also refer as class B scavenger receptor that is involved in metabolism, atherosclerosis, angiogenesis, immunity and behavior[142,143]. Previous research has established CD36 as a fatty acid translocase (FAT) with significant involvement in lipid balance regulation and the transportation/uptake of long chain fatty acid (LCFAs) into the muscle and adipose tissues of both rodents and humans[104,129]. It translocases fatty acids in heart, skeletal muscle and adipocyte and is an important substrates for energy production[129]. A previous experiment showed that overexpressed in skeletal muscle revealed increased fatty acid oxidation, reduced circulating fatty acid and triacylglycerols, and fat deposition[144]. However, knocking down CD36 resulted in decrease adipocytes, oxidative muscle transport of LCFAs and high level of cholesterol, triacylglycerol and fatty acids[110]. This suggests that CD36 has a crucial role in maintaining the balance of lipoproteins and lipids in the body. Previous studies with mice lacking CD36 systemically[110] and in endothelial cells (ECs)[113] showed that CD36 is necessary for normal LCFAs uptake in various organs. Studies in wild type fat cells showed the lack of high affinity component of LCFA transport in adipocytes of CD36 null mice[110].

The importance of endothelial CD36 is well established as a gatekeeper to LCFA uptake[113,114] as previous studies showed that knocking down CD36 abolishes lipid uptake. Intracellular LCFA accumulation in endothelial cells is strongly linked to endothelial dysfunction (ED)[130,145] indicating the CD36 as transporter contributes to ED with increase in fatty acid (FA) accumulation in visceral adipose arteries (VAA). However, the mechanism facilitating this LCFA uptake still needs further investigation. Our past work demonstrated the impact of visceral obesity on endothelial function where VAA exhibit robust ED while subcutaneous adipose arteries (SAA) retain endothelial function/NO production in obese humans and rodent models of diet-induced obesity[69].

Therefore, we hypothesized that adipose tissues (AT) where those arteries are located are influencing ED by transporting LCFAs via CD36 and reportedly impairing endothelial Kir2.1. Here, we performed *ex vivo* study to examine if knocking down of CD36 could restore endothelial function in a Kir2.1 and eNOS-dependent fashion in VAA of obese mice by restoring flow induced vasodilation (FIV). We hypothesized that inhibition of Kir2.1 current in VAA was driven by CD36 and contributes to ED.

3.2 Method

3.2.1 Animal preparation

The research was conducted following approval of animal model from the Institutional Animal Care and Use Committee at the University of Delaware. Ten-week-old male and female mice were separated into two diet groups with lean control groups kept on a normal laboratory diet and next an obese group as WT and CD36 KO mice on high-fat Western die (42% of calories) for 8 weeks. Mice were euthanized by cervical dislocation, followed by thoracotomy and subsequently, mesenteric and subcutaneous AT arteries were isolated from WT and CD36 knockout mice and cleaned to assess flow-induced vasodilation via pressure myography *ex vivo*.

3.2.2 Pressure myography.

After 8 weeks of diet, VAA and SAA from lean and obese WT or CD36 mice were isolated. Arteries were connected to glass pipettes from both sides in a special chamber designed for video microscopy purposes[69,146]. This chamber was positioned on a microscope with a camera to observe the artery on a screen, and the diameter of the arteries were measured using a video system. The chamber received a continuous flow of oxygenated Krebs buffer at 37°C. The arteries were gently filled with Krebs buffer from both sides using reservoirs kept at the same height of 60 cmH2O to maintain equal pressure. Arteries were kept at normal pressure for one hour before being constricted with endothelin-1 (ET-1) to 40-50% of its baseline diameter **(Table 1).** Once the pre-constriction within the specified range was achieved, the reservoirs were adjusted equally and in opposite directions to start luminal flow through the vessel gradually. The flow with each increase was maintained for 5 minutes before assessing the diameter of the vessel. To ensure that the smooth muscles in the blood vessels were working properly throughout the experiment, we tested their ability to relax in response with papaverine (100 μM) before ending of each procedure. In case diameter of the artery didn't reach at least 80% of its original size after adding papaverine, we didn't include any measurements from that artery in our analysis **(Table 1).** After observing how the blood vessels initially responded to flow for 30 minutes, $BaCl_2$ (30 μM) and/or LNAME (100 μM) were introduced into the circulating solution. Then, the flow protocol was repeated. The extent of dilation in each artery in response to flow was calculated as a percentage change in dilation (%dilation) from the baseline diameter relative to the constriction induced by ET-1, as indicated by Equation 1.

$$\%\text{Dilation} = \frac{\text{baseline diameter} - \text{diameter after preconstriction to ET-1}}{\text{baseline diameter} - \text{diameter in response to flow } \Delta X} \times 100$$

3.2.3 Statistical analyses

The Kruskal–Wallis test was used followed by the Dunn’s post hoc test to compare multiple groups. Initial tests were set to $P < 0.05$ before implementing post hoc corrections. Two-way repeated measures ANOVA followed by Bonferroni post hoc tests was used to detect significant differences. We conducted tests to assess for significant differences between male and female mice and if not significant, data were analyzed combinedly. (n = 5; 3 male and 2 female mice/group).

