2023

Associations Between Adipose Tissue Glycocalyx Quality, Nox Activity, and Microvascular Blood Flow

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ASSOCIATIONS BETWEEN ADIPOSE TISSUE GLYCOCALYX QUALITY, NOX ACTIVITY, AND MICROVASCULAR BLOOD FLOW.

By

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A Thesis submitted to the Interdisciplinary Medical Sciences Division in partial fulfillment of the requirements for graduation with Honors in the Major

Degree Awarded: Summer, 2023
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Introduction

In the United States, cardiovascular disease (CVD) is recognized as the number one cause of death for the general population (He et al., 2021). In 2017, CVD comprised 23% of all U.S. deaths, 24.2% of male deaths, and 21.8% of female deaths, accounting for a total of 647,457 deaths that year (Heron, 2019). In 2017, CVD was most prevalent in older populations, as it was the number one cause of death for individuals aged 65-81 years at 25-28.6% of total deaths, and the second leading cause of death for individuals aged 45-64 years at 20.8% of total deaths (Heron, 2019). In 2023, this trend remains true, with the majority of deaths in individuals aged 65 years and older resulting from CVD (Virani et al., 2021). Since 2017, CVD related deaths have increased to approximately 659,000 deaths per year, causing approximately one in every four deaths (Virani et al., 2021). CVD is not only the number one cause of death in the U.S., but it is also very costly. From 1996 to 2016, CVD related spending increased by $108 billion, from $212 billion in 1996 to $320 billion in 2016 (Birger et al., 2021). Currently, the U.S. spends approximately $363 billion per year on CVD, including medical care and medications (Virani et al., 2021).

Cardiovascular disease is linked to a number of risk factors that are continuously increasing in prevalence due to changing American lifestyle habits. These risk factors include but are not limited to: hypertension, hypercholesterolemia, sedentary lifestyles, poor nutrition, smoking, and substance and alcohol abuse. High blood pressure is attributed to an increase in death rates, with the high blood pressure attributed deaths increasing by 34.2% from 2009-2019 (Tsao et al., 2022). High cholesterol is another CVD risk factor and currently, 10.5% of adults 20 years of age and older have high total cholesterol (Tsao et al., 2022). The U.S. is seeing a decrease in active lifestyles in
individuals post high school, as well as an increase in sedentary behavior by adults (Tsao et al., 2022). U.S. citizens are also consuming more saturated fats, low quality carbohydrates, and added sugars, due to dietary changes and the increased prevalence and accessibility of fast food (Tsao et al., 2022). In the U.S., overall tobacco use is decreasing, however 14% of adults aged 18 and over still use tobacco products and smoke daily (Tsao et al., 2022). Due to the development of e-cigarettes and their gaining popularity, e-cigarette use by adolescents has increased by 18.1% from 2011 to 2020 (Tsao et al., 2022). Alcohol, cannabis, opioid, and stimulant abuse also contribute to CVD risk, as abusers of these products have been found to have a higher risk of developing CVD (Gan et al. 2021). The combined CVD risk factors of high blood pressure, high cholesterol, and smoking are prevalent in approximately 46.5% of the American population, making them highly impactful on CVD development (Fryar et al., 2012).

There is also a link between CVD, type 2 diabetes (T2D), and obesity. From 1999-2018 in the U.S., obesity prevalence increased nationally, with the frequency of obesity reaching 42.4% among adults in 2018 (Tsao et al., 2022; Hales et al., 2020. Currently, 2.4 million deaths can be attributed to obesity worldwide (Tsao et al., 2022). Diagnosed diabetes affects approximately 28.2 million individuals, pre-diabetes affects approximately 113.6 million individuals, and undiagnosed diabetes affects approximately 9.8 million individuals in the U.S. (Tsao et al., 2022). In 2019, diabetes was determined to be an underlying cause of death in the deaths of 87,647 individuals (Tsao et al., 2022).

Obesity and T2D are connected, as insulin resistance is a key factor in the development of obesity-related cardiometabolic disease (Tsao et al., 2022). Insulin
resistance and diabetes are also linked to hyperglycemia, hyperlipidemia, and endothelial dysfunction, resulting in an increased risk of developing CVD. Hyperglycemia and hyperlipidemia, specifically, have been identified as potential factors relating to CVD development via oxidative stress, which is delineated as an overproduction in reactive oxygen species (ROS). An overproduction of ROS not only creates a vicious cycle of increasing levels of hyperglycemia and hyperlipidemia, as ROS concentrations stimulate these two states, but ROS overproduction also contributes to endothelial dysfunction and altered insulin signaling pathways. Therefore, it is beneficial to investigate the relationship between insulin resistance and oxidative stress, and how the relationship between the two states is affected by obesity.

As a result of increasing levels of risk factors and the prevalence of CVD in the United States, researchers, physicians, and medical staff are in agreement that the key to reducing CVD related mortalities is to increase an individuals' social determinants of health (SODH). That involves reducing poverty, increasing access to healthcare, increasing access to holistic nutrition, making education accessible, and creating safe environments that foster increased physical activity, growth, and development. Making positive dietary changes, including following a Mediterranean diet, reduces CVD risk by 26% for at risk individuals (Delgado-Lista et al., 2022). Furthermore, frequent exercise helps to reduce CVD risk factors (Lin et al., 2020). Pharmacy-based interventions, where pharmacists guide patients on pharmaceutical intervention and provide CVD education also helps to reduce CVD (Tsuyuki et al., 2016). However, the most beneficial way of reducing CVD and CVD associated risk factors is by providing culturally-competent and holistic preventative care (Brewer et al., 2017). By tailoring preventative primary care,
physicians and teams are able to reduce heart healthcare costs and cardiovascular related hospitalizations (Mundt et al., 2015). Also, by utilizing preventative care measures, health care providers could prevent between 50,000-100,000 CVD related deaths annually (Farley et al., 2010).

Despite researchers and medical staff having ways to reduce CVD risk factors and treat CVD once it appears, there are still gaps in knowledge of how CVD presents itself at the biological level. However, researchers have identified endothelial cells as playing a key role in CVD development, as a decline in endothelial cell function can lead to cardiovascular and cardiometabolic disease (Incalza et al., 2018). Therefore, to better understand the relationship between endothelial dysfunction and CVD development, as well as the relationship between endothelial dysfunction and other cardiometabolic diseases, a combined approach to studying how various tissue types affect endothelial cell function must be carried out. More specifically, the impact of ROS on endothelial dysfunction and the factors culminating in the overproduction of ROS need to be identified. Therefore, the purpose of this study is to pinpoint factors that lead to the overproduction of ROS within endothelial cells located in adipose tissue. To examine this, quantitative data will be collected from populations of obese and lean individuals participating in Cesar Meza and Dr. Robert Hickner's study aimed to link vascular and metabolic dysfunction via NADPH oxidase (Nox). The overall research question this larger study explores is: does increased Nox-derived ROS production in obesity impair blood glucose profiles by reducing insulin-mediated suppression of lipolysis? As the present study is a sub study of the overall research project, this study addresses the
question: how does glycocalyx quality correlate with adipose tissue Nox activity and adipose tissue microvascular blood flow?
**Literature Review**

**Obesity and Lipolysis**

Insulin is an important biological molecule, impacting metabolic and vascular functions within the body. Under normal conditions, after a meal is consumed, insulin secretion is stimulated, resulting in hyperinsulinemia and the storage of excess nutrients, in the form of triglycerides, within adipose tissue. As a result of the hyperinsulinemic state, lipolysis, the breakdown and release of fat molecules, is suppressed, resulting in increased adipose cell size, within adipose tissue. Under abnormal conditions, in individuals with obesity, after a meal is consumed, insulin secretion is still stimulated. However, insulin does not function well to suppress lipolysis in individuals with obesity, as individuals with obesity are insulin resistant, or lower blood glucose levels, therefore resulting in hyperglycemia. As a result, NADPH oxidase (Nox), may become activated, stimulating lipolysis via a reactive oxygen species (ROS) signaling pathway, further reducing the effects of insulin on adipose cells.

