DETERMINING THE IMPACT OF TWO-WEEKS OF DIETARY SALT LOADING ON RESTING AND POST-EXERCISE VASCULAR ENDOTHELIAL HEALTH

by

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Abstract

Elevated sodium intake is one of the leading factors underlying cardiovascular disease. A majority of Canadians consume sodium far in excess of daily requirements, increasing the prevalence of many types of cardiovascular disease and damages to vascular function. Several aspects of vascular function are regulated through an arterial layer called the glycocalyx. The glycocalyx is a sugar and protein rich layer that is responsible for storage of sodium, mechanical sensing of blood flow and serves as a protective boundary for the cells that make up blood vessels. Damage to this layer has been linked to decreased responsiveness of blood vessels as well as reduced overall vascular health. Damage can be caused by sudden increases in blood flow, such as from trauma or intense exercise.

The purpose of this study was to examine the effects of high sodium intake on multiple measures of cardiovascular and vascular health in healthy individuals, as well as the response to lower limb exercise. Healthy individuals (N = 13) participated in a randomized crossover study receiving either: a) 12g per day dietary salt (NaCl) supplement, or b) 12g per day of a sugar placebo for a two-week period. Conditions were separated by a two-week washout period. Measurements of vascular health included flow-mediated dilation of the brachial and popliteal artery as well as ELISA analysis of plasma hyaluronan and C-reactive protein as measures of glycocalyx integrity and inflammation, respectively.

This study determined that sodium loading had a substantial negative effect on flow-mediated dilation in both the brachial and popliteal arteries. Despite this, there was no significant change to the response to exercise in either the brachial or popliteal arteries. There was a mild increase in plasma hyaluronan following exercise, but no change arose from elevated sodium intake either pre- or post-exercise. These results indicate that while high sodium intake has a rapid negative influence on vascular function, it does not exert a comparable effect on the integrity of the glycocalyx either at rest or in response to exercise. These findings are a first step forward towards determining the mechanisms underlying sodium-induced vascular dysfunction as well as determining what factors can influence the glycocalyx.

Keywords: Cardiovascular health, Cardiovascular Disease, Vascular function, Glycocalyx

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Chapter 1: Introduction

Cardiovascular Disease

Cardiovascular disease (CVD) is among the leading causes of death in the world, representing 29.6% of all deaths (Balakumar et al., 2016; Roth et al., 2020). Cardiovascular diseases can be both acute and chronic and affect single or multiple components of the cardiovascular system, notably the heart and arteries. Cardiovascular health is in large part determined by the function of the endothelial cells that make up the lining of the blood vessels, called the endothelium (Alexander et al., 2021). Diseases that afflict the cardiovascular system include coronary artery disease, stroke, aneurysms and hypertension (Roth et al., 2020). Due to the complexity and importance of the cardiovascular system, dysfunction at key points can have significant impacts throughout the rest of the body. End organ damage because of long-term CVD can spread throughout the body and resultant complications can be fatal (He et al., 2020; National Academies of Sciences, Engineering, and Medicine, 2019).

Overall Risk Factors

Many factors lead to an increased risk of developing CVD with age, smoking and elevated blood cholesterol levels being significant predictors (Goldsborough et al., 2022). Individuals with Diabetes Mellitus type I and II also have demonstrated elevated rates of CVD (Wilson et al., 1998; Yusuf et al., 2004). Yusuf et al. (2004) further found that lifestyle factors such as alcohol consumption 3 or more times per week and regular physical inactivity have also been correlated with increased rates of CVD. Like excessive alcohol consumption, dietary factors have become an important area of focus for reducing CVD incidence (Yusuf et al., 2004). A relationship between excess sugar consumption and increased risk has largely been established independent of the increased risk brought by diabetes or similar diseases. Multiple epidemiological studies and meta-analyses support this idea (Yang et al., 2014; Malik and Hu, 2019; Dehghan et al., 2017). The risks associated with general fat consumption have been called into question. The results of earlier studies showing elevated risk with increased saturated fat consumption have not been substantiated in more modern

trials and cohort studies (Dehghan et al., 2017). Whilst dietary cholesterol also lacks a conclusive association with an increase in CVD prevalence, elevated endogenous levels of low-density lipoprotein has been linked to increased rates of CVD (Packard et al., 2021). As such, the overall impact of the diet depends on the substance, with some well associated and others lacking quality evidence. One dietary nutrient that has garnered attention is sodium, which has both evidence for and against when considering its role in CVD onset and progression (Tan et al., 2018; O'Donnell et al., 2020; He et al., 2021)

Dietary Sodium as a Risk Factor

Sodium chloride (NaCl), often referred to as dietary salt, in food is the largest source of sodium (Na) in people's diets. Though it is recognized as an essential nutrient, dietary sodium intake is a significant driver of CVD and related conditions (National Academies of Sciences, Engineering, and Medicine, 2019). The association between elevated levels of salt consumption (>5g per day) and increased risk of hypertension and CVD has been established since the early 1900s (Ambard, 1904). The INTERSALT study examined four populations without high levels of sodium intake as determined by 24-hour urinary excretion. The study compared the Yanomamo and Xingu peoples of Brazil and rural populations of Kenya and Papua New Guinea to population centres across the globe. In the groups with low sodium intake, blood pressure (BP) does not increase with age and there seems to be an almost complete absence of hypertension. Hypertension was highest in the rural Kenyan sample, with 5% of the population affected, a fraction still much lower than the compared groups (Fries, 1976; Carvalho et al., 1989). It was also found that age-related changes in BP from salt intake were dependent on initial BP, elevating with higher initial value (Law et al., 1991). Similar to INTERSALT, it was also observed within the Solomon Islands that islanders following a hunter-gatherer lifestyle had lower BP than whites from the mainland United States (Law 1997). This contrasts with undeveloped tribes with access to salt who experience an age-dependent increase in BP similar to populations in western countries as well as increased rates of hypertension (Fries, 1976; He and MacGregor, 2007). The response to sodium is not identical across all persons. Some individuals exhibit a heightened BP increase in response to high sodium intake and greater decreases when intake is reduced. This has been termed "salt-sensitivity" and is an indicator of increased CVD risk (Franco and

Oparil, 2006). Alternatively, “salt-resistance” is indicated by a diminished response to such changes in Na^+ intake.

The salt-sensitivity of individuals has been determined using several methods. The BP response to a one-week period of both high and low-salt diets is currently considered to be the standard and is considered to be the most reliable method for assessment (Elijovich et al., 2016). Protocols involving the response to administration of diuretics and saline infusions have also been used, though these methods have low accuracy and reproducibility (Kurtz et al., 2017). Sensitivity is not a discrete variable, with varying degrees dependent on several factors. The cutoff for sensitivity is also not universal and is an arbitrarily selected value determined by the investigators, which can confound results (Kurtz et al. 2017). Sensitivity and resistance can be conferred through multiple means, including age, genetics, diet, kidney function and diabetes or combinations thereof (Elijovich et al., 2016). Racial differences in salt sensitivity can be removed when supplementing dietary potassium, likely due to socioeconomic differences in diet (Kurtz et al., 2021). In two notable studies, Weinberger found that salt sensitivity is present in both normotensive and hypertensive populations, but that normotensive salt resistant individuals have increased survival (Weinberger et al., 1986; Weinberger et al., 2001).

Current Recommendations for Limiting Sodium intake

The link between excessive sodium intake and increased BP has been known for over a century (Ambard, 1904). In the later stages of research in the area, the recommendation for a reduction or limitation of salt (NaCl) intake to below 2 grams per day (0.8g Na) to limit hypertension was suggested (Fries 1976). In Canada, the advised daily limit of sodium is 2300 mg, and the recommended consumption value is 1500 mg per day. Despite this, the average Canadian consumes 2760 mg per day, largely from processed and preserved foods (Health Canada 2018). A meta-analysis by He et al. found that reduction in daily salt intake

by 4.4g (1.8g Na) led to a 4.18 mmHg and 2.06 mmHg decrease in systolic and diastolic BP, respectively. This effect was found to be dependent on hypertensive status, being larger in those with hypertension compared to normotensives: -5.39 vs. -2.42 mmHg systolic and -2.82 vs. -1.00 mmHg diastolic in hypertensives and normotensives respectively (He et al., 2013). The findings of Aburto et al. (2013) are largely similar; reducing salt intake below 2 grams per day (0.8g Na^+) reduced systolic BP by 3.47 mmHg and diastolic BP by 1.81 mmHg (Aburto et al., 2013). A large body of substantiating evidence has been built to support the idea of improved cardiovascular health from reducing sodium intake (Cook et al., 2007; He and MacGregor, 2011; He et al., 2013; Joffres et al., 2007; Sacks et al., 2001). The "Trials of Hypertension Prevention" I and II studies were two randomized trials that examined the effect of seven non-pharmacological treatments on reducing BP in individuals aged 34-50. Long-term follow-ups were then conducted 5 and 10 years after the trial and demonstrated that those with reduced sodium intake had a 25% reduced risk of a cardiovascular event (Cook et al., 2007). Furthermore, there is a 20% reduction in cardiovascular events after a reduction in salt of approximately 2.0-2.3g per day (0.8-0.9g Na) (He and MacGregor, 2011). Work by Joffres et al. (2007) also estimated the effect of a 1840mg reduction in dietary sodium and determined that on average systolic and diastolic BP would decrease by ~5mmHg and 2.7mmHg, respectively. This reduction would reduce the relative incidence of hypertension by 30% (Joffres et al., 2007). Overall, the body of evidence is so robust that national authorities have released statements recommending a general reduction in sodium and salt consumption amongst Canadians (He et al., 2013; Health Canada 2018). Despite this, not all meta-analyses agree on the strength of the correlation. Adler et al. (2014) determined that there was weak evidence in hypertensives for reductions in cardiovascular disease resulting from decreased sodium intake (Adler et al., 2014). In addition, multiple meta-analyses by Graudal and colleagues (2011, 2014, 2015) claim a "U-shaped" relationship towards risk and cautioned against recommendations of sodium restriction (Graudal et al., 2011; Graudal et al., 2014; Graudal et al., 2015). These recommendations among other similar conclusions made by other research groups have caused growing controversy.

Controversy Over Sodium Intake

While it is ubiquitous that excess sodium has negative effects, the quantity where excess is defined is debated by some. Multiple recent studies have characterised current recommendations for sodium intake as “controversial” (Mente et al., 2021; O’Donnell et al., 2014). The primary conclusion from these studies is that cardiovascular disease risk is U or J-shaped, with increased risk lying on either side of typical western sodium consumption (Mente et al., 2021, Graudal et al., 2014). The methodologies, namely spot urinary Na^+ measurement, used to determine this U or J-shaped relationship with disease risk have been criticized as unreliable and misleading since they involve inaccurate measurements and lack of control for confounding variables (Campbell et al. 2023; He et al., 2021; Cappucio et al., 2019; He et al., 2019). Spot or single time point urinary Na^+ measurement varies by time of day, duration, collection volume, sodium in the last meal consumed, as well as other body systems associated with CVD outcome and is considered to be unreliable to determine sodium balance (Campbell et al., 2023; Cogswell et al., 2016). Ensuring proper control for these factors is challenging, increasing uncertainty in the measurement (He et al., 2021). Also, researchers have demonstrated that the relationship between sodium intake and CVD risk changes depending on which formulas are used to extrapolate kidney sodium excretion from spot urine samples (He et al., 2019). Specifically, the Kawasaki formula causes what some see as a spurious J-shaped relationship (He et al. 2021). Also, some studies contributing to increased risk under low-sodium intake included individuals with kidney disease and diabetes (Ekinci et al., 2011; O’Donnell et al., 2011; Pfister et al., 2014). These diseases impact water and electrolyte balance and contribute to the severity and progression of cardiovascular disease (Thomas et al., 2011; Ekinci et al., 2011). They can also impact the validity of using spot urine assessments since kidney function is impaired. Additionally, studies examining this relationship have not accounted for some factors such as potassium intake, which alters the cardiovascular effects of sodium (O’Donnell et al., 2014; He et al., 2021).

When considering the entire body of research in the field, there is a large amount of compelling evidence that current average levels of sodium intake are excessive. Current and historical research finds substantial reductions in risk factors, CVD incidence and mortality from lower sodium intake. Furthermore, the evidence put forth recommending against a

general reduction in sodium intake is low in both quality and quantity by comparison. Though some questions about precise mechanisms remain, a known area of significance is the layer of cells that lines the inside of blood vessels, called the vascular endothelium where the interaction with dietary sodium may be complex.

Vascular Endothelium Structure and Function

The endothelium as a whole is a single-cell thick layer present on all organs. When this layer is directly interacting with blood, it is named the vascular endothelium (Figure 1). It has been known that the vascular endothelium played a role in the regulation of vascular tone since 1980, when it was demonstrated that the removal of the innermost layer of rabbit aortas prevented relaxation of the vessel in response to acetylcholine (Furchgott and Zawadzki, 1980). This layer is created during the construction of new blood vessels in response to several signalling molecules, most significantly vascular endothelial growth factor (VEGF) (Apte et al., 2019). After these cells are created, they are long lived and relatively quiescent with less than 3% proliferating per month in mice (Li et al., 2023). The integrity and health of endothelial cells is important for several blood vessel characteristics, such as vasoconstriction and vasodilation, movement of white blood cells, and mechanical sensing and transmission of blood hydrodynamics (Languille and O'Donnell, 1986; Sumpio et al., 1988; Piechoka et al., 2021). This sensing of hydrodynamics and other important vascular functions has been linked to a layer on the surface of endothelial cells, often referred to as the glycocalyx (Olde engberink et al. 2015). The proper function of the endothelium is not immutable. Changes to the environment surrounding the cells as well as within them can lead to loss of function, with one of the most common causes being high sodium intake (Cahill and Redmond, 2016, He et al., 2013).