3.3 Results

3.3.1 The removal of CD36 prevents obesity-induced endothelial dysfunction in VAA.

It is well established that there is loss of NO production in obesity with the consequence of ED. However, not all vasculature exhibits ED with obesity as the arteries embedded in VAT are robustly impaired whereas those arteries embedded in SAT retain endothelial function[21,69,133]. In addition, previous studies demonstrated that ED in VAA induced by obesity is mediated through the impairment of endothelial Kir2.1 channels and these channels are crucial upstream regulators of NO production and subsequent vasodilation[68,69]. Conversely, endothelial Kir2.1 channels in SAA remain functional during obesity and facilitate NO-mediated vasodilation comparable to lean control. This contrast in endothelial and Kir2.1 function during obesity indicates the requirement of further investigation to VAA.

While we don't fully understand how CD36 contributes to the early stages of disease and its role in ED, it is known to play a part in the development of atherosclerosis and its function in blood vessel walls is well-known[127,128,147]. In our experiment, we used a commonly used mouse model that develops obesity when fed a high-fat diet. This model reflects how obesity affects the function of blood vessels in VAT and SAT, similar to what happens in obese humans. Using this model, we studied how CD36 contributes to the problems caused by obesity in both blood vessels and the Kir2.1 channels within VAA. As described earlier, both WT and CD36 KO mice were on a high-fat Western diet for 8 weeks. This duration was selected because it aligns with the time when noticeable ED in arteries was usually seen in obese mice[68,69]. At the same time, a lean control group was given a normal rodent diet. The endothelial function in VAA and SAA were assessed using pressure myography. This method was used to evaluate the endothelial response to flow which is an established stimulus known to activate Kir2.1 channels and consequently leads to the production of NO[73,74,138]. VAA and SAA were isolated, cannulated, and pressurized in special chambers. The baseline diameters of all artery types were measured, and there were no notable differences between the groups (**Table 1**). The arteries were then preconstricted with endothelin-1 before increasing the flow. After constricting the arteries, we gradually increased the intraluminal flow within the vessel using a pressure gradient[69,146]. The findings revealed that arteries isolated from lean WT mice and SAA isolated from obese WT mice usually dilate in response to the intraluminal flow. This expansion reached an average increase ranging from about 77% to 84% compared to the baseline diameter, which was what we expected. In contrast, VAA from obese WT mice showed weaker expansions in response to flow, reaching only about 45% of the baseline size at the maximum flow level. Moreover, knocking down CD36 globally didn't change how SAA from obese mice or arteries from lean mice responded to flow when compared to WT mice. But, in contrast, VAA from obese mice without CD36 showed a substantial improvement in how they responded to flow, reaching about 85%

of their baseline size at maximum flow rate. This response was significantly improved compared to VAA from obese WT mice and resembled the response observed in arteries from lean WT mice (**Figure 3.1).** These findings provide evidence that CD36 plays a role in mediating ED in VAA during obesity. These findings are further supported when observing the mean absolute diameter changes in response to an increase in flow **(Table 3).**

Table 1: Vessel inclusion criteria

Artery	**Ave. constriction to ET-1 ± SEM (%)**	**Ave. dilation to papaverine ± SEM (%)**
SAT – WT Lean	46.6 ± 3.2	98.7 ± 0.8
VAT – WT Lean	47.1 ± 4.8	98.8 ± 0.7
SAT – WT Obese	48.7 ± 2.1	98.6 ± 0.7
VAT – WT Obese	49.0 ± 4.2	98.7 ± 0.8
SAT – CD36 KO Lean	44.9 ± 2.5	95.5 ± 4.0
VAT – CD36 KO Lean	41.6 ± 3.0	98.0 ± 0.9
SAT – CD36 KO Obese	44.3 ± 2.7	97.9 ± 1.4
VAT – CD36 KO Obese	46.5 ± 2.3	97.7 ± 1.5

Nonstandard abbreviations: ET-1, endothelin-1; SAT, subcutaneous adipose tissue; VAT, visceral adipose tissue; KO, knockout

3.3.2 CD36 ablation restores flow-induced vasodilation in VAA of obese mice via recovery of Kir2.1/eNOS function.

To confirm if removing CD36 restores the function of VAA endothelium by rescuing Kir2.1/eNOS, we investigated whether blocking Kir channels with Ba2+ or inhibiting eNOS with LNAME would decrease the flow-induced dilation of arteries from AT of CD36 KO mice. First, we assessed arteries from lean and obese CD36 KO mice for dilation in response to flow. Then, we treated them with inhibitors for 30 minutes and reassessed to see if their dilation changed. This method has been used before and has been shown to reduce dilation in both vascular bed from lean WT mice and in SAA from obese WT mice[68,69]. However, in contrast, the residual dilatory response to flow in VAA from WT obese mice did not change by blocking Kir2.1 or eNOS. This indicates that there is an impairment with this signaling pathway in VAA from obese WT mice. Blocking both Kir2.1 and eNOS, either alone or together, caused a significant decrease in dilation to flow in arteries from CD36 KO mice (**Figure 3.2**). This suggests that removing CD36 didn't change Kir2.1 function in vasculature from lean mice or SAA from obese mice. The response to flow before treatment was the same as in normal mice and blocking Kir2.1 or eNOS had a similar effect.