![Figure 1. Potential mechanisms by which Nox stimulates lipolysis within adipose cells. Nox may stimulate ROS by either an ANP or β-adrenergic signaling pathway, leading to the breakdown of stored fats into three fatty acids and a glycerol molecule, which then enter circulation for usage.](image-url)
The Nox stimulated lipolysis, in turn, breaks down the stored triglycerides into a glycerol molecule and three free fatty acids. Based on the proposed model, the glycerol molecule released may serve as a precursor molecule for hepatic gluconeogenesis, resulting in hyperglycemia. The state of hyperglycemia within the body may work to create a cycle with Nox production, by further stimulating Nox in adipose tissue to increase lipolysis. At the same time, it is also possible that endothelial cells are affected by adipose Nox. The free fatty acids released during lipolysis are sometimes reused by the adipose cells for continued fat storage, but in some cases, the free fatty acids may increase oxidative stress within endothelial cells by releasing endothelial Nox mediated ROS, leading to possible endothelial dysfunction. Adipose Nox may also indirectly affect endothelial Nox, and therefore affect the overproduction of ROS within endothelial cells through hepatic glucose, in which the glucose produced via lipolysis stimulates endothelial Nox, creating a vicious cycle.
Figure 2. A linear depiction of the vicious cycle of Nox stimulated lipolysis and obesity. When insulin is suppressed in individuals with obesity, adipose Nox may stimulate the breakdown of fat into three fatty acids and a glycerol molecule. The free fatty acids may work to produce Nox-mediated ROS, resulting in oxidative stress and endothelial dysfunction. The glycerol molecule released via lipolysis may be taken up by the liver for hepatic gluconeogenesis, resulting in a state of hyperglycemia, which compounds insulin resistance and obesity.

Figure 3. The vicious cycle of Nox stimulated lipolysis in individuals with obesity.

Endothelial Cells

Endothelial cells make up the interior lining of blood vessels, the lumen, and are responsible for separating blood cells from extravascular tissues, thus maintaining homeostasis, regulating blood fluidity, and controlling inflammation (Meza et al., 2019; Incalza et al., 2018). Endothelial cells are also responsible for the mechanisms that control vasodilation and vasoconstriction of the surrounding smooth muscle cells, which make up the exterior layer of the blood vessels, in times of stress and relaxation (Meza et al., 2019).
Figure 4. The composition of a blood vessel. Endothelial cells line the interior of the vessel and are in direct contact with blood cells. Vascular smooth muscle cells line the exterior of the vessel, and depending on the molecular pathways activated within the endothelial cells, the cells will either maintain their structure, vasodilate, or vasoconstrict.

Within endothelial cells, a multitude of biomolecules and pathways exist that control vasodilation and vasoconstriction of the vascular smooth muscle cells. Out of this multitude, four hold significant importance: endothelial nitric oxide synthase (eNOS), nitric oxide (NO), NADPH oxidase (Nox), and reactive oxygen species (ROS). Vascular shear stress is increased when blood flow exerts pressure on the endothelial lining. Under normal conditions, increased shear stress or acetylcholine (Ach) activate eNOS through a multistep signaling pathway (Meza et al., 2019). L-arginine and O₂ further activate eNOS, resulting in the production of L-citrulline and NO (Meza et al., 2019). NO is responsible for vasodilation of the vascular smooth muscle cells, and if an adequate amount of NO is produced and diffuses into the smooth muscle cells, the muscle cells will dilate (Gielis et al., 2011). Under normal conditions, Nox, located in the plasma
membrane of the endothelial cells, produces hydrogen peroxide (H$_2$O$_2$) and superoxide (O$_2^-$), which are ROS molecules, and releases them outside of the cell (Incalza et al., 2018). Some H$_2$O$_2$ reenters the cell through channel proteins, but is then removed by glutathione peroxidase (GPx) (Meza et al., 2019; Figure 5). Therefore, the majority of ROS molecules go on to act as signaling molecules for a variety of cellular activities within the body, such as cell adaptation, cell division, and allosteric activation of proteins (Alhayaza et al., 2020).

**Figure 5.** Under normal conditions, NADPH oxidase (Nox) produces superoxide (O$_2^-$) and hydrogen peroxide (H$_2$O$_2$), which are released outside the cell. Some H$_2$O$_2$ may enter the cell through channel proteins, but the H$_2$O$_2$ is then degraded by glutathione peroxidase (GPx), allowing normal endothelial functions to continue. To trigger the vasodilation pathway, either acetylcholine (Ach) activates its cell surface receptor or shear stress occurs, enabling endothelial nitric oxide synthase (eNOS) activation. Then O$_2$ and L-arginine act upon eNOS to produce L-citrulline and nitric oxide.
(NO). NO, once produced, diffuses into the surrounding smooth muscle cells, triggering vasodilation (Image adapted from Meza et al., 2019).

Under abnormal conditions, endothelial cells experience an increase in ROS molecules, also known as oxidative stress, resulting in eNOS uncoupling and endothelial dysfunction (Mahmoud et al., 2017; Meza et al., 2019). Although shear stress and Ach can continue to stimulate eNOS activation, oxidative stress is associated with eNOS uncoupling, which is characterized by the splitting eNOS dimers into two monomers that do not produce NO (Meza et al., 2019). In turn, the uncoupled eNOS molecules generate O₂⁻ (ROS), rather than NO, that reacts with available NO to produce peroxynitrite (ONOO⁻). The production of ONOO⁻ leads to further eNOS uncoupling, as well as reduced bioavailability of NO, therefore causing endothelial dysfunction. As a result, oxidative stress and the increased production of ROS within the endothelial cells results in endothelial dysfunction, due to the prevention of NO synthesis and reducing the bioavailability of NO present within the endothelial cells (Mahmoud et al., 2017).
Figure 6. Under conditions of oxidative stress, NADPH oxidase (Nox) produces superoxide (O$_2^-$) and hydrogen peroxide (H$_2$O$_2$), which are released outside of the cell. H$_2$O$_2$ enters the cell and contributes to endothelial nitric oxide synthase (eNOS) uncoupling, as glutathione peroxidase (GPx) is not present to remove it from the endothelial cell. Shear stress and acetylcholine (Ach) continue to act as eNOS stimuli, which is facilitated by O$_2$ and L-arginine. However, the accumulation of reactive oxygen species (ROS) within the endothelial cell leads to the uncoupling of eNOS, causing the dimer to separate into its monomers. This uncoupling prevents the synthesis of nitric oxide (NO) and instead creates O$_2^-$, as well as peroxynitrite (ONOO-) via extra NO within the cell, leading to further eNOS uncoupling and endothelial dysfunction (Image adapted from Meza et al., 2019).