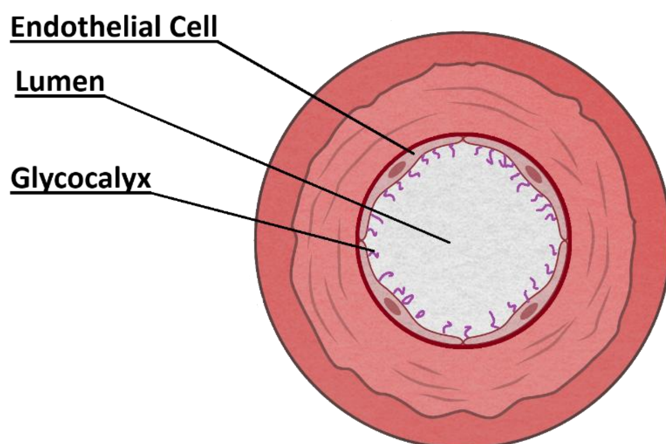

Figure 1. Diagram of an artery showing the vascular endothelium and glycocalyx projecting into the lumen of the vessel.

High Sodium Intake Causes Vascular Endothelium Dysfunction

The complex balance of factors that induce constriction and dilation is also regulated by the endothelium and maladaptive changes to these factors may contribute to development of CVD with decreased vascular endothelium function often preceding the development of CVD (Cahill and Redmond, 2016). Studies on Dahl rats determined that salt-sensitive rats given an 8% by weight NaCl diet for 8 weeks experienced a significantly decreased endothelial relaxation response (Lüsher et al., 1987). An 8% salt (NaCl) diet in mice was also linked to maladaptive remodelling of the heart and aorta, as well as altered proteins associated with regulation of vascular function (Vinaiphat et al., 2023). It is notable that especially in short-term studies done on the larger conduit arteries like the aorta, this maladaptation can be inconsistent, with both decreases and increases in endothelial nitric oxide synthases having been observed (Ni and Vaziri, 2001; Ying and Sanders, 1999). The negative effects become more consistent when looking at muscle arterioles and smaller arteries of mice and rats where multiple studies have found decreased vessel dilation and NO

bioavailability in response to blood flow and vasodilatory compounds (Boegehold, 1993; Boegehold, 1995; Weber and Lombard, 2000).

There have been several studies that support sodium intake causing endothelial dysfunction in humans as determined by the ability of the artery to dilate or constrict with changes in shear stress. Dickinson et al. 2009 gave participants “low-salt” 50 mmol Na^+/day (2.922g NaCl/day) and “usual-salt” 150 mmol/day Na^+ (8.766g NaCl/day) diets for 2 weeks. The low-salt condition had increased endothelial dependent dilation of the brachial artery (i.e. flow-mediated dilation (FMD)) 4.89 ± 2.42% compared to 3.37 ± 2.10% (Dickinson et al., 2009). Jablonski et al. (2009) found that low sodium intake was associated with 52% higher flow-mediated dilation (FMD) in adults (Jablonski et al., 2009). This was followed by a 2013 study where 4 weeks of decreased sodium intake amongst participants with elevated BP improved brachial artery FMD by 68% and improved resistance artery dilation by 42% independent of changes in BP (Jablonski et al., 2013). Dupont et al. (2013) demonstrated that normotensive individuals who consumed 300-350 mmol Na^+ per day saw a relative decrease of 32% in FMD response of the brachial artery (Dupont et al., 2013). In individuals with salt-resistant and salt-sensitive hypertension, high salt diets impaired relative brachial artery FMD response by 34.3% and 38.1% respectively when compared to a low salt diet (Matthews et al., 2015). A 7-day 300 mmol Na^+ also attenuated brachial artery low-flow mediated constriction from -0.58±0.99% to 0.17±1.23%, in which dilation is considered a maladaptive change (Shenouda et al., 2020).

Another method of assessing in vivo arterial tone is to measure the speed of the pulse along arteries which is increased or decreased with concomitant arterial tone changes. Pulse-wave velocity under high-sodium conditions is also increased independent of increases in BP, indicating a negative change in vascular tone (Todd et al., 2010). Sodium concentrations above 145 mmol in plasma have been linked to endothelial cell stiffness, reiterating that plasma sodium can cause endothelial dysfunction and changes to vascular function (Oberleithner et al., 2007). The relationship between sodium load and arterial stiffness is also apparent in children and young adults (Brady et al., 2022). In addition, Babcock et al. (2020) found that the BP response to submaximal exercise was increased with high dietary sodium intake, likely due to impaired endothelial function (Babcock et al., 2020). The health of the

vascular endothelium may also have an impact on the function of the immune system by changing the expression of binding proteins and chemoattractants on immune cells (Li et al., 2022; Wilck et al., 2017). Thus overall, excess sodium intake negatively alters vascular function and exaggerates physiological responses to stresses that likely have long-term health consequences. One notable proposed mechanism for this is reduced production and bioavailability of nitric oxide.

Blood Pressure Regulation by the release of Nitric Oxide

Nitric oxide is an important signaling molecule and is a vital regulator of BP. In the cardiovascular system, NO is synthesized primarily by the endothelial nitric oxide synthase (eNOS) enzyme (Ignarro et al., 1987; Rees et al., 1989; Wood et al., 2013). An enzymatically active form of the eNOS protein is also found in erythrocytes which has been suggested as another mechanism for the pathway to regulate BP, though this remains debated (Leo et al., 2021). The expression of neuronal nitric oxide synthase (nNOS) has also been observed at low levels in vascular smooth muscle cells and may allow for some vascular responsiveness when eNOS is dysfunctional. This is separate from its role in BP regulation via the central nervous system (Schwarz et al., 1999). Activity of nitric oxide synthases in endothelial cells is decreased by a high-salt diet (Li et al., 2009). Furthermore, knockout of the eNOS enzyme in mice lead to a 15% increase in mean arterial pressure relative to control (Leonard et al., 2006). The lack of bioavailable NO has also been implicated in changes to adhesion molecule dynamics, which has notable negative outcomes on the development of some types of CVD.

NO Changes Adhesion Molecule Expression and Activation

Atherosclerotic plaques are created through the accumulation of low-density lipoprotein (LDL) within the intima layer of the arteries, which then triggers inflammation and the activation and migration of immune cells to the intima. The activation of leukocytes and adherence to the vascular wall is an early event in atherosclerosis and a contributing factor in end organ damage from hypertension (Ferri et al., 1998). After migration, phagocytes then uptake oxidized LDL which induces a morphological change in phagocytes. These altered phagocytes are then referred to as foam cells and may leave the arterial wall or

undergo apoptosis within the vascular wall where they accumulate, potentially forming the cores of atherosclerotic plaques (Chistiakov et al., 2017).

Decreased bioavailability of NO has been linked to the development of atherosclerosis since, when in abundance, it inhibits this process by preventing lymphocyte function-associated antigen (LFA-1) CD11a/CD18 and macrophage-1 antigen adhesion molecule (Mac1) CD11b/CD18 from binding to endothelial cell surfaces (Kubes et al., 1991). NO also regulates the surface expression of these proteins (Arndt, et al. 1993) by inactivating vascular cell adhesion molecule 1 (VCAM-1) gene expression in vascular endothelial cells. In regulating all these processes, NO plays a role in the modulation and dampening of inflammation and immune activation within the blood vessel wall. As such, alterations to the bioavailability of NO because of sodium consumption may have downstream impacts on adhesion molecule expression and activation (Khan et al., 1996).

The Effects of Sodium on Blood Pressure Regulation via the Renin-Angiotensin Aldosterone System

In addition to direct effects on the vascular beds throughout the body, dietary sodium filtration, reabsorption and excretion are under hormonal regulation. The renin-angiotensin-aldosterone system (RAAS) is a major regulator of arterial BP and electrolyte levels, including sodium (Patel et al., 2017). Sodium excretion and reabsorption occurs primarily in the kidneys where epithelial sodium channels (ENaC) are a key Na^+ transporter within the nephron (Hamm et al., 2010). Aldosterone increases expression of ENaC on endothelial cell surfaces in the kidney and causes increases in systemic cell stiffness due to the increased Na^+ uptake. The cellular expression of ENaC is regulated at several levels and is significantly influenced by hormonal processes such as the RAAS (Hamm et al., 2010). Improper activation of ENaC causes increased reuptake of sodium in the kidneys instead of excretion. The resultant increased sodium level in the blood causes hypertension and kidney damage (Kakizoe et al., 2009). Furthermore, it increases sensitivity to changes in sodium and potassium levels (Takeda, 2004). As detailed in Cappucio et al. (1985), hypertensive individuals on a sodium-restricted diet experience a significant decrease in BP, whereas

normotensives do not. This occurs through a sodium-dependent decreased response of the renin-angiotensin system (Cappucio et al., 1985). This manifests as suppressed plasma renin activity, as well as suppressed plasma angiotensin II and aldosterone levels (Takeda, 2004). An increase in aldosterone produced within endothelial cells then occurs, which stimulates Na^+/K^+-ATPase activation and gene expression in vascular smooth muscle cells (Takeda, 2004). The incorporation of ENaC and Na^+/K^+-ATPases caused by this natively produced aldosterone then increases the stiffness of the endothelium, decreasing NO production and over time likely contributing to systemic damage to the vasculature (Fels et al., 2009). Chronic infusion of angiotensin II also increases vascular presence of IL-17, a cytokine which promotes inflammation (Maduhr et al., 2010). In vitro treatment of vascular endothelial cells with an aldosterone antagonist also prevents the negative effects of sodium on the endothelial glycocalyx. This suggests that aldosterone activity plays a role in the maladaptive response of the endothelial glycocalyx to sodium (Oberleithner et al., 2011). Sodium-induced changes to the RAAS are not limited to electrolyte balance, and the changes to inflammatory responses indicates the immune system is another potential avenue for maladaptive change.

Sodium alters immune function and increases pro-inflammatory markers

The interaction between the vasculature and immune system can promote or prevent atherosclerosis development and sodium intake seems to modulate this relationship. Specifically, individuals on a high sodium diet show an increase in monocyte presence and activation (Machnik, 2009). Rats fed high sodium diets accumulate sodium within the skin which serves as a non-osmotic storage compartment for sodium (Titze et al., 2003). This leads to increased movement of macrophages into this niche where production of vascular endothelial growth factor-C (VEGF-C) by these cells as well as decreased eNOS production. There is also evidence that mononuclear phagocyte system cells, including macrophages act to buffer high-sodium diets (Machnik, 2009).

Other consequences of high sodium levels include the induction of T cell differentiation, specifically promoting T-helper 17 cells which may induce hypertension

through the production of proinflammatory IL-17 (Madhur et al., 2010) whereas decreasing sodium intake has been associated with a decrease in inflammatory IL-6 and IL-23 and an increase in IL-10 production, an anti-inflammatory cytokine (Yi et al., 2015). In the deoxycorticosterone acetate (DOCA-salt) rodent model, rats have one kidney removed and receive injections of deoxycorticosterone acetate and a high salt (NaCl) diet. They then develop sodium dependent symptoms of cardiovascular remodeling similar to those in humans. One mechanism for this is the overexpression of immune signaling pathways, especially the NLRP3 inflammasome and the IL-1ß cytokine causing inflammation leading to subsequent damage of the cardiovascular system (Krishnan et al., 2015).

C-reactive protein (CRP) is a protein that is partially responsible for mediating the systemic inflammation response (Gabay and Kushner, 1999; Black et al., 2004). Plasma concentrations of CRP can vary extensively and rapidly, able to rise several hundred-fold within 24-48 hours following an acute inflammatory challenge, such as trauma or sepsis (Devran et al., 2012). Although some inflammatory markers are elevated with high sodium intake, C-reactive protein, a systemic low-grade inflammation marker, has not been conclusively linked to high sodium intake. Studies have found both positive and negative correlations between sodium intake and CRP levels under multiple different types of hypertension, making a blanket association impossible (Yilmaz et al., 2012; Gruppen et al., 2016).

There are a number of mechanisms through which excessive sodium intake causes damage to the vascular system: Stiffening of the endothelium, changes to electrolyte balance, alterations to immune function and inflammation, and beyond. One important area to all these functions is an extracellular projection from the vascular endothelium, called the glycocalyx. It is known that the glycocalyx plays a significant role in many endothelial functions and serves as a significant location for sodium sequestration but also may limit exposure of the endothelium cell themselves to excess sodium (Oberleithner et al. 2011). As a result, this layer requires investigation.

The Composition and Roles of the Glycocalyx

The glycocalyx is a negatively charged glycoprotein and carbohydrate rich extension of every human cell. Originally termed in 1962, the glycocalyx was found to exist as a coating on muscle and endothelial cells (Bennett, 1963). It is made up predominantly of units of glycosaminoglycans (GAG), glycolipids and proteoglycans. The primary GAGs are chondroitin sulfate, heparan sulfate and hyaluronan (Gao and Lipowsky, 2010). The vascular glycocalyx is present on the luminal side of the blood vessel and plays a role in the mechanotransduction of signals, acts as a sodium storage compartment and can serve as a barrier for the diffusion of solutes (Oberleithner et al. 2011; Olde Engberink et al. 2015).

The role of the glycocalyx in mechanotransduction was determined through enzymatic removal of the heparan sulfate present on the outside of endothelial cells. Florian et al. (2003) first noted that the removal of this layer prevented the shear stress induced production of NO_2 and NO_3 but did not prevent their production from bradykinin. These findings are well supported by other studies using selective enzymatic removal which determined that specifically heparan sulfate, hyaluronan and sialic acid residues are responsible for the conduction of shear stress to the endothelium (Mochizuki et al. 2003; Pahakis et al. 2007). Increased epithelial Na^+ channel (ENaC) activity and resulting increased intracellular Na^+ concentration within endothelial cells has been shown to increase the stiffness of the cortical cytoskeleton and reduce the response to conducted shear stress (Jeggle et al., 2013). This effect then manifests functionally as reduced endothelial dependent dilatory function or a reduced flow mediated dilation and may contribute to long term blood pressure alterations.