This suggests that removing CD36 had no impact on the function of the endothelium or Kir2.1 channels in vessels from lean mice or SAA of obese mice. This is because the initial response to flow was similar to that of WT and it was similarly affected by Ba2+ and LNAME. However, in VAA of obese mice, CD36 removal restored the interaction between Kir2.1 channels and eNOS, which was impaired in WT. Hence, this restoration of the Kir2.1/eNOS axis effectively reversed the dysfunction observed in VAA of obese mice. Altogether, these findings indicate that CD36 plays a role in disrupting Kir2.1 and eNOS signaling pathways that mediates obesity-induced ED in VAA. Additionally, there were no effects of Ba^{2+} or LNAME on baseline arterial diameters within artery groups **(Table 2).**

Table 2. Baseline AT artery diameters +/- inhibitors

Artery	Ave. baseline diameter ± SEM (µm)	Ave. baseline diameter following incubation with Ba^{2+} ± SEM (µm)	Ave. baseline diameter following incubation with LNAME ± SEM (µm)
SAT – WT Lean	123.3 ± 13.1	121.4 ± 14.2	122 ± 13.5
VAT – WT Lean	120.8 ± 9.5	119.2 ± 10.0	118.6 ± 10.8
SAT – WT Obese	115.3 ± 15.8	114.3 ± 16.1	112.8 ± 18.4
VAT – WT Obese	117.7 ± 24.9	114.6 ± 22.8	115.0 ± 23.0
SAT – CD36 KO Lean	125.5 ± 8.0	124.1 ± 7.3	122.9 ± 7.7
VAT – CD36 KO Lean	138.6 ± 15.1	135.9 ± 16.1	135.8 ± 15.8
SAT – CD36 KO Obese	145.1 ± 10.7	144.5 ± 10.7	144.4 ± 13.0
VAT – CD36 KO Obese	134.1 ± 13.5	130.5 ± 12.5	129.8 ± 12.4

A two-way ANOVA was used when comparing baseline diameters across diet and genotype. One-Way repeated measures ANOVAs were used to determine the effects of inhibitors within each artery group. Nonstandard abbreviations: SAT, subcutaneous adipose tissue; VAT, visceral adipose tissue; KO, knockout