The Glycocalyx

Lining the interior luminal portion of endothelial cells lies a protective and dynamic gel-like coating called the glycocalyx (Weinbaum et al., 2007). The glycocalyx is a sugar-protein complex, composed of glycoproteins, proteoglycans and
glycosaminoglycans anchored to the endothelial cells, with additional plasma proteins attaching to the sugar complexes (Jedlicka et al., 2020). The glycocalyx, also referred to as the “endothelial surface layer” (ESL), serves the primary function of maintaining the endothelial barrier, therefore reducing the development of endothelial dysfunction (Jedlicka et al., 2020). Depending on the location of blood vessels and the shear stress experienced by those vessels, the density of the ESL varies, as sugar-protein complex synthesis and shedding are stress dependent (Nieuwdorp et al., 2005).

The glycocalyx comes into direct contact with blood cells, fluid, and biomolecules that pass along through veins, capillaries, and arteries, making it the first protective line of defense for the endothelium. Therefore, the glycocalyx protects endothelial cells by initiating blood flow resistance, preventing damage from oxidants, immune cells, and harmful biomolecules, and regulating endothelial cell permeability and osmosis (Zheng et al., 2022). Degradation of the glycocalyx, by the shedding of the sugar-protein complexes, endangers the integrity of endothelial cells, as a reduction in glycocalyx quality results in a minimal protective barrier, allowing harmful substances and stress to affect endothelial cells. Therefore, the density of the glycocalyx is indicative of endothelial function, with a thick glycocalyx density relating to good endothelial function. Although the pathways resulting in glycocalyx degradation are unknown, reduced glycocalyx quality has been linked to T2D, hypertension, hyperglycemia, and other metabolic and vascular diseases (Jedlicka et al., 2020; Zheng et al., 2022).
Figure 7. Glycocalyx structure and placement on endothelial cells. When a low density of glycocalyx proteins are present, there are less protein complexes present to protect the endothelial cells from the movement of red blood cells through capillaries, veins, and arteries. Therefore, low glycocalyx density is thought to be indicative of poor endothelial function. When a high density of glycocalyx proteins are present, it is indicated that endothelial cells are better protected, therefore promoting good endothelial function.

The Glucose/ROS Cycle

Researchers have described ROS and glucose as forming a cyclic pathway, in which an increase in one causes an increase in the other, resulting in a vicious cycle of heightened ROS and glucose production that can result in hyperglycemia. Therefore, increased glucose metabolism causes an increase in ROS production, while an increase in ROS production causes an increase in glucose uptake and glycolysis (Alhayaza et al., 2020). Hyperglycemic states increase ROS production by resulting in the breakdown of regulatory molecules, such as eNOS, as well as by inhibiting and diverting other necessary regulatory pathways (Alhayaza et al., 2020). When oxidative stress occurs and
ROS levels are not regulated, due to a lack in antioxidant capabilities of endothelial cells, glucose uptake and metabolism increases (Alhayaza et al., 2020). It has been reported that higher glucose administration over the course of multiple days led to increased ROS production (Alhayaza et al., 2020). Over the course of three days, two groups were administered either 400 mg/dL of glucose or 100 mg/dL of glucose; after three days, ROS production in the 400 mg/dL group was significantly higher than the 100 mg/dL group (Alhayaza et al., 2020). In another study of the effect of glucose on ROS levels, Parasanthan and Jain (2020) investigated glucose-6-phosphate-dehydrogenase (G6PD) deficiencies and their effect on transforming growth factor-β (TGF-β) mediated Nox production. In human aortic endothelial cells (HAEC) lacking G6PD but exposed to high glucose, Nox activity and ROS levels were significantly higher, as compared to their normal HAEC counterparts, showing that high glucose exposure has a direct impact on increasing ROS levels (Parsanathan & Jain, 2020). In another study identifying melatonin's effect on glucose and ROS levels, Tiong et al. (2020) found a positive correlation between ROS levels and glucose administration. The investigators exposed human umbilical vein endothelial cells (HUVEC) to glucose and measured an increase in ROS concentration within the HUVEC (Tiong et al., 2020). However, when the HUVEC were exposed to high glucose and melatonin, the cells still experienced an increase in ROS concentration, due to the increase in glucose uptake, but they also experienced mitochondrial dysfunction and underwent apoptosis as a result of the melatonin exposure (Tiong et al., 2020). Overall, there is a clear positive correlation between glucose uptake and ROS production.
Clinical Implications

Obesity increases the risk of developing cardiometabolic diseases, as obesity poses both metabolic and vascular complications. Increased Nox activity is a plausible underlying component contributing to these metabolic and vascular impairments, as upregulated Nox activity has been observed in adipose tissue and the endothelium of individuals with obesity. Further, a novel role of Nox as a positive regulator of adipose tissue lipolysis may be a key mechanism leading to hyperglycemia and endothelial dysfunction. On the metabolic level, individuals with obesity are insulin resistant, in which insulin no longer functions to adequately suppress lipolysis within adipocytes. Increased Nox activity in adipocytes has been shown to stimulate lipolysis pathways, releasing ROS, glycerol, and free fatty acids into the circulation. The released glycerol may then be taken up by the liver for hepatic gluconeogenesis, in which glucose is produced, resulting in hyperglycemia and further insulin resistance. On the vascular level, the free fatty acids released by lipolysis may be taken up by endothelial cells, resulting in the overproduction of ROS within those cells. Heightened ROS levels may then lead to endothelial dysfunction, as the overproduction of ROS impairs the process of vasodilation of the smooth muscle cells surrounding the endothelial cells.

In individuals with obesity, states of hyperlipidemia and hyperglycemia, due to impaired lipolysis and the overproduction of ROS, result in heightened insulin resistance and oxidative stress within the system. As a result, a vicious cycle is formed, where increased adipocyte ROS production leads to hyperlipidemia and hyperglycemia, which, in turn, contribute to ROS production and oxidative stress in the vascular endothelium. A key component of this cycle is Nox in adipose tissue, as observations have supported Nox...
as being a stimulus for lipolysis. Therefore, the primary aim of the parent study is to address the pathways by which Nox regulates lipolysis.

The glycocalyx, a gel-like glyco-protein complex that lines the luminal side of endothelial cells, serves to protect the endothelium from shear stress and harmful substances. A healthy glycocalyx may help to mitigate the effects of heightened lipolysis hyperglycemia on endothelial cells by protecting against elevations in circulating glycerol, lipids, and glucose. However, there is very little known about the mechanisms of glycocalyx function. The effects of insulin and lipolysis on the glycocalyx are unknown, and therefore, this study addresses how Nox-mediated lipolysis and hyperinsulinemia affect the glycocalyx in vivo.
Methods

For this study, data will be collected during Cesar Meza’s study, which is focused on investigating Nox as a possible link between metabolic and vascular dysfunction. To study this, the parent study will utilize two unique procedures, a hyperinsulinemic-euglycemic clamp and adipose tissue microdialysis. A clamp procedure works to “clamp” a participant’s blood sugar levels at a certain level, and for this study, the clamp is used to mimic the body’s response to a meal by infusing insulin at a steady rate and glucose at varying rates. By stimulating a state of hyperinsulinemia, lipolysis should be suppressed, however this process is impaired due to insulin resistance in individuals with obesity. Therefore, the clamp is used to see if insulin derived lipolysis suppression is less in individuals with obesity, as compared to normal weight individuals. If there is reduced lipolysis suppression in obese individuals, there should be higher rates of glycerol based hepatic gluconeogenesis that can be measured via microdialysis. In microdialysis, probes with a semipermeable membrane are inserted into human tissue, either muscular or adipose. For this procedure, microdialysis probes are inserted into the subcutaneous adipose tissue of the abdomen. During the procedure, the probes facilitate the flow of a perfusate solution into the tissue, as well as collect a dialysate solution. The dialysate is a metabolized version of the perfusate, which also contains other biological molecules found in the adipose tissue surrounding the probes, including glycerol and ROS, allowing for the monitoring of lipolysis and blood flow.