Historically, sodium storage was thought of as a “two-compartment” model where storage in the intracellular and extracellular spaces is regulated by excretion from the kidney (Olde Engberink et al., 2019). However, 24-hour urinary sodium excretion has been shown to vary considerably from daily sodium intake without the expected corresponding change in weight (Titze et al., 2002). This indicates that there must be additional storage where sodium is present in such a way that it is osmotically inactivated. Sodium storage within the skin glycocalyx has been shown to occur in rats. Sprague-Dawley rats fed 8-week diets of 0.1% or 8% NaCl showed increased GAG content within the skin and increased non-osmotic sodium

storage within the skin on the high-salt diet. This occurred without a commensurate increase in plasma sodium concentration, indicating significant Na^+ storage within the compartment commensurate with the increase in GAGs (Titze et al. 2004).

It is likely that once sodium storage reaches a limit, continued high extracellular sodium concentrations then decrease endothelial glycocalyx thickness and increase cell stiffness by approximately 50% and 130% respectively (Oberleithner et al., 2011). The glycocalyx also stiffens, becoming less able to carry shear force to the cell surface (Korte et al., 2012) which would contribute to endothelial vasodilatory dysfunction. Finally, the ability of negatively charged GAG to non-osmotically store sodium suggests that it plays a role in BP regulation through sequestration of sodium (Olde Engberink et al., 2015).

Quantification of the Glycocalyx and Factors Affecting Glycocalyx Integrity

In relation to the integrity of the glycocalyx, several factors may degrade or build this layer over the long term. Decreased oxidative stress and improved antioxidant defences have been proposed as protective mechanisms (Majerczak et al., 2017; Lee et al., 2019). In the acute setting, shedding of the glycocalyx may be caused by increased shear resulting from trauma or acute exercise (Majerczak et al., 2017; Rahbar et al., 2015; Rehm et al., 2007), which may decrease the ability of the glycocalyx to modulate vascular tone and decrease its sodium buffering capacity. Integrity of the glycocalyx can be assessed through measuring plasma concentrations of the component GAGs, commonly syndecans 1 and 4, heparan sulfate and hyaluronan. When measured using biomarkers, increased presence is associated with decreased integrity (Hahn et al., 2021). Overall, the integrity of this layer of the endothelium may be key in modulating vascular tone and arterial BP whilst also modulating sodium bioavailability through its sequestration.

As mentioned above, shear stresses are one of the principal factors that influence both the health and function of the glycocalyx (Vittum et al., 2024; Weinbaum et al., 2003). Shear stress is generated from the flow of blood over the glycocalyx, which transfers this force to actin within the cytoskeleton, triggering cellular responses such as the release of NO and the production of glycocalyx components and other signalling compounds (Piechocka et al.,

2021; Thi et al., 2004; Wang et al., 2020). This response to shear stress is protective in nature, as areas with inconsistent or disturbed flow patterns have decreased glycocalyx thickness and increased intima-media ratios (Gouverneur et al., 2006). When cell cultures are exposed to laminar flow, which is characteristic of regions resistant to atherosclerosis, glycocalyx formation is promoted (Giantsos et al, 2013; Koo et al., 2013). These *in vitro* findings are supported in humans by Majerczak et al. (2017), where 20 weeks of regular exercise, which is known to increase shear stress, resulted in improved glycocalyx integrity (Green et al., 2017; Majerczak et al., 2017). However, brief stimuli of sufficient intensity may interact with the glycocalyx and potentially induce shedding that would reduce normal function of this barrier with implications in vascular endothelial function and short-term blood pressure regulation (Vinaiphat et al. 2023). One such stimulus is acute exercise which may be used to probe glycocalyx function in the applied setting.

Exercise Effects on Glycocalyx Shedding Flow-Mediated Dilation, and C-Reactive Protein

While long-term exercise has been demonstrated to increase glycocalyx integrity, sufficient length bouts of strenuous exercise can function as a method to indirectly measure changes in glycocalyx integrity and function using the quantity of shed components in the blood (Lee et al. 2015; Majerczak et al. 2016; Majerczak et al. 2017). Length of exercise is an important factor, as a short bout of maximal exercise is not sufficient to cause shedding, whereas steady state cycling for 30 minutes at 60% of peak power output and 45 minutes at 85% of peak power output in a high-intensity interval format were sufficient to induce glycocalyx shedding (Majerczak et al., 2017; Sapp et al., 2019; Kröpfl et al., 2021). One of the consequences of utilizing exercise as a tool for determining glycocalyx integrity is that the acute exercise response will have impacts beyond causing shedding (Kröpfl et al., 2021; Majerczak et al. 2017). Acute exercise has been linked to a decreased FMD response post-exercise that typically returns to baseline after approximately one hour (Dawson et al., 2013). Increasing exercise intensity above 50% of maximum heart rate also results in greater decreases in FMD response, independent of changes to baseline artery diameter and shear (Birk et al. 2012). This response may be triggered in part by disruption of the endothelial glycocalyx. As highlighted, this can lead to a loss of mechanotransduction and downstream

NO signalling and production as a result (Florian et al., 2003; Yen et al., 2015; Majerczak et al., 2017; Sapp et al., 2019). This is supported by Grandys et al. (2023) in which the reduced flow-mediated dilation developed by female athletes was accompanied with an increase in circulating glycocalyx biomarkers, reflecting degradation of the glycocalyx (Grandys et al. 2023). However the interaction of glycocalyx integrity and sodium related changes in vascular function both chronically and acutely following exercise has not been explored and may be related to many exercise factors that contribute to glycocalyx shedding.

Another acute exercise response that may impact glycocalyx integrity and shedding is the acute inflammatory response. The acute exercise response of CRP has been variable between studies. Bizneh and Jaafari observed an increase in concentration from 1.98 to 2.46 mg/L immediately following circuit training at 35% of maximum, whereas Markovitch et al. found no change following 30 minutes of exercise at 50% of maximal oxygen uptake (Bizneh and Jaafari, 2011; Markovitch et al., 2008). This variability extends into the 24 hours following exercise, though different exercise lengths and relative intensities may explain some of the inconsistency (Davis et al., 2008; Murtagh et al. 2005; Scharhag et al. 2005). Some increase in CRP may not be caused directly by the exercise response, possibly being caused by minor damage to the musculature from the exercise (Scharhag et al. 2005). Meta analyses suggest that a positive relationship between exercise and CRP is most consistent after intense exercise (Brown et al. 2015; Kasapis and Thompson, 2005). It is, however, accepted that over longer time periods exercise has been shown to reduce plasma and serum CRP (Kasapis and Thompson, 2005).

Exercise as a probe to access the involvement of the glycocalyx in acute changes in endothelial function

Though research into the effects of sodium and exercise on vascular endothelial function (e.g. FMD) individually has been performed, there has been little investigation on how high sodium intake alters the FMD response following exercise or into how high sodium intake alters glycocalyx integrity. Since sodium is stored inactively within the glycocalyx, damage to its structure could release sodium into circulation, further worsening the post-exercise reduction in FMD (Oberleithner et al., 2011; Olde Engberink et al., 2015; Olde Engberink et al., 2019). Since the increase in shear from exercise is predominantly within the

exercising limbs, measurement of FMD in the brachial and popliteal arteries could allow for the determination of both local and systemic effects (Green et al. 2017). An increased reduction in exercising limb FMD following high sodium intake could support the hypothesis that localized sodium release from the glycocalyx may further exasperate the reduced FMD response following exercise. This could be further rationalised as a consequence of increased serum Na^+ being associated with decreased FMD (Dupont et al. 2013).

Objectives

The primary goal of this study was to determine if a period of high sodium (NaCl) intake would affect f and their response to a lower limb exercise bout. As well, the work examines whether sodium intake further reduces the typical post heavy intensity exercise reduction in FMD previously shown both locally in the exercising limb (popliteal) and systemically in the non-exercising limb (brachial). As well, some potential mechanisms will be explored by determining levels of circulating biomarkers of glycocalyx damage and immune activation at rest and post-exercise and if high sodium intake had any impact on this relationship. Finally, the work will involve assessing whether there is a sodium-dependent correlation between possible changes to circulating glycocalyx biomarkers and changes to endothelial function as measured by flow-mediated dilation. Based on existing literature, the following hypotheses were proposed:

1. Sodium (NaCl) loading will significantly reduce FMD of the brachial and popliteal arteries reflective of decreased nitric oxide bioavailability in accordance with existing research.

2. FMD post-exercise will be reduced to a greater extent in the exercised limbs compared to the non-exercised limbs due to glycocalyx removal.This effect will be further exacerbated under high sodium ingestion condition.

3. Markers of glycocalyx shedding, specifically plasma hyaluronan concentration will be elevated following exercise, with a greater exercise-induced increase following

sodium loading. The relationship between FMD and the degradation of the glycocalyx will be visible. Increased presence of biomarkers within the plasma are expected to be reflective of reduced vasodilatory capability, visible as an attenuated FMD response in the exercising limbs due to the increased glycocalyx shedding from increased blood flow. Plasma CRP concentration will not change from either sodium loading or exercise, indicating that any observed inflammatory response is driven by other factors, consistent with published literature.

Chapter 2: Methods

Ethical and Biohazard Approval Statements

Ethical approval and a Biohazardous Materials Use Application for the study were granted by the Thompson Rivers University Research Ethics Board (File No. #103480, #103494). All experiments were performed within the CL-2 facility at Thompson Rivers University. This study conformed to the Declaration of Helsinki and written informed consent was given by every participant.

Participant Information and Demographics

The study included 13 healthy participants who met all inclusion criteria. Participants were assessed via a questionnaire and deemed ineligible for participation if they had diagnosed diabetes, cardiovascular disease or hypertension; were a current smoker, were currently taking any prescription medications or had any medical condition which could limit or otherwise make dangerous strenuous physical activity. Participants were recruited from the Thompson Rivers University campus as well as social media. Nine male and 4 female participants participated. Two additional participants were recruited but were unable to complete the study and their characteristics are not reported.

Participant physical characteristics are reported in Table 1.

Study Design

A randomized cross-over design study was conducted involving a high dietary sodium (NaCl) intake period or a placebo period. These were specifically an additional 12 grams per day of dietary salt (4.75g Na^+) for a two-week period, or 12 grams per day of a sugar placebo for a two-week period, each given in identical flavourless, unmarked capsules. This quantity of sodium is comparable to the quantities used in previous studies in the area (Yi et al., 2015; Babcock et al., 2020; Decker et al., 2022). Each supplementation period was separated by a minimum two-week washout period where no additional supplement was taken. Participants were not informed which trial they were assigned to in either period and were asked to maintain their regular diets throughout the duration of the study.

After an initial session where physical measurements and assessment of maximal aerobic exercise capacity ($\dot{V}O_{2peak}$) testing was undertaken (described below); all subsequent visits followed an identical procedure. Participants were asked to continue their normal diet during the loading period and abstain from alcohol, caffeine and exercise for >24 hours before each testing session. Participants were also asked to fast for >4 hours prior to the testing session. For logistical reasons, the menstrual cycles of female participants were not monitored as part of this study. Hashimoto et al. (1995) found that there can be significant variation in female FMD between the follicular, luteal and menstrual phases, though D'Urzo et al. (2017) have found that this effect is not consistent across all women (D'Urzo et al. 2017, Hashimoto et al. 1995). Participants were rested lying supine on a cushioned table in the lab for 20 minutes prior to any physiological measurements. Pre-exercise measurements of flow-mediated dilation (FMD) of the brachial and popliteal arteries were taken, as well as a venous blood sample. A 45-minute exercise bout was then performed on a cycle ergometer at a work-rate determined by their $\dot{V}O_{2peak}$ ramp increment test. If the participant was unable to maintain the targeted intensity, the work rate was lowered until they were able to maintain the intensity for the full duration. This lowered intensity was then used for all subsequent testing sessions. No participant required a reduction ≥10% in work rate from estimated values. Approximately one minute post-exercise completion, another venous blood sample was drawn. Post-exercise measurement of brachial FMD was performed 5 minutes post-exercise and popliteal FMD was measured approximately 15 minutes post-exercise.

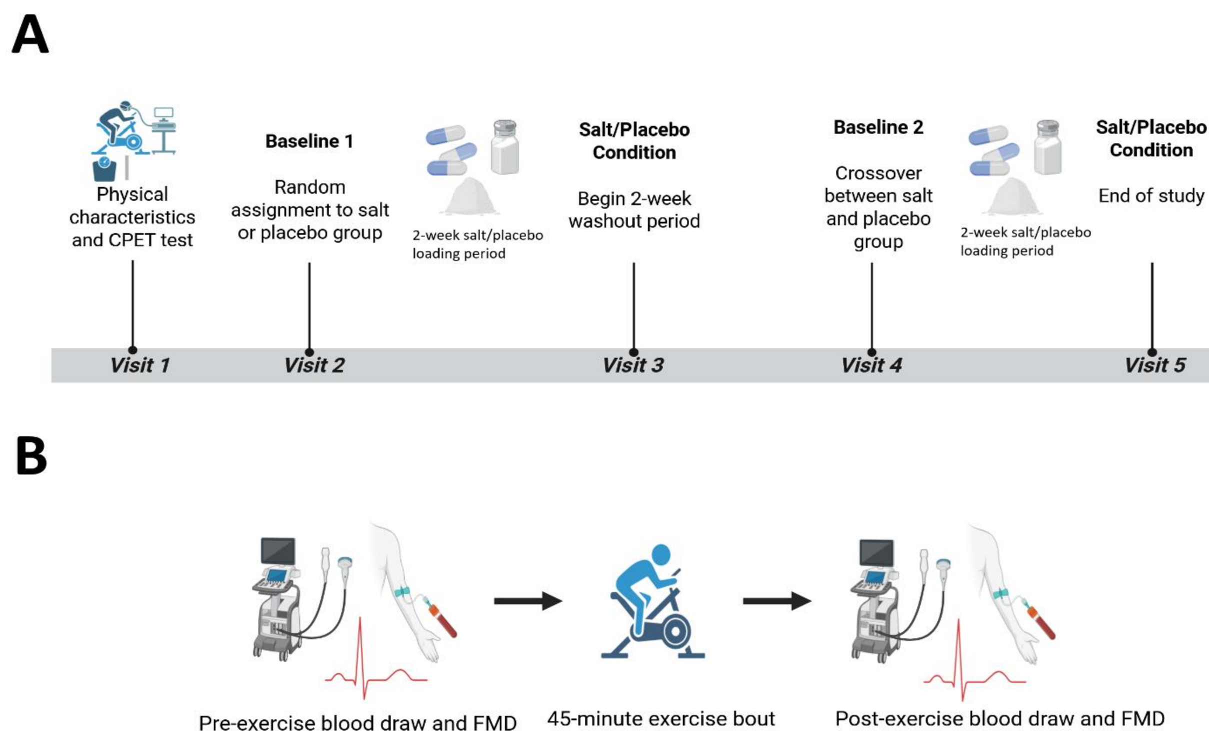

Figure 2. Diagram of overall study design (A) and intra-day testing timeline (B). Participants had physical characteristics and VO_{2peak} measured during the initial visit. All subsequent visits followed the following procedure: Participants had a pre-exercise blood sample as well as brachial and popliteal FMD measured. Then participants performed a 45-minute exercise bout. After the bout, brachial and popliteal FMD were re-measured, and another blood sample drawn.