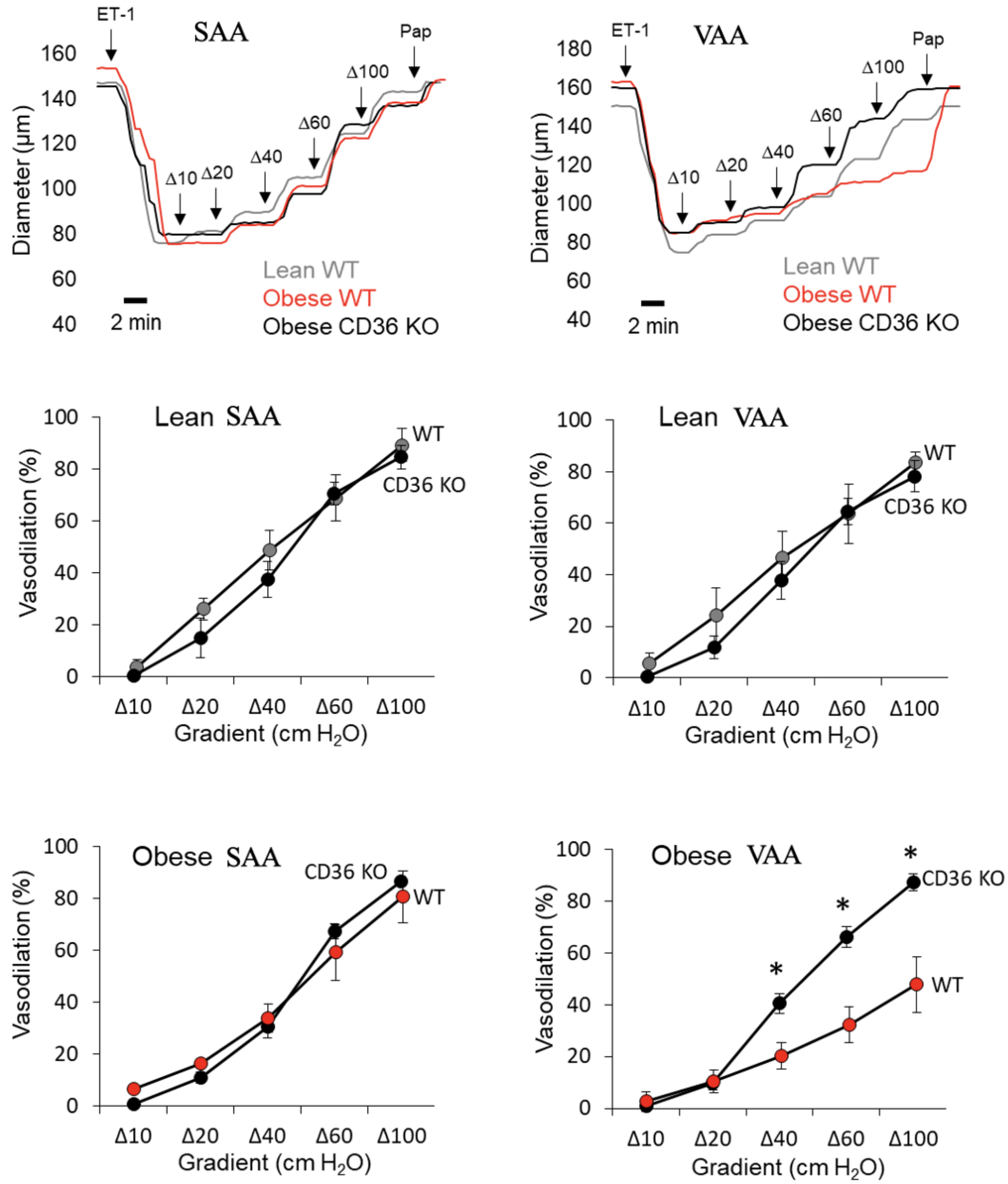


Figure 3.1. Flow-induced vasodilation is restored in visceral arteries of obese mice lacking CD36. VAA and SAA from WT and CD36 KO mice were isolated and cannulated for pressure myography and the endothelial response to flow was measured. Representative traces show the dilatory response to an increase in intraluminal flow in SAA and VAA of lean and obese WT or obese CD36 KO mice. Pre-constrictions to low doses of endothelin-1 (ET-1; ≤ 120 pM) were complete in under 2 minutes. Papaverine (Pap; 100 µM) was used to confirm

the functionality of smooth muscle at the end of each protocol. Flow-induced vasodilation was assessed between WT and CD36 KO mice in SAA of lean mice, VAA of lean mice, SAA of obese mice and VAA from obese mice. A two-way ANOVA followed by Bonferroni post hoc tests was used to detect significant differences (*) (n = 5; 3 male and 2 female mice/group).