For this sub study, another procedure, the GlycoCheck, is utilized. The GlycoCheck is a camera and computer analysis software duo that analyzes endothelial and capillary function through the identification and measurements of the glycocalyx
sugar-protein complex. The presence of a dense glycocalyx is thought to indicate good endothelial function, and a low glycocalyx density is thought to indicate poor endothelial function (Figure 7). Therefore, the GlycoCheck serves to link obesity focused lipolysis and endothelial dysfunction, as it bridges the gap between lipolysis activated Nox in adipose cells and oxidative stress in endothelial cells. Furthermore, the combination of the Clamp, microdialysis, and GlycoCheck procedures allows for the furthering of knowledge surrounding the connection between glucose, Nox, ROS, and endothelial dysfunction, therefore connecting metabolic and vascular dysfunction on the biological level.

**Participant characteristics**

Males and females (18 - 45 years; BMI: 18.5 - 29.9 kg/m²) of all races and socioeconomic backgrounds from the Leon County area will be studied. These individuals are sedentary, in which they do not engage in strenuous activity for more than 150 minutes per week, as well as have maintained a steady weight (<±2 kg) for the preceding six months. Participants will be non-smokers, lack a known history of diabetes or CVD, and will not be taking medications for hypertension, hypercholesterolemia, hyperglycemia, or on NSAIDs. During a screening visit, participants must have a blood pressure below 140/90 mmHg and a fasting blood glucose below 126 mg/dL.

**Participant Timeline**

Each participant will undergo three separate visits. For the visits, participants will be instructed to refrain from alcohol or caffeine use, and limit strenuous activity for at least 24 hours leading up to each scheduled visit.

**Visit One**

Visit one will be for the signing of the consent forms and a screening to determine eligibility. During the screening process, participants will undergo anthropometric
measurements (height, weight, and waist and hip circumference), resting blood pressure, and a finger stick blood sample to determine fasting blood glucose level and blood lipids. Concentrations of total cholesterol, high-density lipoprotein (HDL), low-density lipoprotein (LDL), and triglycerides will be determined from the same blood glucose fingerstick. Participants will also complete physical activity and medical questionnaires during their screening visit to ensure there are no medical conditions that would prevent them from participating in the various testing procedures. Participants who do not meet the criteria will be denied from the study. Eligible participants will be enrolled and scheduled to complete the following testing procedures.

**Visit Two**
Visit two will include a body composition exam (DXA), a flow mediated dilation (FMD) test of the brachial artery via ultrasound, and a VO₂ max test to determine aerobic fitness, that will be completed within a week of visit three. During this time, participants will also be instructed on how to keep a food and beverage log that will need to be completed for the three days leading up to the third and final visit.

**Visit Three**
Visit three is the main testing day that will include the use of microdialysis procedures, a hyperinsulinemic-euglycemic clamp, and GlycoCheck testing. The present study will focus on data collection only during visit three, as visit three is the only time that GlycoCheck system will be used.

<table>
<thead>
<tr>
<th>Visit Day</th>
<th>Procedures Involved</th>
<th>Duration of Visit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Visit One</td>
<td>Screening: informed consent, anthropometrics, fasting blood glucose and lipids, medical history and physical activity</td>
<td>1 hour</td>
</tr>
</tbody>
</table>
Table 1. Visit timeline for participants.

<table>
<thead>
<tr>
<th>Visit Two</th>
<th>Visit Three</th>
</tr>
</thead>
<tbody>
<tr>
<td>DXA, FMD, VO₂ max test. Food log instructions.</td>
<td>Microdialysis procedures, hyperinsulinemic-euglycemic clamp, the GlycoCheck, and blood collections.</td>
</tr>
<tr>
<td>1.5 hours</td>
<td>8 hours</td>
</tr>
</tbody>
</table>

Visit Three Testing Day Procedures

Figure 8. Timeline of visit three testing procedures. Day three includes microdialysis and hyperinsulinemic-euglycemic clamp procedures, GlycoCheck testing, and blood draws.

Microdialysis

To examine lipolytic regulation and microvascular blood flow in vivo, microdialysis procedures will be carried out in abdominal adipose tissue. After completing an overnight fast, participants will undergo the microdialysis procedure upon arrival to the lab, in which three microdialysis probes (CMA 20: 10 mm * 0.5 mm 20 kilodalton cutoff membrane, 5 cm probe shaft length, inlet tubing and outlet tubing 20 cm; CMA/Microdialysis, Stockholm, Sweden) will be inserted into the subcutaneous abdominal adipose tissue (at the level of the naval) approximately 3 cm apart. Each probe
will be inserted using an 18 gauge needle under sterile technique and with the use of local numbing via an ethyl chloride spray (Gebauer Company, Cleveland, OH).

Figure 9. This is an example of the microdialysis technique utilized by Cesar Meza and Dr. Robert Hickner. The area in which the probes are inserted is first sterilized via a betadine solution. Then, using a sterile 18 gauge needle and local anesthetic, the skin is marked to determine the location of the microdialysis probes. Afterwards, using another sterile 18 gauge needle with a catheter, the probe guide is inserted into the previously made hole in the skin; once the guide is inserted into the tissue and the needle is withdrawn, the probe is inserted through the use of the guide and the guide is removed. Then, sterile gauze is applied to the insertion points and taped down using steri-strips for the duration of the microdialysis procedure, to ensure the probes remain within the participants tissue.

**Probe conditions.**
Each probe will be perfused with a solution at a rate of 2 μL/minute using CMA/107 micro-infusion pumps (CMA/Microdialysis, Stockholm, Sweden). Each probe will contain a solution consisting of 0.9% sodium chloride (saline) and 5 mM ethanol (for blood flow monitoring), as well as 100 μL Amplex Ultrared, 1 U/mL horseradish
peroxidase (HRP), and 10 U/mL SOD for measurement of \textit{in vivo} \text{H}_2\text{O}_2 (ROS) production. To assess Nox-specific ROS production, apocynin, a local Nox inhibitor, will be used. To determine if Nox influences lipolysis through \(\beta\)-adrenergic or ANP mediated pathways, isoproterenol (\(\beta\)-adrenergic agonist) and ANP (a local non-canonical lipolysis agonist) will be perfused. Apocynin will be perfused following the stimulation of lipolysis via the addition of isoproterenol and ANP. These probe conditions will occur under fasted conditions, before the clamp, as well as under clamp conditions, during the clamp. Between the fasted and hyperinsulinemic microdialysis procedures, a two hour washout period will be utilized, in which saline is perfused into the adipose tissue, to prevent tachyphylaxis of the \(\beta\)-adrenergic and ANP receptors.

A description of the three probe conditions:

1. **Probe 1** will serve as the control probe to compare unstimulated lipolytic rates against lipolytic rates stimulated by isoproterenol (Probe 2) or ANP (Probe 3). Probe one will only contain 0.9% sodium chloride (saline), 5 mM ethanol, 100 \(\mu\)L Amplex Ultrared, 1 U/mL horseradish peroxidase (HRP), and 10 U/mL SOD.

2. **Probe 2** will assess Nox contributions to \(\beta\)-adrenergic stimulated lipolysis. The control solution will be perfused with the addition of isoproterenol at a concentration of 10 \(\mu\)M.