Determination of Physical Characteristics, VO_2 Max and Targeted Exercise Intensity

Prior to condition assignment, an initial testing session involved obtaining baseline measurements of physical characteristics and determination of VO_{2peak} (Figure 2). VO_2 max was determined by a ramp increment test performed until voluntary exhaustion on an electrically braked cycle ergometer (Tacx Neo, Garmin, Olathe, United States). The ramp increment protocols were a 5-minute 50W warmup for males and females, 50+5W/15s ramp for males, 50+3W/12s ramp for females. Participants were instructed to maintain approximately 60 revolutions per minute (RPM) and the test was considered complete once a participant reached voluntary exhaustion. An individual was determined to have exerted

sufficient effort if they achieved a peak respiratory exchange ratio of >1.10 as well as reaching 85% of age predicted heart rate max, in accordance with recommended guidelines (Balady et al. 2010). Gas exchange data was taken breath-by-breath using a metabolic cart (Quark CPET, COSMED, Italy). Before each test, the device was calibrated according to manufacturer protocol, using a 3L air syringe and a calibrated gas mixture.

Participants were equipped with a heart rate monitor (Garmin, United States). $\dot{V}O_{2peak}$ was determined as the peak 30 second average. Ventilatory thresholds 1 and 2 were determined using the V-slope method as described in Beaver et al. (1986). VT1 was additionally verified by identifying non-linearity in a ventilatory equivalent vs. $\dot{V}O_2$ graph. VT2 was additionally verified as the point at which end-tidal O_2 began to rise and end-tidal CO_2 began to fall, representing hyperventilation. We then used these thresholds to determine the intensity of a 45-minute exercise bout at subsequent experimental sessions, targeted at 1/3 between VT1 and VT2. This intensity was chosen as it is comparable to existing intensities and durations that are known to cause glycocalyx shedding in existing studies (Lee et al., 2019; Sapp et al., 2019, Kröpfl et al., 2021). The work rate was determined by taking the work rate at the timepoints of VT1 and VT2 and using the formula:

$$1/3 \times (Workrate_{VT2} - Workrate_{VT1}) + Workrate_{VT1} \qquad (1)$$

Measurement of Haemodynamics and Brachial and Popliteal Endothelial Function by Flow-Mediated Dilation

ECG leads were placed inferior to the clavicles and on the left hip. Blood pressure was monitored using a continuous non-invasive arterial pressure monitor (CNAP Monitor® 500 HD, CNSystems, Graz, Austria). Heart rate was derived from the ECG output from the ultrasound (Philips EPIQ 5G, Amsterdam, the Netherlands). BP and ECG were exported to AcqKnowledge data acquisition software (BIOPAC, California, USA). Resting heart rate and BP were taken as a 30 second average after resting supinated for 20 minutes in a dimly lit lab.

Brachial artery endothelial function assessed using the FMD technique was taken in accordance with established guidelines (Thijssen et al. 2019). The participants' arm was placed in a supinated extended position. The left brachial artery was imaged using ultrasound

(Philips EPIQ 5G, Amsterdam, the Netherlands) in duplex mode with a 12MHz linear array transducer held in place above the cubital fossa by a trained sonographer. The pulsed-wave Doppler frequency was set at 3.5MHz and did not vary between sessions. To reduce variability, the recording location was marked on the participant's skin and previous clips were used for landmarking between sessions. Baseline diameters were then recorded for 30 seconds. An occlusion cuff was placed around the upper forearm of the participant and inflated to > 200 mmHg for 5 minutes and then rapidly deflated to induce reactive hyperemia within the brachial artery. Diameters were recorded for 15 seconds prior to deflation of the cuff until 2 minutes after deflation. Videos were exported in compressed DICOM format. Brachial artery borders were measured from rest and post reactive hyperemia video clips using software that tracked the edges of the arterial walls (CAROLAB, Creatis, France). Internal diameter was measured from intima to intima using the integrated edge detection within CAROLAB. Baseline diameter was determined as the average diameter over 15 consecutive heart cycles taken at rest. The highest average diameter over 3 consecutive heart cycles was used to determine maximum dilation. Absolute FMD was calculated using the formula:

$$Max\ Diameter\ -\ Baseline\ Diameter \qquad (2)$$

Relative FMD% was calculated using the formula:

$$\left(\frac{Max\ Diameter - Baseline\ Diameter}{Baseline\ Diameter}\right) \text{x } 100 \qquad (3)$$

Popliteal artery FMD was measured in a similar manner. Diameter was determined using the same ultrasound and software as the brachial artery. The participant was placed in a prone position and the left leg extended. Baseline diameter was recorded for 30 seconds and the location marked. A cuff was then placed around the upper calf of the participant and inflated to > 200 mmHg for five minutes. The framerate of recorded clips varies, being lower with increased depth, resulting in popliteal artery FMD clips having a lower framerate than those of a brachial FMD. Video clips were taken of the artery in the proximal popliteal fossa, before the bifurcation into the tibial and fibular arteries. Diameter was continuously recorded

for 15 seconds pre-release to 2 minutes post-cuff release. Peak diameter and FMD% were determined using the same methods and formula as the brachial artery.

To account for changes in arterial diameter from post-exercise expansion as well as location, allometric scaling of FMD was used in accordance with Atkinson and Batterham, 2013. To determine if allometric scaling was required, the slope of the linear regression of the natural log of baseline and peak diameters was determined. If the β deviated from 1 and the upper confidence interval was <1, then allometric scaling is required. For this study the common slope was determined to be 1.073±0.017. This is higher than is typical, though this may be a result of a small sample size which has been cited to lead to variation in statistics estimates (Lolli et al. 2017). The foundational studies for the development of this method had sample sizes of N = 48 and N = 50, larger than the N = 13 for this study (Atkinson et al. 2013, Atkinson and Batterham 2013). Since the slope was ~1, allometric scaling was not applied.

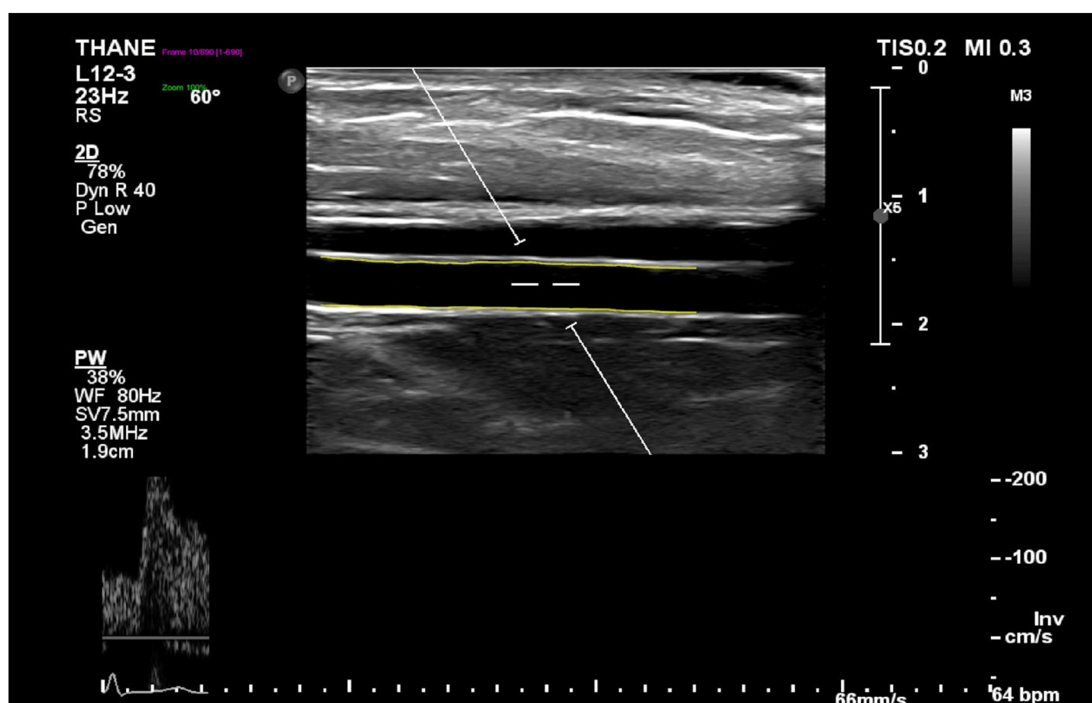

Figure 3. Image of brachial artery analysis being performed in CAROLAB.

Blood Velocity Measurements:

Peak single heart cycle blood velocity and velocity time integral (VTI) were determined using doppler velocity data gathered during each FMD. The sample volume of the pulsed Doppler signal was placed to surround the lumen of the artery and the velocity scale corrected for insonation angle. The insonation angle was kept at ≤60° to ensure accurate velocity measurements (Thijssen et al. 2011). A cine loop of the 15 seconds post-ischemia was exported in DICOM format and imported into Fiji. Fiji is an expanded distribution of ImageJ2 which includes several supplementary packages for image analysis, notably the BioFormats expansion which can be used to import DICOM clips (Schindelin et al. 2012, Linkert et al. 2010). This clip was then converted to a grayscale and the threshold adjusted until the entire peak envelope was highlighted before then being converted to a binary black/white image. The region of interest (ROI) featuring the velocity/time curve was then cropped for analysis. Each analyzed heart cycle was determined using the R-R interval from an ECG trace which was visible within each cine loop. The area under the envelope was then shaded, resulting in a highlighted pixel area. This area was then compared to a calibrated region known to represent a VTI value of exactly 200 cm. To guarantee consistency, this calibrated area was measured for each video clip using its present time and velocity scale resolutions. The peak single beat and total value over 15 seconds post cuff release were obtained.

Blood velocity was determined using the same thresholding function in ImageJ2 (Figure 4), calibrated to the integrated scale bar within the doppler signal region of interest. In accordance with recommendations, blood viscosity is not assumed to change between participants or testing conditions. The mean shear rate area under the curve was calculated using the following equation (Thijssen et al. 2019):

$$\textit{Shear Rate} = 8 \times (\textit{Mean blood velocity})/(\textit{average arterial diameter}) \qquad (4)$$

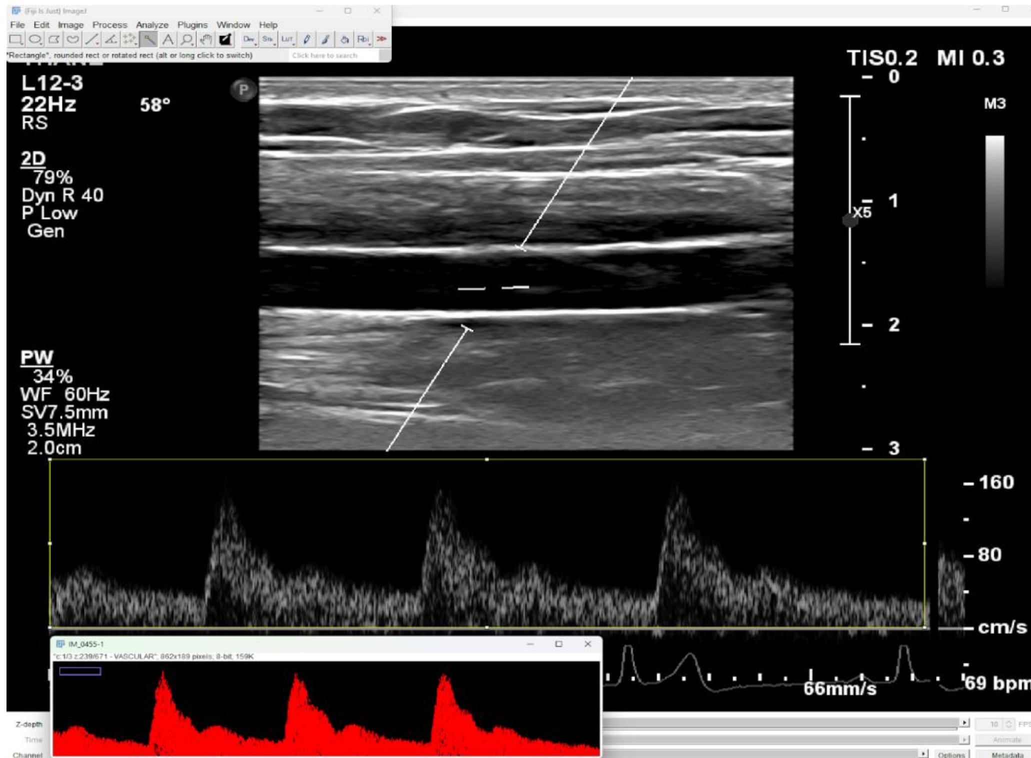

Figure 4. Example of thresholding of the doppler ROI within Fiji showing the sample video and a selected and cropped region for analysis.