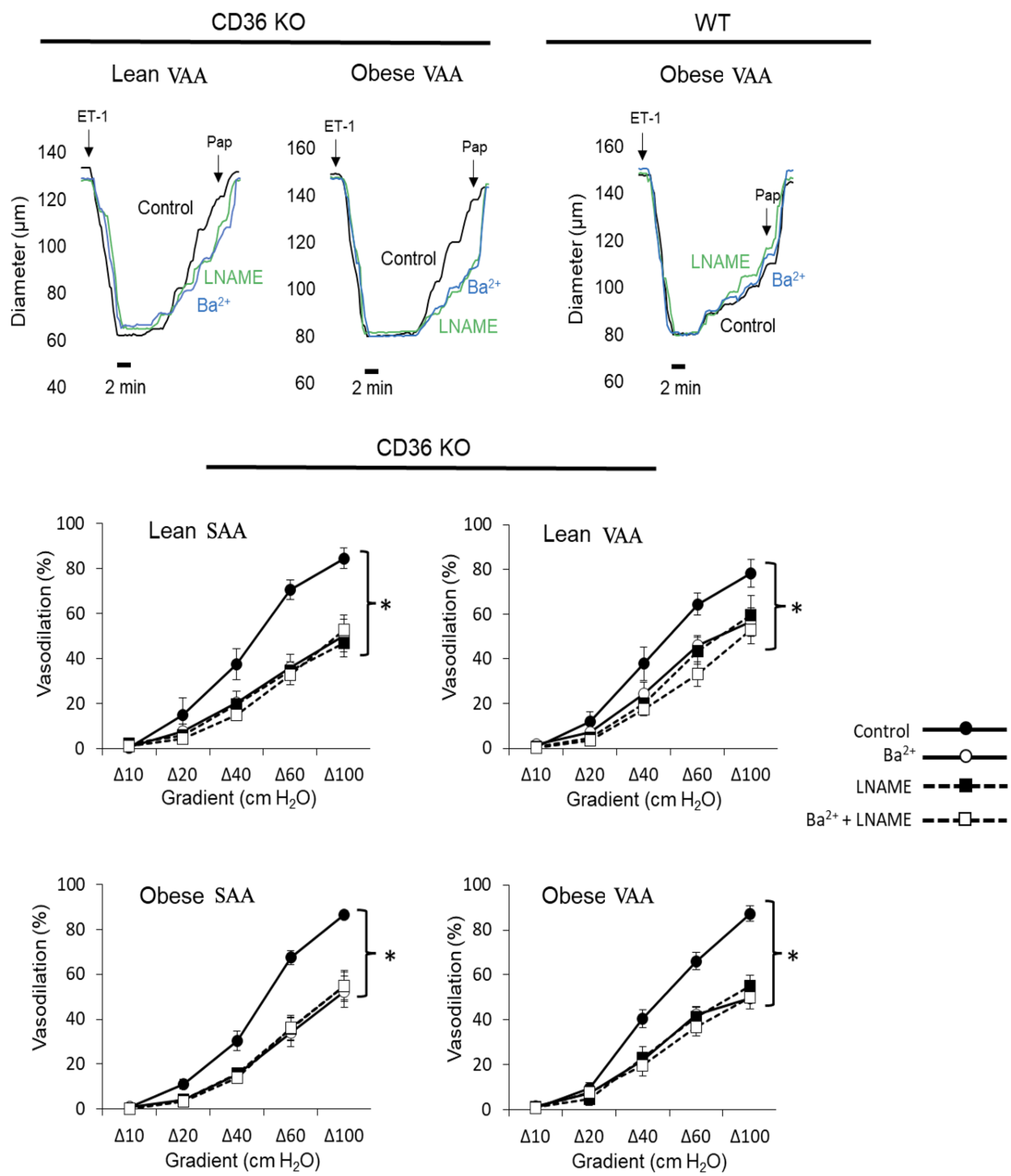


Figure 3.2. CD36 ablation restores flow-induced vasodilation in VAA of obese mice via recovery of Kir2.1/eNOS function. Representative traces show the dilatory response to an increase in intraluminal flow in VAA of lean and obese CD36 KO mice and VAA of obese WT mice. Pre-constrictions to low doses of endothelin-1 (ET-1; ≤ 120 pM) were done for 2 minutes. Papaverine (Pap; 100 μM) was used to confirm the functionality of smooth muscle at the end of each protocol. The effects of Kir and eNOS inhibition on FIV was measured in SAA of CD36 KO mice, VAA of lean mice, SAA of obese mice and VAA from obese mice. Following initial responses to intraluminal flow, VAA and SAA from CD36 KO mice were incubated with $BaCl_2$ (30 μM; Kir channel inhibitor), LNAME (100 μM; eNOS inhibitor), and then a combination of both inhibitors for 30 minutes prior to reassessing dilations to flow. The inhibitors were washed out of the circulating bath chamber between each assessment of the dilatory response to flow. A repeated measures two-way ANOVA followed by Bonferroni post hoc tests was used to detect significant differences between the initial response to flow and pharmacological treatments (*) (n = 5; 3 male and 2 female mice/group).

Table 3. Mean arterial diameter increases in AT arteries in response to flow +/- inhibitors.

Artery	Absolute change in artery diameter at Δ100 flow gradient ± SEM (µm)	Absolute change in artery diameter at Δ100 flow gradient with Ba^{2+} ± SEM (µm)	Absolute change in artery diameter at Δ100 flow gradient with LNAME ± SEM (µm)
SAT – WT Lean	35.5 ± 9.8	†16.5 ± 10.0 (p = 0.037)	†14.1 ± 12.3 (p = 0.040)
VAT – WT Lean	43.3 ± 6.4	†25.6 ± 4.0 (p = 0.021)	†23.9 ± 5.9 (p = 0.031)
SAT – WT Obese	47.6 ± 5.3	†31.8 ± 8.8 (p = 0.044)	†33.6 ± 11.3 (p = 0.046)
VAT – WT Obese	*26.7 ± 4.3 (p = 0.025)	24.8 ± 4.6	27.0 ± 9.4
SAT – CD36 KO Lean	47.0 ± 2.6	†27.2 ± 4.7 (p = 0.002)	†25.9 ± 4.4 (p < 0.001)
VAT – CD36 KO Lean	45.5 ± 6.3	†28.7 ± 3.4 (p = 0.008)	†31.4 ± 5.3 (p = 0.009)
SAT – CD36 KO Obese	45.5 ± 4.8	†32.8 ± 4.5 (p = 0.006)	†35.8 ± 4.3 (p = 0.011)
VAT – CD36 KO Obese	#54.8 ± 6.9 (p = 0.014)	†29.6 ± 2.7 (p = 0.003)	†32.5 ± 1.7 (p = 0.008)

Mean increases in diameters in response to flow were assessed relative to the pre-constricted diameter in each artery group. A two-way ANOVA followed by Bonferroni post hoc testes was used when comparing baseline diameters across diet and genotype (*significantly different vs. VAT – WT Lean; #significantly different vs. VAT – WT Obese). One-Way repeated measures ANOVAs followed by Bonferroni post hoc tests were used to determine the effects of inhibitors within each artery group (†significantly different vs. diameter changes in the absence of inhibitor within an artery group). Nonstandard abbreviations: SAT, subcutaneous adipose tissue; VAT, visceral adipose tissue; KO, knockout.