3. **Probe 3** will assess Nox contributions to ANP stimulated lipolysis. The control solution will be perfused with the addition of ANP at a concentration of 10 \(\mu\)M.

**Microdialysis protocol and timeline.** Each phase of the microdialysis procedure has a specific duration, includes the addition of specific medications, and requires a specific amount of dialysate collection.
### Table 2. Specific microdialysis protocol timeline.

Each phase, including the equilibration period, denotes the expected duration of the period, the amount of sample vials taken, including what those samples are, and which solution is perfused into each probe during that period. Apo is the abbreviation for apocynin, a Nox inhibitor. Iso is the abbreviation for isoproterenol, a β-adrenergic receptor agonist.

*The microdialysis timeline begins at minute 45 of the overall timeline. The duration of each individual phase within the overall visit timeline is clarified in the duration row.

^A two-hour washout window is utilized in Phase 3 to prevent tachyphylaxis of β-adrenergic and ANP receptors.

#The hyperinsulinemic-euglycemic clamp will begin 30 minutes into Phase 3. A dose of 12 mU·m·2·min will be infused to achieve steady state plasma glucose levels prior to starting Phase 4.

**Description of the perfusion phases/timelines:**

1. **Phase 1 (fasted)** and **Phase 4 (hyperinsulinemic)** are to assess lipolytic rates without local Nox inhibition. Interstitial glycerol concentrations during these phases will be compared between probes to compare lipolytic rates between unstimulated and stimulated conditions.
2. Phase 2 (fasted) and Phase 5 (hyperinsulinemic) are to assess Nox contributions to lipolysis through separate signaling pathways: β-adrenergic or ANP-mediated. Interstitial glycerol concentrations will be compared within each probe before and after local Nox inhibition. The reduction in glycerol upon local Nox inhibition will be attributed to Nox-mediated effects on lipolysis.

3. The objective of Phase 3 is to wash out the effects of prior β-adrenergic and ANP stimulation, preventing desensitization of the receptors after fasted conditions. This will allow for subsequent receptor agonism during hyperinsulinemic conditions.

**Dialysate collection and analysis.**
Dialysate, the solution that perfuses out from the adipose tissue via the probe into a collection vial, will be collected as duplicates for each probe condition every 15 minutes.
Figure 10. Once the probes are in place, a pump containing a syringe filled with perfusate is connected to the inlet tubing of the probe and a collection vial is connected to the outlet tubing of the probe. At a rate of 2 μL/minute, perfusate solution is pushed into the probe to the dialysis membrane at the distal end of the microdialysis probe. Diffusion occurs between the perfusate solution and the interstitial fluid over this membrane. Molecules from the surrounding interstitial fluid and cells enter the liquid in the probe via the semipermeable membrane and is then termed dialysate, which is collected in the collection vials for later analysis.

After collection, the dialysate sample will be analyzed immediately using a TD-700 fluorometer (Turner Designs, Sunnyvale, CA) to measure H$_2$O$_2$ (ROS) concentration. To prepare the sample for analysis, 30 μL of dialysate will be transferred from an aluminum-wrapped collection vial to a fluorometer vial within a dark room, as ROS is light sensitive. The fluorometer then uses an excitation wavelength of 550 nm and emission wavelength of 570 nm to assess the fluorescence intensity of a stable resorufin molecule that is generated when H$_2$O$_2$ (ROS) from the participant’s tissue interacts with the Amplex Ultrared and HRP from the perfusate.

After the microdialysis collection is complete, the dialysate will be further analyzed via an ethanol assay to identify the rate of ethanol removal from the probe,
therefore identifying the rate of blood flow. Results will be expressed as an outflow:inflow ratio of [ethanol dialysate] to [ethanol perfusate]. The outflow:inflow ratio is inversely related to blood flow, with a larger ratio denoting impaired blood flow.

The dialysate will be further analyzed by a CMA/600 analyzer to calculate concentrations of interstitial glycerol, glucose, lactate, and pyruvate from the collected dialysate. Lactate and pyruvate concentrations will provide insight into the metabolic quality of the adipose tissue. Lactate also serves to link metabolic and aerobic pathways, as it is a major gluconeogenic substrate released by adipose tissue and taken up the liver for gluconeogenesis. It can also enhance the suppressive effects of insulin on lipolysis through a G-protein coupled receptor cascade. Glycerol concentrations will serve as markers of lipolysis within the adipose tissue.

Hyperinsulinemic-euglycemic Clamp

To assess insulin sensitivity and explore the metabolic effects of Nox mediated lipolysis in obesity, a hyperinsulinemic-euglycemic clamp will be used. A [6,6-\(^2\)H]glucose stable isotope tracer will be utilized during the clamp procedure to assess hepatic gluconeogenesis and connect increased lipolysis and gluconeogenesis.

For the hyperinsulinemic-euglycemic clamp, participants will arrive at the lab after an overnight fast. Two IVs will be inserted into the participant, one into the antecubital vein and one into the contralateral vein of the hand. The antecubital IV is used for administering glucose and insulin, and the hand IV is used for blood draw collections. The hand containing the IV will be kept warm via the use of a warming box, set at 40°C, to ensure good blood flow and allow for arterialized blood sampling.

At 90 minutes prior to the start of the clamp procedure, a primed continuous infusion of [6,6-\(^2\)H]glucose will be started (priming dose 22.5 μmol • kg\(^{-1}\), infusion rate...
0.25 μmol · kg-1 · min-1). At thirty minutes into stage three of the microdialysis protocol, a primed steady dose of insulin will be initiated together with a 20% glucose solution. The insulin administered, consisting of a solution composed of 100 U/mL Humulin R (insulin), 0.9% sodium chloride (saline), and 5 mL of added participant blood, will be infused at a dose of 12 mU · m² · minute to select for an expected plasma insulin concentration near 25 μU/mL. These doses are associated with half-maximal suppression of lipolysis but exist below the complete suppression of hepatic glucose output. The glucose administered will consist of a 20% glucose solution that will be given accordingly, with adjusted infusion rates, to maintain a blood glucose level of 90 mg/dL. The mean rate of glucose infusion during the final 30 minutes of steady-state plasma glucose concentrations will be defined as the glucose disposal rate (GDR).

Once the clamp begins, small blood draws will be taken every five minutes to monitor the participant’s blood glucose levels, and to determine the amount of glucose needed to be perfused. To analyze these samples, a YSI 2900 STAT glucose analyzer (Yellow Springs Instruments Inc.) will be used. Additional blood collections will occur before and during the clamp to measure plasma glucose, insulin, c-peptide, free fatty acids (FFAs), glycerol, and ANP concentrations.

After the clamp concludes, the participant will be given a meal to stabilize the body’s creation of insulin to prevent hypoglycemia from occurring.
**Figure 11.** This is a diagram of the clamp procedure. On one side of the participant, a two port IV is inserted into the antecubital space by a certified medical provider. One port administers insulin at a fixed rate while the other port administers a 20% glucose solution that varies in dosage, depending on the participant’s blood glucose level. On the other side of the participant, another IV is inserted for blood draws, which are taken every 5 minutes to measure blood glucose level. Based upon the glucose level taken from the blood, the amount of glucose administered via the IV will either be altered or kept constant.