Mean blood flow post-release was determined used the formula:

$$\pi\left(\frac{Baseline\ Diameter}{2}\right)^2 \times\ Mean\ Blood\ Velocity \times\ 60s \qquad (5)$$

Blood sampling and Preparation:

Five millilitre venous blood samples were taken *via* venipuncture prior to exercise and immediately upon exercise completion. Samples were collected into anticoagulant tubes coated with ethylenediaminetetraacetic acid (K_2EDTA) (#367863, BD, Mississauga, Canada) and immediately centrifuged for 10 minutes at 3000 x g and 4°C to isolate plasma. Plasma was then drawn off and stored at -80°C until analysis.

Quantification of Glycocalyx Shedding

Circulating biomarkers of glycocalyx shedding were determined using enzyme-linked immunosorbent assays (ELISA) (DY3614, DY2780, DY1707, R&D Systems, Minneapolis, United States). All plate preparation and analyses were performed according to the manufacturer specified protocol. Capture antibodies were reconstituted in 1.0mL of 0.22µL filtered phosphate-buffered saline (PBS). Detection antibodies and standard were reconstituted 0.22µL filtered PBS+1.0% bovine serum albumin (BSA) or PBS+5% Tween-20, depending on the manufacturer recommended protocol. The capture and detection antibodies, standards and streptavidin-HRP were then diluted to the working concentration in the assay's recommended reagent diluent according to the lot-specific certificate of analysis (COA). A 96-well polystyrene microplate (DY990, R&D Systems, Inc. Minneapolis, United States) was then immediately coated with 100µL per well of capture antibody before being sealed and left overnight to incubate. The following day, each well was aspirated and washed with wash buffer (PBS + 0.05% Tween-20). This wash step was repeated 3 times. The wells were then blocked using 300µL of their respective diluents to prevent non-specific binding and left to incubate for one hour at room temperature. After the incubation the wash step was then repeated. Individual standards were reconstituted in reagent diluent according to the COA and serial diluted according to the manufacturer specified instructions for each kit. For the syndecan-1 and CRP assays, plasma samples were diluted in phosphate-buffered saline (PBS) with 1.0% BSA added. When assaying for hyaluronan, samples were diluted in PBS+0.05% Tween-20. Plasma samples were diluted (20:1) and (5:1) for syndecan-1 and hyaluronan respectively. Due to the large variability in possible CRP concentrations, if an individual's CRP levels fell below the assay range, all the participant's samples were reanalyzed at a 1:7000 dilution. One hundred microlitres of each diluted sample was added to each well before being covered and incubated for two hours at room temperature. The wash step was then repeated again 3 times. One hundred microlitres of diluted detection antibody was then added to each well and incubated for two hours at room temperature. The wash step was then repeated again 3 times. One hundred microlitres of diluted detection antibody was then added to each well and incubated for 2 hours at room temperature before the wash step was repeated. One hundred microlitres of streptavidin-HRP was added to each well and the plate covered and incubated out of direct light for 20 minutes. The wash step was then

repeated. One hundred microlitres of a 1:1 mixture of Color Reagent A (H_2O_2) and Color Reagent B (Tetramethylbenzidine) (DY999, R&D Systems) was then added to each well and incubated at room temperature for 20 minutes. Fifty microlitres of 2M H_2SO_4 stop solution was then added to each well to stop the reaction and agitated to ensure proper mixing. Optical density was measured immediately using a microplate reader set at 450 nm. All samples were assayed in duplicate and all samples from each participant were surveyed on a single plate. % Coefficient of variation (%CV) was determined from the duplicate readings of each sample.

Statistical Analysis

Statistical analysis was primarily performed in JASP data analysis software (Version 0.19.3; JASP Team, 2024). Hyaluronan and C-reactive protein ELISAs were analysed using a 2-way repeated-measures analysis of variance to examine the effect of exercise and condition. FMDs were analysed using a 2-way repeated measures ANOVA to examine exercise and condition effects separately for the brachial and popliteal arteries. Post-hoc analysis using Holm corrections was done to make comparisons between time points within and between conditions when significant main effects or interactions were apparent. Significance was set at $p < 0.05$ and all values are reported as mean ± standard deviation. Percentage coefficient of variation for each parameter was calculated as the standard deviation of the two measures divided by the mean of the two measures multiplied by 100. Repeated measures correlation (rmcorr) (package version 0.7.0) was performed in R (R version 4.3.3) using the RStudio IDE (Bakdash and Marusich, 2024; R Core Team, 2024).

Chapter 3: Results

Table 1. Table of participant baseline physical characteristics. All reported values are mean ± standard deviation.

Measurement (N=13)	Value
Age (Years)	22.9 ± 2.0
Systolic Blood Pressure (mmHg)	121 ± 6
Diastolic Blood Pressure (mmHg)	75 ± 5
Height (cm)	180 ± 6.4
Weight (kg)	76.8 ± 8.5
BMI ($kg \cdot m^{-2}$)	23.6 ± 1.7
$VO_{2\ Max}$ ($mL\ O_2 \cdot kg^{-1} \cdot min^{-1}$)	38.1 ± 7.2

Technical Variation

Pre-exercise measures from each baseline session were used to determine inter-day % coefficient of variation (%CV) for each individual. Intra-assay %CV for hyaluronan and C-reactive protein ELISAs was done using the duplicate wells of each sample (Table 2). The inconsistency in FMD% may seem high in relation to many biochemical assays but is in the acceptable range for this assay (Thijssen et al. 2011, Thijssen et al. 2019). Observed variability may be a result of physiological differences within the participant as well as minor inconsistencies in assay location or technique (Thijssen et al. 2019).

Table 2. Inter-day mean, standard deviation and % coefficient of variation for baseline brachial and popliteal artery diameters, %FMD, and hyaluronan and C-reactive protein ELISAs from conditions baseline 1 and 2. Intra-assay %CV for hyaluronan and C-reactive protein ELISA was determined from duplicate wells for each sample.

Measurement	Mean	Standard Deviation	%CV
Brachial Baseline Diameter(mm)	3.70	0.47	2.54
Brachial FMD %	9.13	2.91	15.07
Popliteal Baseline Diameter (mm)	5.58	0.66	3.14
Popliteal FMD%	5.48	2.37	19.82
Hyaluronan (ng/mL)	27.69	6.91	2.66
C-Reactive Protein (mg/L)	1.49	1.45	2.15

Flow-mediated Dilation of the Brachial and Popliteal Arteries

Baseline brachial artery diameter was not significantly different following the salt loading (main effect for condition, p = 0.573) (Figure 5A/5B). When collapsed over conditions, baseline diameter was significantly elevated following exercise (p = 0.017) (Table A.1).

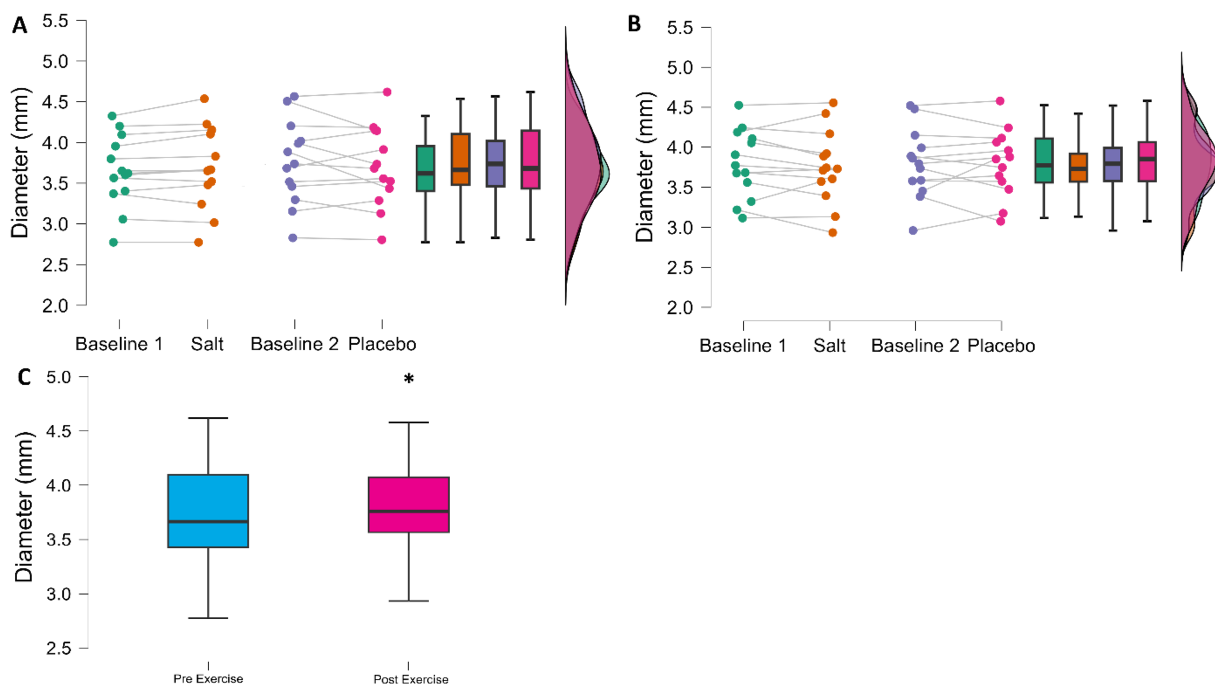

Figure 5. Baseline diameter of the brachial artery. No significant difference in artery diameter was found between conditions (p = 0.573), diameter was significantly increased post-exercise (p = 0.017). Significant comparisons are marked with an asterisk. a) Pre-exercise b) Post-exercise c) Pre- and Post-exercise collapsed over conditions.

Brachial absolute FMD was significantly lowered following 2 weeks of salt intake (main effect for condition, p = 0.007) but not altered by the exercise bout (main effect for exercise, p = 0.128) (Figure 6A/6B, Table A.2). Post-hoc analysis showed that absolute FMD decreased with salt intake (p = 0.008), but not after placebo intake (p = 1.000) (Table A.3). There was no significant condition x exercise interaction (p = 0.371).

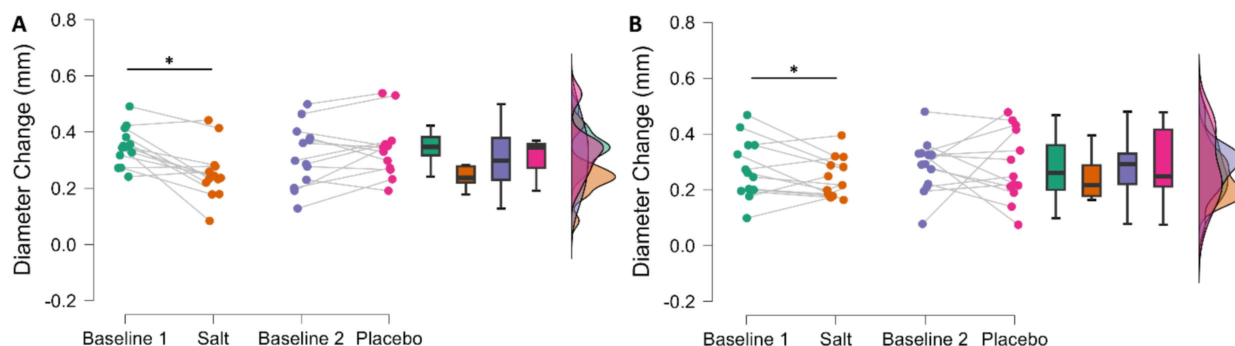

Figure 6. Absolute flow-mediated dilation of the brachial artery under each 2-week loading protocol a) Pre-Exercise and b) Post Exercise. Salt was significantly lowered when compared to Baseline 1 (p = 0.007). Exercise did not significantly affect absolute brachial FMD (p = 0.128). Significant comparisons are marked with an asterisk.

Similar to absolute FMD, brachial relative FMD was significantly altered by salt intake (main effect for condition, p = 0.031). However, there was no significant effect of the exercise bout (main effect for exercise, p = 0.105) nor was there a significant condition x exercise interaction (p = 0.407) (Figure 7A/7B, Table A.4). Post-hoc analysis showed that salt induced a reduction in FMD (p = 0.014), whereas placebo had no effect (p = 1.000) (Table A.5).

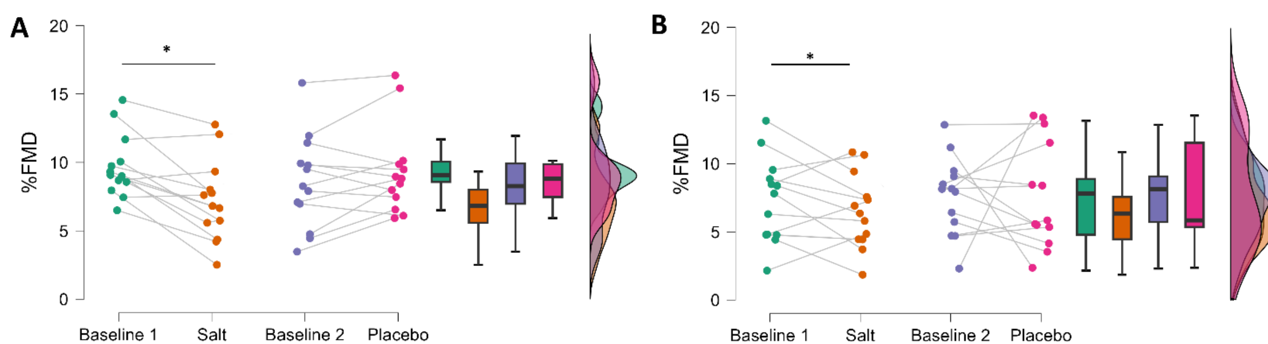

Figure 7. Relative flow-mediated dilation of the brachial artery with 2-weeks of salt or placebo, expressed as percent dilation compared to baseline diameter a) Pre-exercise and b) Post-exercise. %FMD was significantly lowered following salt loading (p = 0.031) but was

not significantly lowered post-exercise (p = 0.105). Significant comparisons are marked with an asterisk.