3.4 Discussion

3.4.1 The endothelial Kir2.1 and flow-induced vasodilation.

Recent research have shown that Kir2.1 plays an important role in vasodilation in response to both chemical and mechanical signals[66,68,74,148]. For example, when certain pathways in the cells are activated, Kir2.1 channels help release K^+ from the endothelium and effectively contributes to EDH. This happens through different processes depending on the type of signal. For instance, when receptor-mediated pathways are stimulated, Kir2.1 channels are activated through a process that involves other channels called calcium (Ca2+)-activated K+ channels (K_{Ca})[66]. Overall, Kir2.1 channels act as "boosters" of the dilatory response in blood vessels. Recently, we found that when blood vessels are exposed to fluid shear, the activation of Kir2.1 channels and their role in endothelium-dependent vasodilation depends on the glycocalyx. The glycocalyx involved in a Akt/eNOS/NO signaling pathway which is a EDRF signaling as opposed to EDH[68,69,74]. However, we're still not sure exactly how the glycocalyx controls the Kir2.1 channels activity or how activating Kir2.1 channels leads to the activation of Akt and ultimately increases eNOS activity.

In contrast to how Kir2.1 and K_{Ca} typically work together in response to receptor-mediated mechanism, they seem to operate separately in response to a mechanical stimulus like shear stress. Our past research showed that blocking Kir2.1 doesn't affect the role of K_{Ca}, and blocking K_{Ca} doesn't stop Kir2.1 from contributing vasodilation. However, when both Kir2.1 and K_{Ca} are blocked together, the ability to dilate in response to flow is significantly reduced[74,149]. It's reasonable to think that when Kir2.1 channels are activated by flow, they might also contribute to EDH or electrical communication with smooth muscle cells, even without involving NO. Recent findings have confirmed that Kir2.1 are expressed at the myoendothelial junction[150] and are known to play a significant role in transmitting signals that control the contraction and relaxation of blood vessels via endothelial-smooth muscle gap junctions[151]. In our research, blocking Kir2.1 channels and eNOS separately or together resulted in similar reductions in vasodilation of arteries in AT in response to flow[68,74,149]. This could mean that either there are very few Kir2.1 channels in the endothelium of AT[66,74], which aren't enough to hyperpolarize the endothelium and in turn, hyperpolarize and relax smooth muscle, or different groups of Kir2.1 channels contribute to different pathways that promotes endothelium-dependent vasodilation.

Although we're not sure how Kir2.1 channels in the endothelium contribute to EDH or EDRF under distinct stimuli, it could be due to its apical expression that is driving EDRF with the glycocalyx in the lumen independent of K_{Ca} or basal expression that is driving EDH with K_{Ca} at the basolateral membrane. Although our studies didn't directly show this, it's still unclear whether a group of Kir2.1 channels is activated when K_{Ca} channels are activated by flow, similar to how they're activated by receptor-mediated pathway that would contribute to flow-mediated EDH. Future research should focus on understanding the location of Kir2.1 channels on the cell surface in relation to their potential roles. Our recent findings indicated that obesity doesn't seem to affect the role of K_{Ca} channels in vasodilation, particularly in VAA. This role was not impacted when Kir2.1 channels were blocked and could be restored by overexpressing of Kir2.1 in the endothelium[68,69]. Therefore, our current study focuses on Kir2.1 channels.

3.4.2 CD36 facilitates obesity-induced endothelial dysfunction in VAA.

The previous study revealed that the endothelium of VAA from obese mice and humans are impaired[68,69] and endothelial Kir2.1 channel function which is a critical component of endothelium-dependent NO production and vasodilation are also disrupted in obesity[137,138]. Also, the circulating free fatty acid[137], a cellular derivative is increased in obesity that inhibits the Kir2.1 current[126,139], we believed that AT-mediated impairment of Kir2.1 is dependent on an increase in endothelial CD36 function. Since, our findings from *in vitro* study indicated that VAT from obese mice robustly decreases endothelial Kir2.1 currents, we hypothesized it was driven by endothelial CD36 and examined this in *ex vivo* mice model.

CD36 is known to play a significant role in causing atherosclerosis, but its involvement in the initial stages of the disease process isn't clear yet. In this study, we used *ex vivo* model to determine the importance of CD36 in mediating obesity-induced

ED in VAA. We found that completely removing CD36 from the body helps restore the function of VAA of obese mice. This is due to restoration of Kir2.1/eNOS signaling in the endothelial response to flow. When Kir2.1 function is lost, it leads to a decrease in the production of NO and causes ED. In our recent study, we demonstrated that overexpression of Kir2.1 specifically in the endothelial cells of VAA was able to restore the endothelial function in the mesenteric of obese mice by restoring the production of NO mediated by eNOS[68]. Since we noticed a comparable improvement in the function of Kir2.1 and eNOS in the VAA in obese mice without CD36, these results indicate that targeting CD36 could be beneficial in preventing the progression of severe disease conditions by improving endothelial function in affected blood vessels.