**GlycoCheck**

To explore the effects of Nox-mediated lipolysis and insulin on adipose tissue microvasculature *in vivo*, the GlycoCheck (Glycocheck, Limburg, Netherlands) system will be used. The GlycoCheck system, a camera and computer software duo, will be used twice during the participant visit, once under fasted conditions and again during the hyperinsulinemic-euglycemic clamp. To measure the microvasculature *in vivo*, the Glycocheck camera will be placed in the participants mouth, sublingually, to measure the participants sublingual microvasculature. Although the GlycoCheck only measures the sublingual vessels, the findings are indicative of the metabolic and vascular activity occurring within the microvessels of adipose tissue cells.
**Figure 12.** This is the GlycoCheck camera that is placed sublingually in the participant’s mouth. The camera is located at the end of the probe and is used to look at the sublingual capillaries. The camera is hooked up to the computer which then records the data.

**GlycoCheck procedure.**
Under both conditions, before the GlycoCheck camera is placed sublingually, it will first be hooked up to the GlycoCheck computer system and calibrated using the GlycoCheck software. The camera will then be cleansed using an 70% isopropyl alcohol wipe. Afterwards, a sterilized CapiScope HVCS Probe Cover (KK Research Technology Limited, Honiton, England) will be placed over the camera, followed by the camera and attached probe cover being placed sublingually.

For data collection, a dynamic setting will be used, in which 24 measurements of the sublingual microvasculature will be obtained. Each measurement will take between 10-20 seconds, as the system requires motion, intensity, and focus to be stable, to obtain quality imaging for software analysis. After reading 12, the participant will be allowed to take a small break to minimize participant discomfort. In total, data collection will take
between 15-20 minutes for each condition, depending on participant sublingual microvessel quality. After the procedure is completed, the GlycoCheck system will be left to analyze the participant data, which will take between 1 to 2 hours.

*Figure 13.* This is the GlycoCheck system during a data collection. The image in the center of the screen is the camera’s visual. To the left of the central image is a box containing scales that measure motion, intensity, and focus; the goal is to keep the scales within the green regions to ensure the best visual possible is being collected for data analysis. To the right of the central image is a region containing a blue bar and a clock countdown. As the blue bar increases in height, more data points are collected.

**GlycoCheck data analysis.**

To process and analyze the fasted and clamp conditions data, the GlycoCheck will automatically group microvessels by their diameter, which range between 4-25 μM.

Based on their grouping, the GlycoCheck will then analyze the microvessels to identify
sublingual density, microvessel red blood cell (RBC) velocity, and perfused boundary region (PBR) averages of each group. Each data point will provide insight into the functional capabilities of the grouped microvessels, as well as identify the integrity and thickness of the glycocalyx through the use of the individual PBR readings. The analyzed data will then be used by the GlycoCheck system to calculate overall blood flow, capillary blood flow (microvessels 4-7μM), large microvessel blood flow (10-25μM), total density, capillary density (microvessels 4-7μM), large microvessel density (10-25μM), PBR, and RBC velocity for each participant.

Figure 14. This is the data collected by the GlycoCheck system post a dynamic setup of 24 separate measurements after it has been automatically analyzed.
After the automatic analysis, the data will then be manually analyzed to assess the glycocalyx thickness of individual microvessels, sized 6-10 μM in diameter, under fasted and clamp conditions. To manually measure the glycocalyx, video imaging saved by the system must be viewed for the passing of white blood cells through the microvessels for. In total, 10 viable videos per condition, fasted and clamp, need to be identified, for a total of 20 videos per vessel diameter per participant. Once a white blood cell passes through, and video quality is satisfactory, the video can be saved and uploaded to ImageJ for further analysis. Once the videos are uploaded to ImageJ, a VascularAnalysis plugin must be used, in which the glycocalyx thickness can manually be measured by determining “pre” thickness, before the white blood cell passes through, and “post” thickness, after the white blood cell passes through. The equation (pre − post)/2, will then be averaged from the ten videos per condition to determine how glycocalyx thickness found on one side of the endothelial cell lumen is affected.

**Statistical Analysis**

As this study addresses the question of how glycocalyx quality correlates with adipose tissue Nox activity and adipose tissue microvascular blood flow, the data collected via microdialysis will be compared to the data provided by the GlycoCheck. Therefore, analysis of GlycoCheck data points, including density, microvessel red blood cell velocity, and perfused boundary region, and microdialysis data points, including glycerol, ROS, and ethanol concentrations, will be carried out for both fasted and hyperinsulinemic conditions. The analysis of these relationships will help address if there are significant connections between the microdialysis and GlycoCheck data, therefore bridging how Nox-mediated lipolysis affects metabolic and vascular functions *in vivo*, including the effects of insulin on the glycocalyx. A paired t-test will also be carried out.
between means for all GlycoCheck data points and microdialysis data points under fasted and clamp conditions, to determine significance. The relationships between the microdialysis data and the GlycoCheck data can also address the questions: Does insulin affect glycocalyx quality? Is a healthy glycocalyx related to ROS? And, are blood flow and endothelial function connected?
Results

Glycerol

Figure 15. Glycerol concentrations under fasted and clamp conditions. Data were analyzed using a paired t-test to identify differences in lipolysis between fasted and clamp conditions. Data are means ± SD. n = 7 participants.

Glycerol concentration, an indicator of lipolysis within adipose tissue, was calculated under fasted and clamp conditions (Figure 15). The data displayed a higher concentration level of glycerol under fasted conditions, as compared to clamp conditions. However, there was no significant difference in lipolysis level between fasted and clamp conditions, as determined by a paired t-test (mean ± SD; Fasted: 43.40 ± 21.52 μM, Clamp: 22.64 ± 9.444 μM; p = 0.0882).

ROS
Figure 16. Reactive oxygen species concentrations under fasted and clamp conditions. Data were analyzed using a paired t-test to identify differences in ROS concentrations between fasted and clamp conditions. Data are means ± SD. n = 8 participants.

Reactive oxygen species concentration was calculated under fasted and clamp conditions (Figure 16). The data displayed a higher concentration level of ROS under clamp conditions, as compared to fasted conditions. However, there was no significant difference in ROS concentration between fasted and clamp conditions, as determined by a paired t-test (Fasted: 1.043 ± 0.4557 μM, Clamp: 1.630 ± 1.059 μM; p = 0.0702).

Ethanol

Figure 17. Ethanol outflow-to-inflow ratio under fasted and clamp conditions. Data were analyzed using a paired t-test to identify differences in blood flow between fasted and clamp conditions. Data are means ± SD. n = 7 participants.

The ethanol outflow-to-inflow ratio, an indicator of blood flow and microvascular health, was calculated under fasted and clamp conditions (Figure 17). The data displayed a slightly higher ethanol ratio under clamp conditions, as compared to fasted conditions. However, as determined by a paired t-test, there was no significant difference in the ethanol ratio between fasted and clamp conditions (Fasted: 0.6943 ± 0.1745, Clamp: 0.7450 ± 0.08118; p = 0.5375).
Flow

**Figure 18.** Microvessel blood flow under fasted and clamp conditions. Data were analyzed using a paired t-test to identify differences in blood flow in all microvessels between fasted and clamp conditions. Data are means ± SD. n = 6 participants.

Flow, the movement of red blood cells (RBC) through microvessels, was calculated for each participant under fasted and clamp conditions (**Figure 18**). The data displayed a higher rate of flow under fasted conditions, as compared to clamp conditions. However, as determined by a paired t-test, there was no significant difference in blood flow between fasted and clamp conditions (Fasted: 396.1 ± 179.8 $\times 10^3$ μm$^3$/sec/mm$^2$; Clamp: 328.8 ± 89.1 $\times 10^3$ μm$^3$/sec/mm$^2$; p = 0.2297).