Brachial FMD% when normalized to VTI was significantly different between conditions (p = 0.010) and exercise conditions (p = 0.011) (Figure 8A/8B, Table A.6). Post-hoc analysis revealed that salt loading induced a significant reduction in normalized FMD as shown in Table A.7, (p = 0.017), whereas placebo intake did not alter normalized FMD (p = 0.880). (Table A.7).

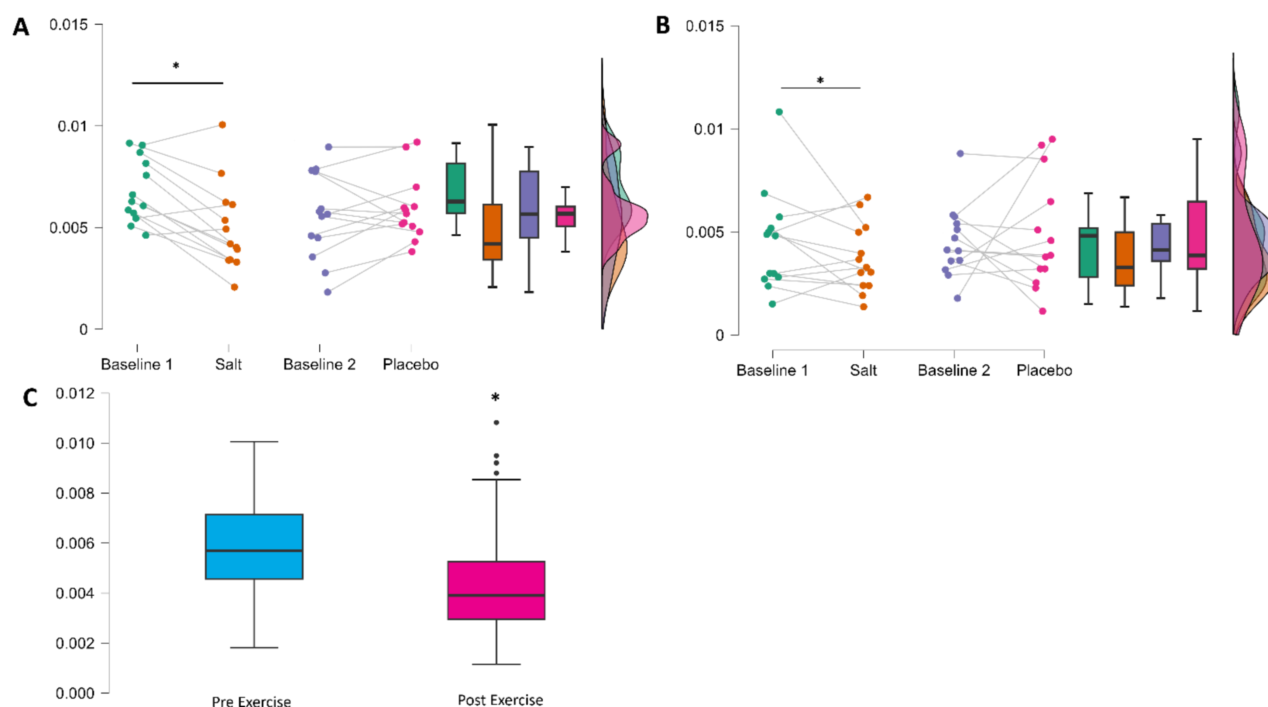

Figure 8. Brachial relative FMD normalized to velocity time integral (VTI) with 2 weeks of salt or placebo a) Pre-exercise and b) Post-exercise. Data was normalized using VTI as a denominator. Salt supplementation reduced normalized FMD both pre-exericse and post-exercise. c) When averaged over all conditions, post-exercise is significantly lowered compared to pre-exercise. Significant comparisons are marked with an asterisk.

Popliteal artery baseline diameter was not significantly different between conditions (main effect for condition, p = 0.383) or exercise (main effect for exercise, p = 0.961). There was no significant Condition x Exercise interaction (p = 0.773) (Figure 9A/9B, Table A.8).

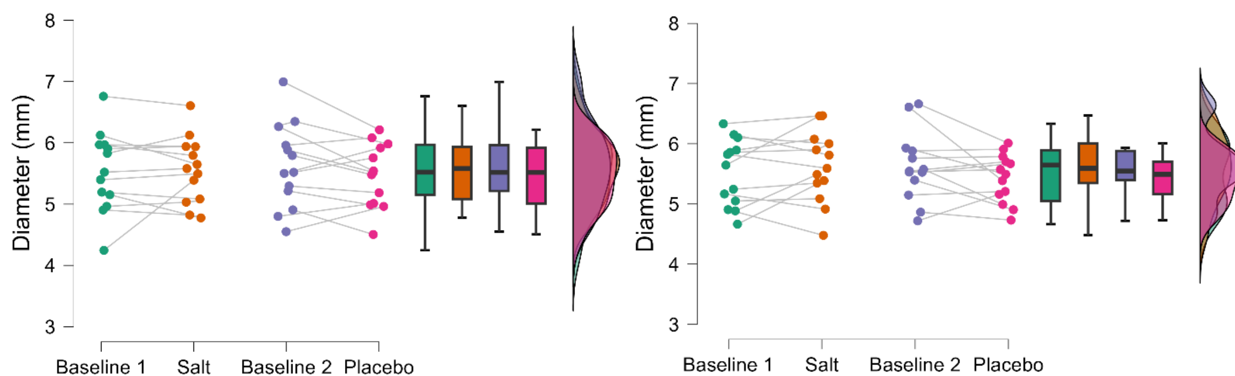

Figure 9. Baseline diameter of the popliteal artery. No significant differences were observed between conditions (p = 0.961) or following exercise (p = 0.773) a) Pre-exercise b) Post-exercise

Popliteal artery absolute FMD was significantly different between conditions (p = 0.046) and decreased significantly after exercise (p < 0.001). No significant condition x Exercise interaction was found (p = 0.299) (Figure 10A/10B, Table A.9). Subsequent post-hoc analysis for the condition effect found no significant pairwise comparisons (Table A.10).

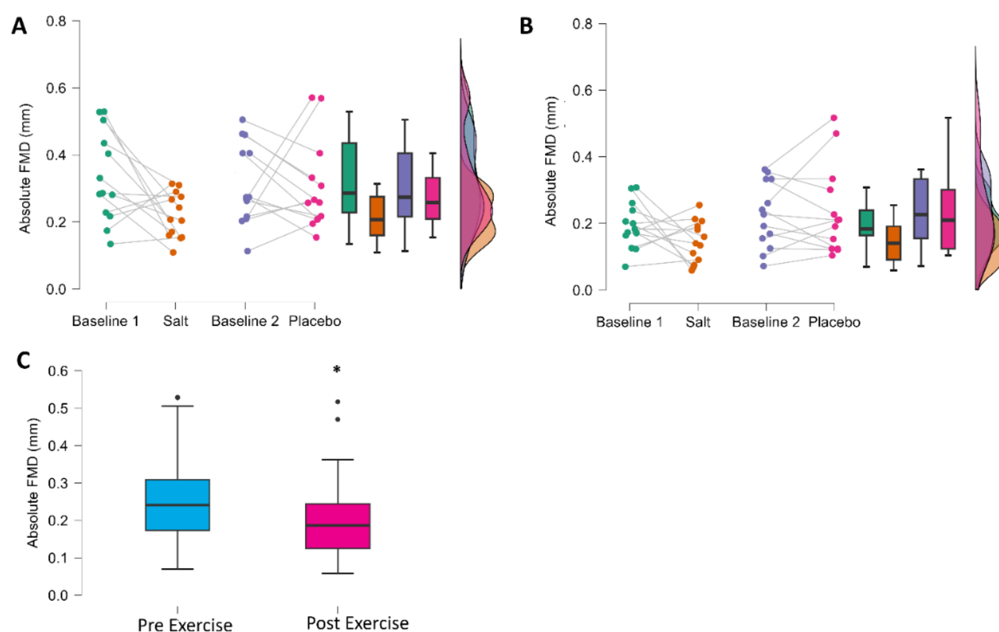

Figure 10. Absolute FMD of the popliteal artery with 2 weeks of salt or placebo a) Pre-exercise and b) Post-exercise. Post-hoc analysis of the condition effect (p=0.046) showed no significant pairwise interactions. When averaged over conditions, post-exercise is

significantly lowered (p < 0.001). Significant comparisons are marked with an asterisk. c) Pre- vs. Post-exercise collapsed over conditions.

Popliteal FMD% was not significantly different between conditions (main effect for condition, p = 0.055) although it was significantly decreased after exercise (main effect for exercise, p < 0.001). No significant condition x exercise interaction was found (p = 0.886) (Figure 11A/11B, Table A.11).

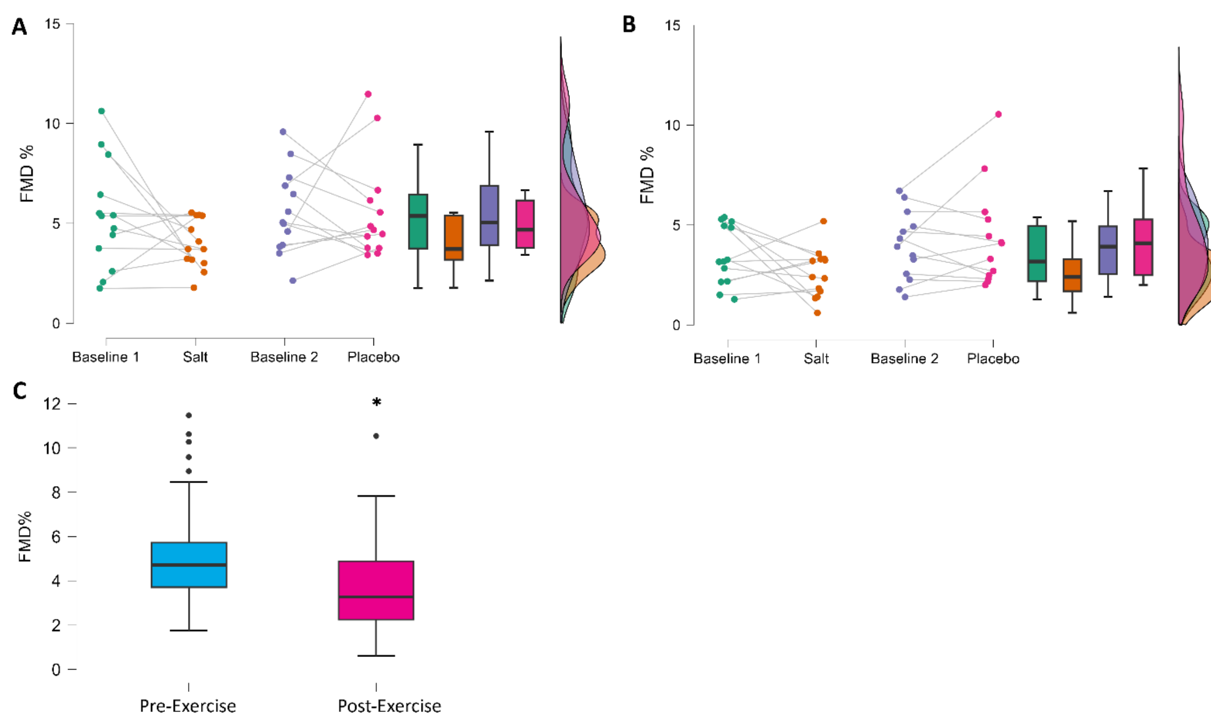

Figure 11. Relative flow-mediated dilation (FMD%) of the popliteal artery with 2 weeks of salt or placebo ingestion, expressed as percent dilation compared to baseline diameter a) Pre-exercise and b) Post-exercise. No significant differences between conditions were found (p = 0.055). When averaged over all conditions post-exercise FMD% is significantly lowered (p <0.001). Significant comparisons are marked with an asterisk. c) Pre- vs. Post-exercise collapsed over conditions.

VTI normalized popliteal FMD% was not significantly different between conditions (p = 0.158) but was significantly different post-exercise (p < 0.001). No condition x exercise interaction was found (p = 0.597) (Figure 12A/12B, Table A.12).