However, despite the finding from *in vitro* studies revealing endothelial CD36 may be the primary cause of Kir2.1 impairment in the presence of VAT, the use of global knockout mice in our *ex vivo* study prevents us from distinguishing between specific cell types responsible for the recovery in VAA endothelial function in the absence of CD36. This is because, apart from being expressed in vascular endothelium, CD36 is also present in various other cell types including adipocytes and immune cells situated in AT[115,118]. Hence, there is possibility that each of these cell types may contribute to inducing ED in obesity. Despite the fact related to presence of CD36 in any cell types, it is driving the dysfunctional changes in vascular endothelium. Together, this study findings provide support for the notion that CD36 acts as a significant contributor upstream of Kir2.1 impairment. However, this *ex vivo* study does not explore more detail pathophysiological mechanism of VAT and SAT involvement in obesity induced ED, but the findings provide support for the concept that CD36 serves as one of the major contributors leading to Kir2.1 impairment.

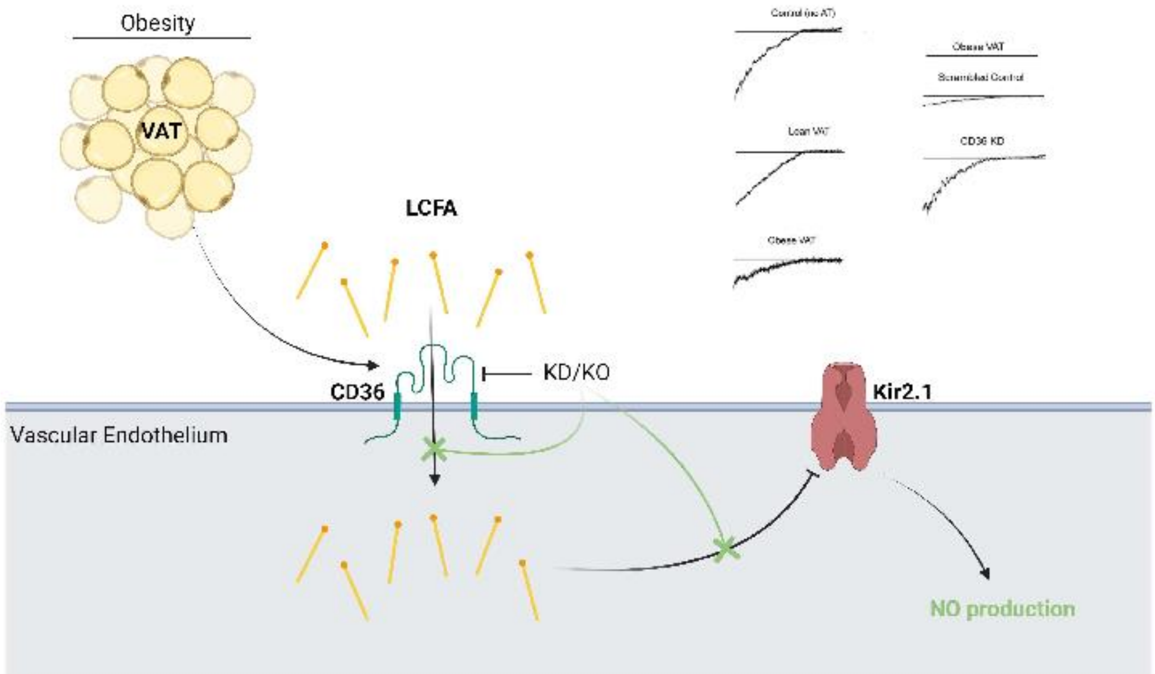
3.5 Conclusion

Our study in vasculature revealed that CD36 could be a target for improving vasodilation by rescuing the Kir2.1 current and restoring the endothelial function. It could prevent CVD progression by restoring the production of NO which is facilitated by Kir2.1 current and subsequently achieve normal vasodilation with flow.

3.6 Future direction

Present study did not explain the changes within the different AT from lean and obese mice and how they are influencing the arteries. Hence, we will investigate the changes in the cellular level to understand exactly what factors in visceral fat controls CD36 in the endothelium and possibly other cells in AT. Futures study will also focus on addressing the mechanism related to CD36 causing obesity-related ED by impairing Kir2.1.

Graphical abstract



Adapted from Visceral adipose of obese mice inhibits endothelial inwardly rectifying Kir2.1 channels in a CD36-dependent fashion © 2024 by Sabita Rokka Published by the American Physiological Society[152].

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Appendix A

IACUC APPROVAL FORMS

University of Delaware
Institutional Animal Care and Use Committee
Application to Use Animals in Research
(New and 3-Yr submission)

Title of Protocol: Obesity-Induced Endothelial Dysfunction in Arteries of Visceral Adipose	
AUP Number: 1372-2023-0	**← (4 digits only — if new, leave blank)**
Principal Investigator: Ibra S Fancher, PhD	
Common Name (Strain/Breed if Appropriate): mouse (C57Bl6) **Genus Species:** Mus musculus	
Date of Submission: Click here to enter text.	