**Red Blood Cell Velocity**

**Figure 19.** Microvessel diameter and red blood cell (RBC) velocity under fasted and clamp conditions. Data were analyzed using a paired t-test to analyze RBC velocity for each microvessel diameter (4-25μm) between
fasted and clamp conditions (E). *P < 0.05 vs Fasted. Data are means ± SD. n = 6 participants.

Red blood cell (RBC) velocity mean was calculated for each microvessel diameter (4-25μm) under fasted and clamp conditions (Figure 19). The RBC velocity at each diameter, for all participants, was grouped by fasted or clamp conditions, and then averaged to determine a mean velocity for each microvessel diameter. As determined by a paired t-test, there were significant decreases in RBC velocity between fasted and clamp conditions (mean ± SD; Fasted: 108.7 ± 7.343 μm/s, Clamp: 103.4 ± 9.951 μm/s; p = 0.0017). The significance lies with microvessels 11μm (p = 0.050), 13μm (p = 0.029), 14μm (0.022), and 22μm (p = 0.006).

Density

![Graph](image)

**Figure 20.** Microvessel density under fasted and clamp conditions. Data were analyzed using a paired t-test to identify differences in microvascular density of capillary microvessels sized 4-6μm (left), microvascular density of large feeding microvessels sized 10-25μm (middle), and microvascular density of all microvessels (right) between fasted and clamp conditions. Data are means ± SD. n = 6 participants.

Microvessel density, the density of microvessels sized 4-25μm per unit area of sublingual tissue, was calculated under fasted and clamp conditions (Figure 20). Microvessels were separated into groups based on vessel size, including small capillaries
sized 4-6μm (left) and large feeding vessels sized 10-25μm (middle), as well as grouped together (right) under fasted and clamp conditions. Microvessel density for capillaries (left) was slightly higher under fasted conditions. However, there was no significant difference in capillary density between fasted and clamp conditions, as determined by a paired t-test (Fasted: 7.470 ± 1.972 mm/mm², Clamp: 6.675 ± 2.108 mm/mm²; p = 0.5455). Microvessel density for feeding vessels (middle) was very similar between both conditions. As determined by a paired t-test, there was no significant difference in large microvessel density between fasted and clamp conditions (Fasted: 6.447 ± 2.103 mm/mm², Clamp: 6.355 ± 1.327 mm/mm²; p = 0.9165). Microvessel density for all microvessel diameters (right) was slightly higher for fasted conditions. However, as determined by a paired t-test, there was no significant difference in microvascular density between fasted and clamp conditions (Fasted: 8.947 ± 2.779 mm/mm², Clamp: 8.450 ± 1.526 mm/mm²; p = 0.7052).

**Figure 21.** Microvessel diameter and density under fasted and clamp conditions. Data were analyzed using a paired t-test to analyze microvascular densities for each microvessel diameter (4-25μm) between fasted and clamp conditions. Data are means ± SD. n = 6 participants.

Microvessel density mean was calculated for each microvessel diameter (4-25μm) under fasted and clamp conditions (**Figure 21**). The microvessel density for each
microvessel diameter, for all participants, was grouped by fasted or clamp conditions, and then averaged to determine a mean density for each microvessel diameter. As determined by a paired t-test, there were significant decreases in specific microvessel densities between fasted and clamp conditions (Fasted: 8.955 ± 7.912 μm, Clamp: 8.445 ± 7.049 μm; p = 0.0212).

**Perfused Boundary Region**

**Figure 22.** Perfused boundary regions (PBR) under fasted and clamp conditions. Data were analyzed using a paired t-test to analyze PBR of capillary microvessels sized 4-6μm (left), PBR of large feeding microvessels sized 10-25μm (middle), and differences in PBR in all microvessels (right) between fasted and clamp conditions. Data are means ± SD. n = 6 participants.

The perfused boundary region (PBR), an indicator of glycocalyx quality, denoted by the lateral movement of RBCs into the glycocalyx of vessels sized 4-25μm, was calculated under fasted and clamp conditions (**Figure 22**). Microvessels were separated into groups based on vessel size, including small capillaries sized 4-6μm (left) and large feeding vessels sized 10-25μm (middle), as well as grouped together (right) under fasted and clamp conditions. PBR for capillaries (left) was approximately equal under fasted and clamp conditions. There was no significant difference in capillary PBR between fasted and clamp conditions, as determined by a paired t-test (Fasted: 0.9094 ± 0.05410


μm, Clamp: 0.9117 ± 0.07659 μm; p = 0.9401). PBR of feeding vessels (middle) was
greater under clamp conditions. However, as determined by a paired t-test, there was no
significant difference in large microvessel PBR between fasted and clamp conditions
(Fasted: 2.722 ± 0.2670 μm, Clamp: 2.875 ± 0.2775 μm; p = 0.1378). PBR for all
microvessel diameters (right) was slightly higher for clamp conditions, but there was no
significant difference in PBR between fasted and clamp conditions, as determined by a
paired t-test (Fasted: 2.294 ± 0.2041 μm, Clamp: 2.406 ± 0.2126 μm; p = 0.1658).

Figure 23. Perfused boundary regions (PBR) between fasted and clamp
conditions. Data were analyzed using a paired t-test to analyze static PBR in all
microvessels between fasted and clamp conditions. *P < 0.05 vs Fasted. Data are
means ± SD. n = 6 participants.

Static PBR, PBR that does not take into account blood flow, was calculated for
each participant under fasted and clamp conditions (Figure 23). The data displayed a
higher PBR under clamp conditions, as compared to fasted conditions. As determined by
a paired t-test, there were significant increases in PBR between fasted and clamp
conditions (Fasted: 2.030 ± 0.1775 μm, Clamp: 2.170 ± 0.2089 μm; p = 0.0420).
Figure 24. Microvessel diameters and perfused boundary regions (PBR) between fasted and clamp conditions. Data were analyzed using a paired t-test to analyze PBR for each microvessel diameter (4-25μm) between fasted and clamp conditions. *P < 0.05 vs Fasted. Data are means ± SD. n = 6 participants.

Perfused boundary region (PBR) mean was calculated for each microvessel diameter (4-25μm) under fasted and clamp conditions (Figure 24). The PBR at each diameter, for all participants, was grouped by fasted or clamp conditions, and then averaged to determine a mean PBR for each microvessel diameter. As determined by a paired t-test, there were significant increases in PBR between fasted and clamp conditions (Fasted: 2.294 ± 0.8007 μm, Clamp: 2.407 ± 0.8634 μm; p<0.0001). The significance lies with microvessels 12μm (p = 0.041), 17μm (p = 0.016), and 18μm (0.034).
Discussion
For this study, the relationships between adipose tissue glycocalyx quality and both adipose tissue Nox activity and adipose tissue microvascular blood flow were explored. Although the overall study that this sub-study belongs to explored both the metabolic and vascular effects of Nox-derived ROS production on the insulin-mediated suppression of lipolysis, this sub-study primarily explored the vascular components. To identify how the vicious metabolic cycle of Nox, ROS, and hyperglycemia related to endothelial dysfunction, the glycocalyx, a protective barrier lining the lumen of endothelial cells, was identified and studied. To do so, the GlycoCheck system was utilized, as it provided in vivo readings of the sublingual microvasculature of participants, allowing the identification of glycocalyx differences under fasted and hyperinsulinemic conditions.