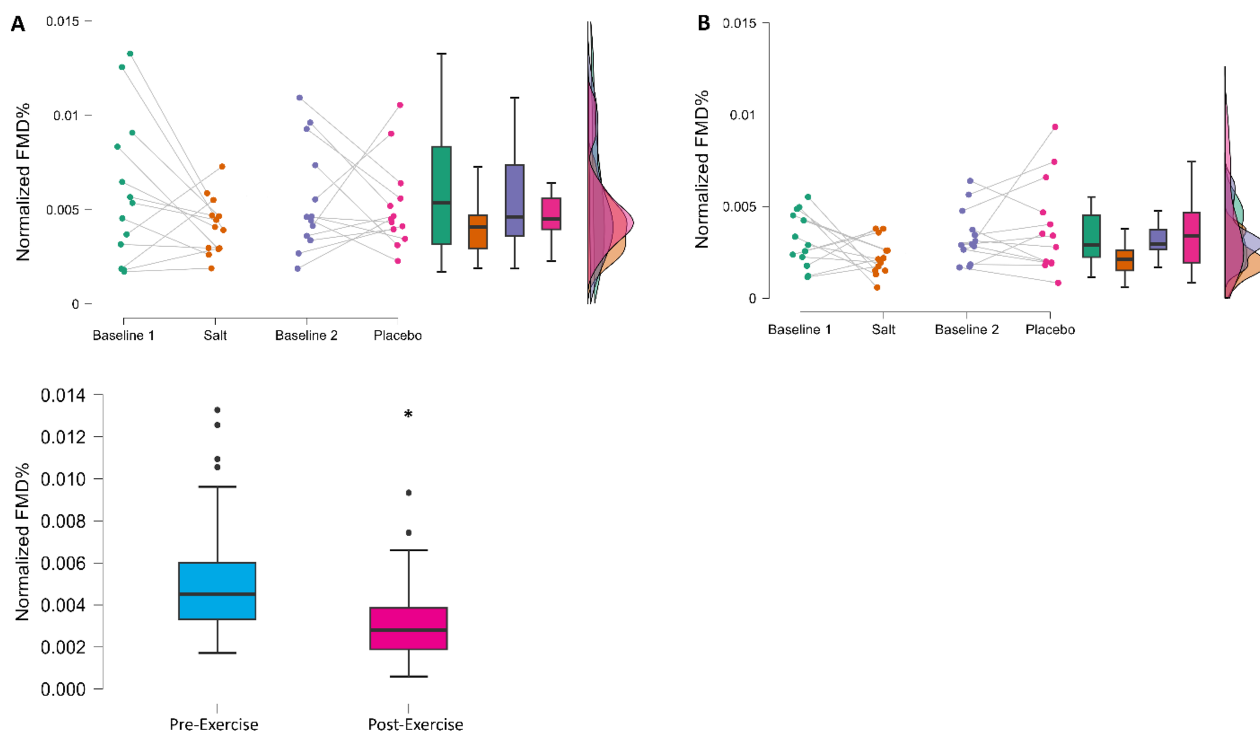

Figure 12. Relative FMD of the popliteal artery normalized to VTI with 2 weeks of salt or placebo ingestion a) Pre-exercise and b) Post-exercise. Data was normalized using VTI as a denominator. No significant differences between conditions were found (p = 0.158). When averaged over conditions, post-exercise is significantly lowered (p < 0.001). Significant comparisons are marked with an asterisk. c) Pre- vs. Post-exercise collapsed over conditions.

Haemodynamic Responses at the Brachial and Popliteal Arteries

Brachial artery 15 second velocity time integral (VTI) hyperemic response was not significantly different between conditions (main effect for condition, p = 0.678) but was significantly increased after exercise (main effect for exercise, p = 0.002). There was no significant condition x exercise interaction (p = 0.325) (Figure 13A/13B, Table A.13).

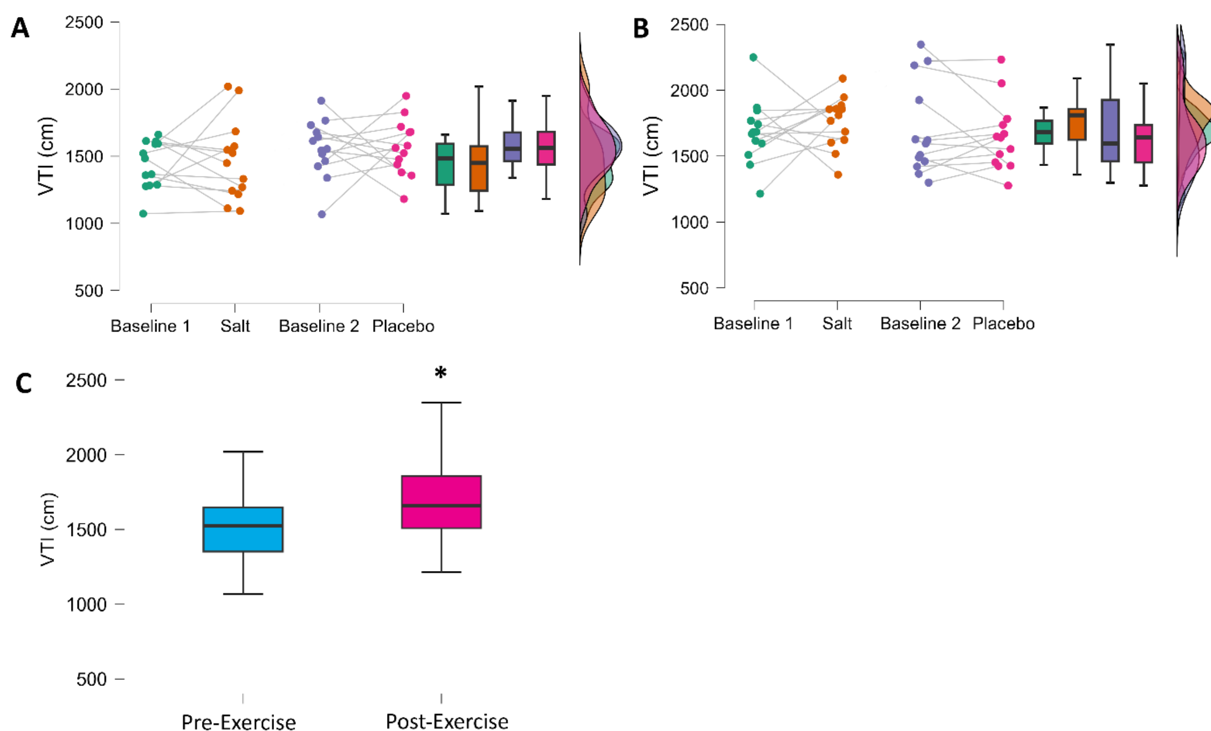

Figure 13. Velocity-time integral (VTI) of the brachial artery within the fifteen seconds after cuff release with 2 weeks of salt or placebo ingestion a) Pre-exercise and b) Post-exercise. Conditions were not found to be significantly different (p = 0.678). When averaged over all conditions, post-exercise is significantly elevated (p = 0.002). Significant comparisons are marked with an asterisk. c) Pre- vs. Post-exercise collapsed over conditions.

Popliteal artery VTI was not significantly altered by salt or placebo intake (main effect for condition, p = 0.274). However, VTI was significantly elevated after exercise (p = 0.018) but was not different across conditions (Condition x Exercise interaction, p = 0.944, Figure 14A/14B, Table A.14).

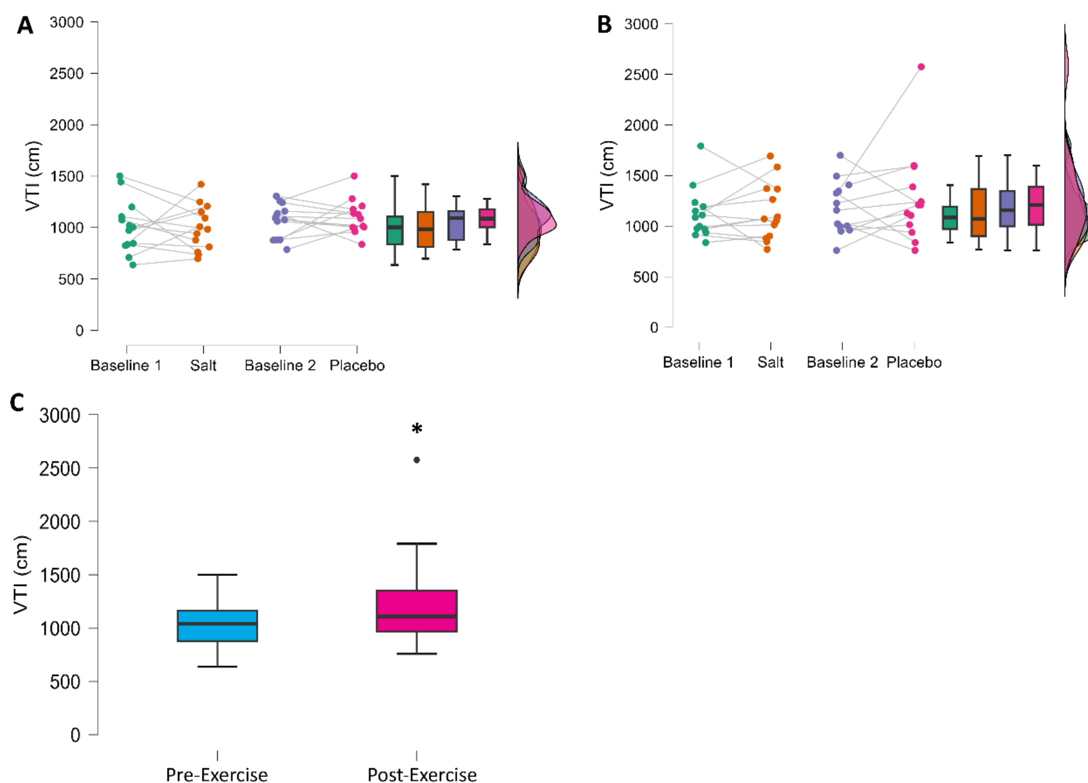

Figure 14. Velocity-time integral (VTI) of the popliteal artery within the first fifteen seconds of post-occlusion reactive hyperemia with 2 weeks of salt or placebo ingestion a) Pre-exercise or b) Post-exercise. VTI was not significantly different between conditions ($p = 0.274$). When averaged over all conditions, post-exercise VTI was significantly elevated ($p = 0.018$). Significant comparisons are marked with an asterisk. c) Pre- vs. post-exercise collapsed over conditions.

Brachial shear rate area under curve (AUC) for the first fifteen seconds of post occlusion reactive hyperemia was not significantly different between conditions (main effect for condition, $p = 0.753$) but was increased after exercise (main effect for exercise, $p = 0.011$, Figure 15A/15B, Table A.15).

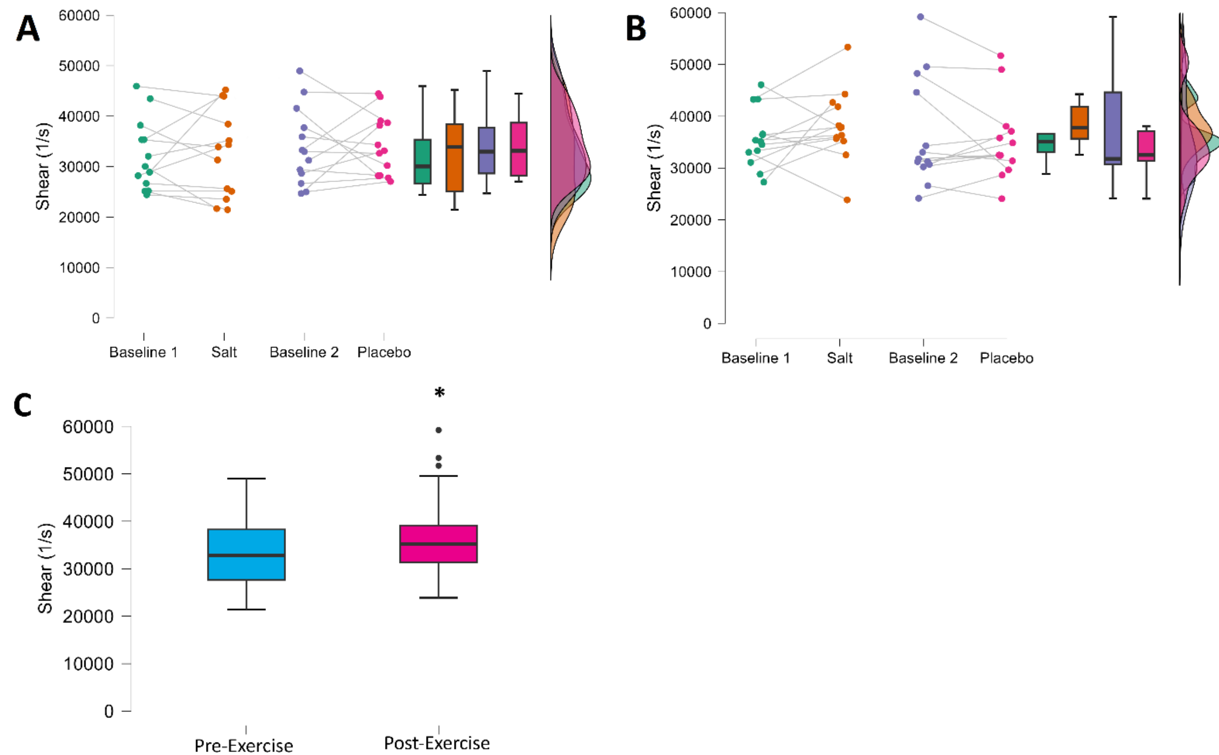

Figure 15. Brachial artery shear area-under-curve (AUC) within the first fifteen seconds of post-occlusion reactive hyperemia with 2 weeks of salt or placebo ingestion a) Pre-exercise or b) Post-exercise. Shear AUC was not significantly different between conditions (p = 0.173) but was significantly elevated following exercise (p = 0.019). Significant comparisons are marked with an asterisk. c) Pre vs. Post-exercise collapsed over conditions.

Popliteal shear AUC was not significantly different between conditions (main effect for condition, p = 0.173) but was also significantly elevated after exercise (main effect for exercise, p = 0.019, Figure 16A/16B, Table A.16).