Official Use Only

IACUC Approval Signature: ___Tall, DVM___

Date of Approval: ____________12/1/2023______________________________

Revised: 05/2020 Page **1** of **34**

Principal Investigator Assurance

1. I agree to abide by all applicable federal, state, and local laws and regulations, and UD policies and procedures.
2. I understand that deviations from an approved protocol or violations of applicable policies, guidelines, or laws could result in immediate suspension of the protocol and may be reportable to the Office of Laboratory Animal Welfare (OLAW).
3. I understand that the Attending Veterinarian or his/her designee must be consulted in the planning of any research or procedural changes that may cause more than momentary or slight pain or distress to the animals.
4. I declare that all experiments involving live animals will be performed under my supervision or that of another qualified scientist. All listed personnel will be trained and certified in the proper humane methods of animal care and use prior to conducting experimentation.
5. I understand that emergency veterinary care will be administered to animals showing evidence of discomfort, ailment, or illness.
6. I declare that the information provided in this application is accurate to the best of my knowledge. If this project is funded by an extramural source, I certify that this application accurately reflects all currently planned procedures involving animals described in the proposal to the funding agency.
7. I assure that any modifications to the protocol will be submitted to by the UD-IACUC and I understand that they must be approved by the IACUC prior to initiation of such changes.
8. I understand that the approval of this project is for a maximum of one year from the date of UD-IACUC approval and that I must re-apply to continue the project beyond that period.
9. I understand that any unanticipated adverse events, morbidity, or mortality must be reported to the UD-IACUC immediately.
10. I assure that the experimental design has been developed with consideration of the three Rs: reduction, refinement, and replacement, to reduce animal pain and/or distress and the number of animals used in the laboratory.
11. I assure that the proposed research does not unnecessarily duplicate previous experiments. ***(Teaching Protocols, including cooperative extension demonstrations, Exempt)***
12. I understand that by signing, I agree to these assurances. Signature of Principal Investigator   8/23/2023 Date

NAMES OF ALL PERSONS WORKING ON THIS PROTOCOL

I certify that I have read this protocol, accept my responsibility and will perform only those procedures that have been approved by the IACUC.

Name	Signature
1. Ibra S Fancher	
2. Halley Wisner	
3. Sabita Rokka	Sabita Rokka
4. Emma Hudgins	
5. Masoumeh Sadeghinejad	Sadeghi
6. Malak Alradi	
7. Erica Johnson	
8. Click here to enter text.	
9. Click here to enter text.	
10. Click here to enter text.	

If after hours participation is required by students on project involving **agricultural animals**, please describe how this is handled and the times and days that students may be on site
Click here to enter text.

The Animal Use Protocol form has been developed to facilitate review of requests for specific research, teaching, or biological testing projects. The review process has been designed to communicate methods and materials for using animals through administrative officials and attending veterinarians to the Institutional Animal Care and Use Committee (IACUC). This process will help assure that provisions are made for compliance with the Animal Welfare Act, the Public Health Service Policy on Humane Care and Use of Laboratory Animals and the Guide for the Care and Use of Laboratory Animals.

Please read this form carefully and fill out all sections. Failure to do so may delay the review of this application. Sections that do not apply to your research must be marked "NA" for "Not Applicable."

This application form must be used for all NEW or THREE-YEAR RENEWAL protocols.

All answers are to be completed using Arial 12 size font.

All questions must be answered in their respective boxes and NOT as attachments at the end of this form.

Please complete any relevant addenda:

Hybridoma/Monoclonal Antibodies ("B")
Polyclonal Antibodies ("C")
Survival Surgery ("D")
Non-Survival Surgery ("E")
Wildlife Research ("F")

If help is needed with these forms, contact the IACUC Coordinator at extension 2616, the Facility Manager at extension 2400 or the Attending Veterinarian at extension 2980.

1. Principal Investigator Information:	
a. Name:	Ibra S Fancher
b. University/Company:	University of Delaware
c. Department:	Kinesiology and Applied Physiology
d. Building/Room:	Ammon Pinozzotto Biopharmaceutical Innovation Center
e. Office Phone:	302-831-7192
f. Lab Phone(s):	
g. Home Phone:	NA
h. Mobile Phone:	724-825-7146
i. E-Mail Address:	ifancher@udel.edu

2. Protocol Status:
a. ☐ New Protocol ***OR*** ☒ Re-submission due to three (3) completed years. If re-submission, enter Protocol Number: **1372**
b. ☒ Research ***OR*** ☐ Teaching or Cooperative Extension
c. ☒ Laboratory Animals ***OR*** ☐ Wildlife ***OR*** ☐ Agricultural Animals If "Wildlife" please complete Addendum "F" For agricultural animal protocols, please list the name and contact information for veterinarian who is on-call. A copy of the protocol should be shared with the veterinarian Click here to enter text.
d. Proposed Start Date: 12/15/2023
e. Proposed Completion Date: 12/14/2026
f. Funding Source: NIH COBRE (PI: Dave Edwards)
g. Award Number if applicable: 2P20GM113125

Appendix B

PERMISSION

Publications: Visceral adipose of obese mice inhibits endothelial inwardly rectifying Kir2.1 channels in a CD36-dependent fashion © 2024 by Sabita Rokka is licensed under CC BY 4.0

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