Microdialysis
To assess the changes identified between fasted and clamp conditions via the GlycoCheck, data was related to the data obtained from the microdialysis procedure. This included control glycerol concentrations, control ROS concentrations, and the control ethanol outflow:inflow ratio under both fasted and clamp conditions. Glycerol concentration slightly decreased under clamp conditions, as compared to the fasted glycerol concentration (Figure 15). The decrease in glycerol, although insignificant, identified that less glycerol was available in the bloodstream under clamp conditions. This can be related to two situations: either lipolysis decreased under clamp conditions due to the infusion of insulin into the participants, or glycerol was removed from the bloodstream for hepatic gluconeogenesis. The ROS concentration increased under clamp conditions, as compared to the fasted ROS concentration (Figure 16). Although this
increase was not deemed significant, the increase in ROS concentrations signifies a possible increase in Nox activity within adipose cells under hyperinsulinemic conditions. This finding may be related to the glycerol concentration data, in which an increase in ROS concentration may signify an increase in Nox-mediated lipolysis activity, which would result in an increase in glycerol concentration within the bloodstream. Because this trend is not seen with the data, the glycerol produced from lipolysis may be taken up by the liver for gluconeogenesis instead of existing freely in the bloodstream, causing hyperglycemia. The ethanol outflow:inflow ratio also slightly increased under the clamp condition, as compared to the fasted condition (Figure 17). Although the slight increase was not significant, the difference identified a small decrease in blood flow when insulin was perfused, as the ethanol outflow:inflow ratio is inversely related to blood flow. This finding does not correspond to the vasodilatory effects of insulin on arterioles, as insulin typically supports an increase in blood flow to transport glycerol and free fatty acids within the vascular system. However, the slight increase in the ethanol ratio, when related to the ROS concentrations, may signify endothelial dysfunction through the uncoupling of eNOS, as a result of increased ROS concentrations, therefore lessening the vasodilatory effects of insulin on arterioles.

**GlycoCheck Flow**

When assessing blood flow via the GlycoCheck system, a decrease in mean flow was exhibited between fasted and clamp conditions, with the clamp flow being lower (Figure 18). This decrease, although insignificant, identifies that on the microvasculature level of capillary and feeding vessels, blood flow decreases. This finding follows the microdialysis ethanol outflow:inflow ratio findings (Figure 17), as the ethanol ratio
slightly increased between conditions, while the GlycoCheck calculated flow decreased between conditions, both identifying that blood flow decreased. This may signify that on the microvessel level, insulin may lack its vasodilatory effects. With the addition of the ROS concentration findings (Figure 16), the ROS mediated endothelial dysfunction pathway can be addressed. As ROS concentration increased and microvascular blood flow decreased across fasted and clamp conditions, the increase in ROS may have caused endothelial dysfunction through the eNOS uncoupling pathway. This could have therefore caused vasoconstriction on the microvasculature level, despite the perfusion of insulin within the system.

**RBC Velocity**

Overall, red blood cell velocity was higher under fasted conditions, as compared to clamp conditions, except for in some larger feeding microvessels (Figure 19). The differences in RBC velocity between conditions was significant, with further significance lying with microvessels 11μm, 13μm, 14μm, and 22μm. The trend seen with decreased RBC velocity under clamp conditions follows the same pattern as flow. Therefore, similar conclusions can be drawn between RBC velocity, insulin, ROS levels, and endothelial dysfunction. The increased ROS concentrations under clamp conditions (Figure 16), combined with the significant decreased RBC velocity, may signify that under clamp conditions, insulin mediated lipolysis malfunctions, leading to the stimulation of Nox-mediated ROS production, resulting in endothelial impairment. As a result, endothelial cells exhibit dysfunction, in which they constrict instead of dilate in the presence of insulin, therefore limiting RBC velocity and, as a result, limiting blood flow.
**Microvessel Density**

When comparing mean density of the individual microvessels sized 4-25μm in diameter, microvessel density was slightly higher under fasted conditions, as compared to clamp conditions, with significance (Figure 21). When comparing the mean microvessel densities of all microvessels between fasted and clamp conditions, there was no significance (Figure 20). The differences between these two comparisons is that one compared the mean of microvessels at each specific diameter (Figure 21), while the other compared the overall mean density of microvessels for all diameters (Figure 20). From the significant data set (Figure 21), it can be concluded that insulin has the greatest effect on the available density of microvessels sized 6-10 μm, as microvessel density decreased for microvessels sized 6-10 μm under clamp conditions. When dividing the microvessels into capillary microvessels (4-6μm; Figure 20) and large feeding microvessels (10-25μm; Figure 20), there were no major differences between fasted and clamp conditions. Capillary density experienced a slight, yet insignificant decrease in microvessel density under clamp conditions, as compared to fasted conditions, while large feeding microvessel density experienced no significant changes in microvessel density between fasted and clamp conditions. As a result of these trends, it is concluded that insulin and Nox-mediated lipolysis have very little to no effect on microvessel density.

**PBR**

Across all findings, PBR increased under clamp conditions. The mean PBR of all individual microvessels sized 4-25μm in diameter were higher under clamp conditions, as compared to fasted conditions, with significance (Figure 24). This significance was found with microvessels sized 12μm, 17μm, and 18μm. When comparing the mean PBR of all microvessels, as well as splitting up the microvessels into capillary and large
feeding microvessels, an increase in PBR was seen between feeding and clamp conditions (Figure 22). Further, when assessing static PBR, there was an increase in PBR between fasted and clamp conditions, with significance (Figure 23). The data shows that under clamp conditions, when insulin is perfused, the perfused boundary region within microvessels increases, which correlates to a decreased glycocalyx density. When connecting the decreased PBR (Figure 23; Figure 24) to the microdialysis data, increased ROS production in adipose tissue (Figure 16) relates to changes in glycocalyx density in microvessels under clamp conditions. This relationship determines that during Nox-mediated lipolysis, when ROS is overproduced, ROS may degrade the density of the glycocalyx structure, therefore diminishing the protective properties of the glycocalyx and leaving endothelial cells unprotected from harmful substances traveling via the blood. The lack of protection from external harmful substances can add to endothelial cell dysfunction, therefore leading to an increased risk in cardiometabolic disease development.
Conclusion

Although the data are preliminary, this study provides novel insight into the link between metabolic and vascular dysfunction via Nox. Thus far, the data has connected how increased ROS production via Nox-mediated lipolysis affects blood flow, RBC velocity, and PBR. The increased ROS production found under clamp conditions may be related to the decrease in blood flow and RBC velocity, as measured by the GlycoCheck. This relationship may be explained through ROS-mediated eNOS uncoupling, resulting in endothelial dysfunction and vasoconstriction of microvessels, therefore causing reduced blood flow and RBC velocity within microvessels. The increased ROS production found under clamp conditions may also be related to the increase in PBR, as measured by the GlycoCheck. This relationship may be explained through ROS impacting the glycocalyx structure through a metabolic process, therefore increasing the region between blood cells and endothelial cells (PBR), and resulting in decreased glycocalyx density. Despite these findings being preliminary, they will provide insight on the pathways responsible for the development of cardiometabolic diseases, including CVD.
Limitations

The main limitation experienced during this study is lack of participants. As there were only six participants who underwent the microdialysis procedures, the hyperinsulinemic-euglycemic clamp, and GlycoCheck testing, the data obtained thus far, despite its significance, is preliminary. Therefore, more testing needs to occur to determine further significance. Furthermore, the metabolic and vascular effects of Nox-mediated lipolysis were not explored within obese populations, due to a lack of obese participant participation. As a result, the breadth of the study was limited, as there was a lack of obese findings to compare to the non-obese findings. To address these limitations, data collection will continue throughout the Summer of 2023.
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