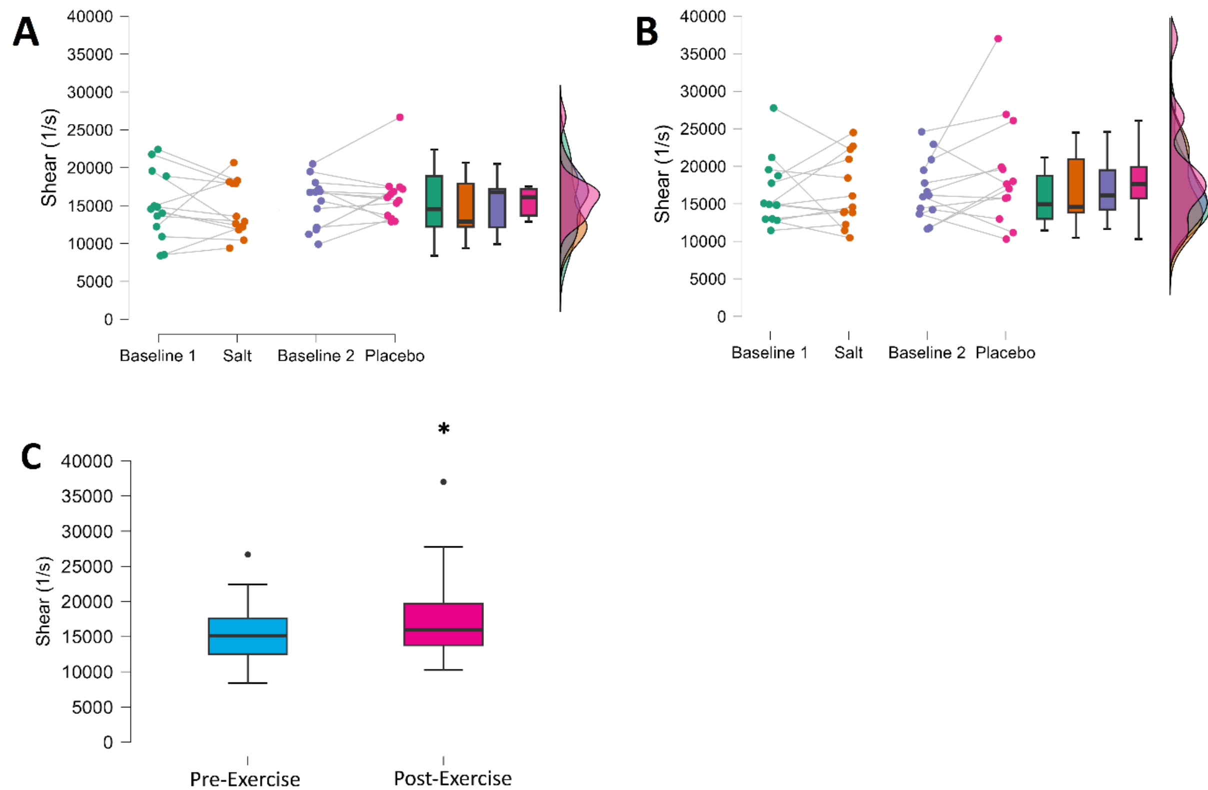

Figure 16. Popliteal artery shear area-under-curve (AUC) throughout the first fifteen seconds of post-occlusion reactive hyperemia with 2 weeks of salt or placebo ingestion a) Pre-exercise or b) Post-exercise. Shear AUC was not significantly different between conditions ($p = 0.173$) but was significantly elevated following exercise ($p < 0.001$). Significant comparisons are marked with an asterisk. c) Pre- vs. Post-exercise collapsed over conditions.

Brachial blood flow during the post-occlusion reactive hyperemia was not significantly different between conditions (main effect for condition, $p = 0.552$). Blood flow was significantly increased after exercise (main effect for exercise, $p < 0.001$) however, there was no condition x exercise interaction ($p = 0.155$) (Figure 17A/17B, Table A.17).

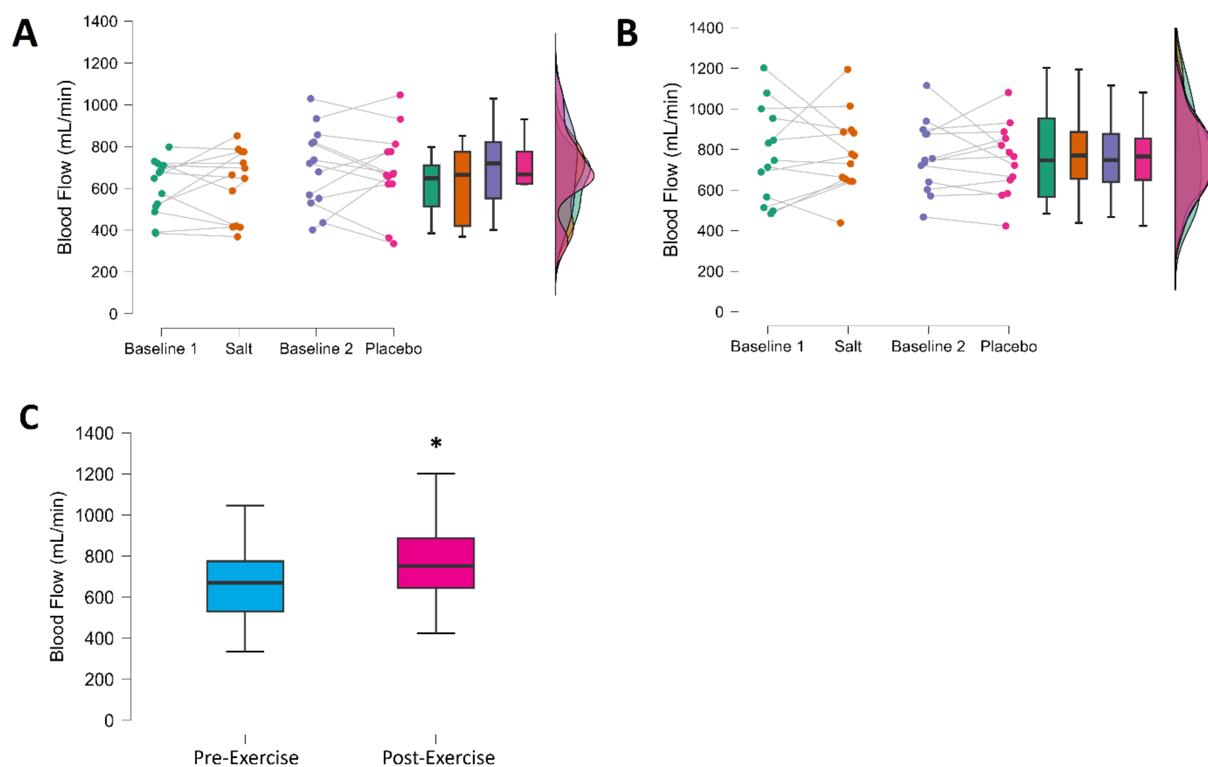

Figure 17. Mean blood flow through the brachial artery throughout the first fifteen seconds of post-occlusion reactive hyperemia with 2 weeks of salt or placebo ingestion a) Pre-exercise or b) Post-exercise. Blood flow was not significantly different between conditions ($p = 0.552$) but was significantly elevated following exercise ($p < 0.001$). Significant comparisons are marked with an asterisk. c) Pre- vs. Post-exercise collapsed over conditions.

Popliteal blood flow was not significantly different between conditions (main effect for condition, $p = 0.610$), though it was significantly increased after exercise (main effect for exercise, $p = 0.023$). No condition x exercise interaction was observed ($p = 0.960$) (Figure 18A/18B, Table A.18).

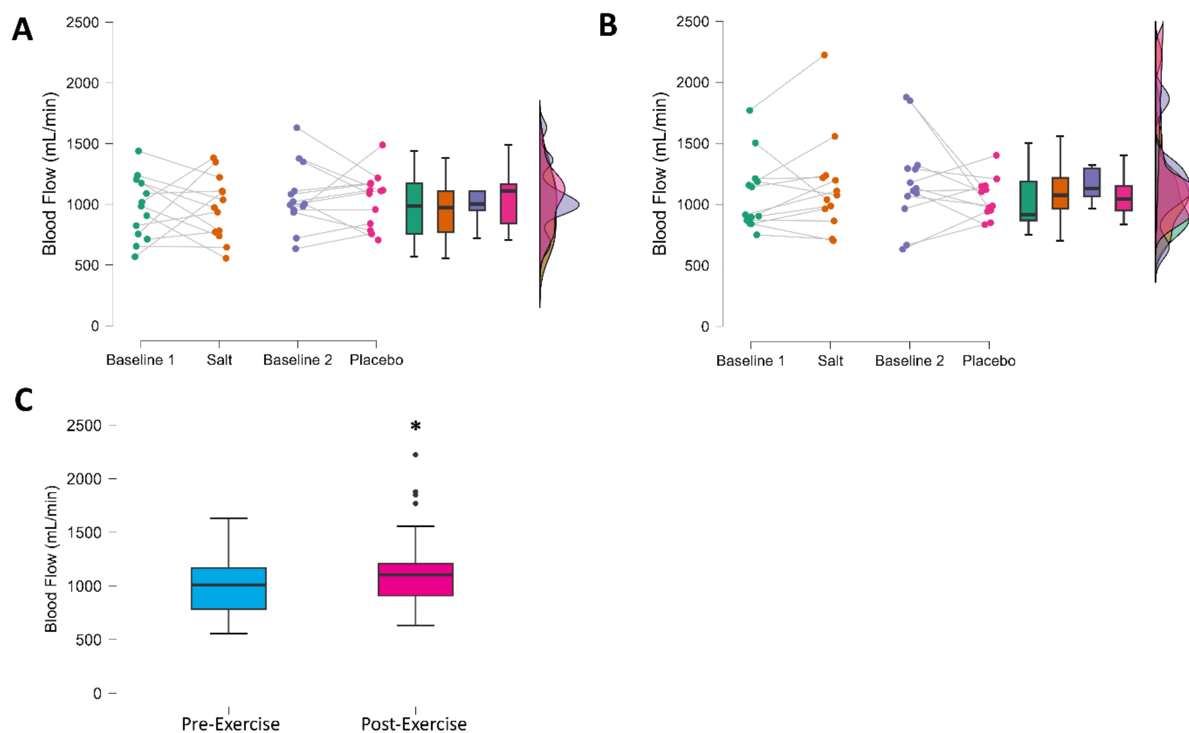

Figure 18. Mean blood flow through the popliteal artery throughout the fifteen of post-occlusion reactive hyperemia with 2 weeks of salt or placebo ingestion a) Pre-exercise or b) Post-exercise. Flow was not significantly different between conditions ($p = 0.610$) but was significantly elevated following exercise ($p = 0.023$). Significant comparisons are marked with an asterisk. c) Pre vs. Post-exercise collapsed over conditions.

Circulating glycocalyx and inflammatory biomarker concentrations

Plasma concentrations of hyaluronan were not significantly different between conditions (main effect for condition, $p = 0.639$) however, they were significantly increased after exercise (main effect for exercise, $p = 0.030$). No significant condition x exercise interaction was found ($p = 0.630$) (Figure 19A/19B, Table A.19).

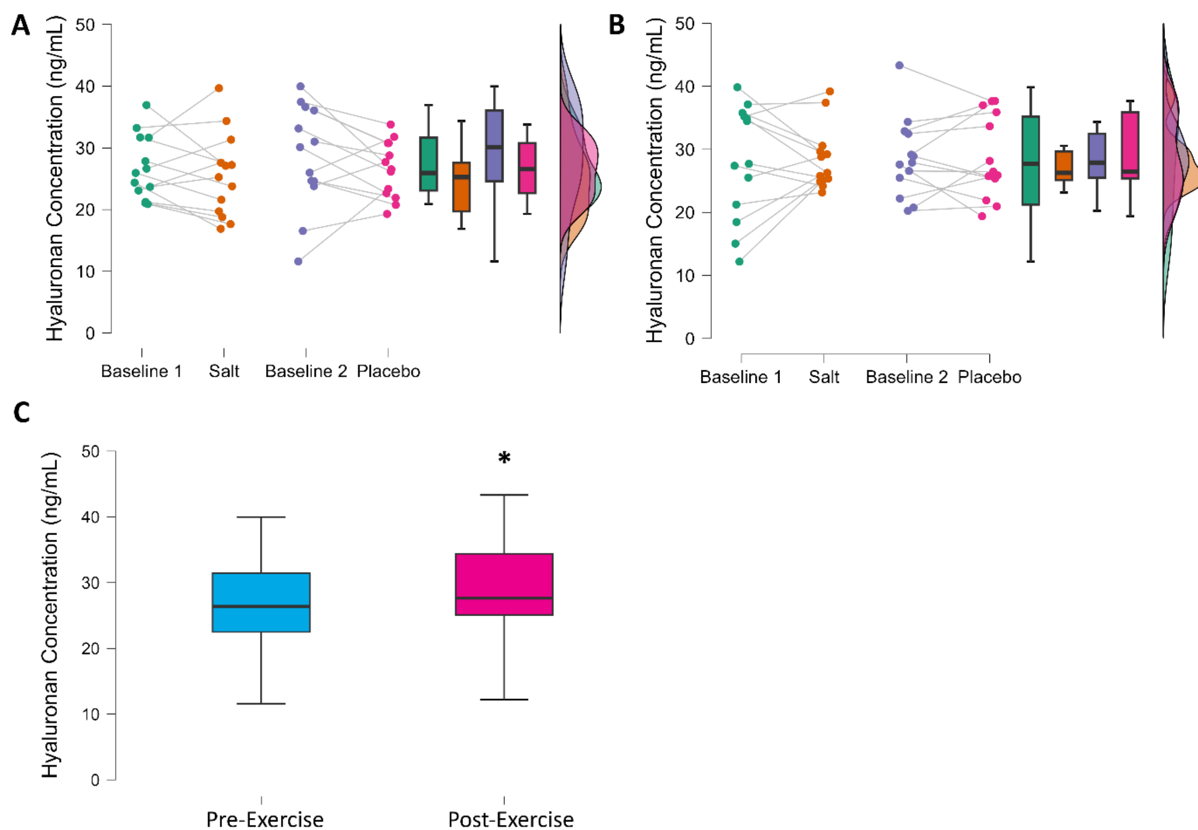

Figure 19. Hyaluronan concentration in plasma with 2 weeks of salt or placebo ingestion a) Pre-exercise or b) Post-exercise. No significant difference was found between conditions ($p = 0.639$). Hyaluronan was significantly elevated following exercise ($p = 0.030$). Significant comparisons are marked with an asterisk. c) Pre- vs. Post-exercise collapsed over conditions.

Plasma C-reactive protein concentrations were not significantly different between conditions (main effect for condition, $p = 0.485$) or following exercise (main effect for exercise, $p = 0.859$) (Figure 20A/20B, Table A.20).

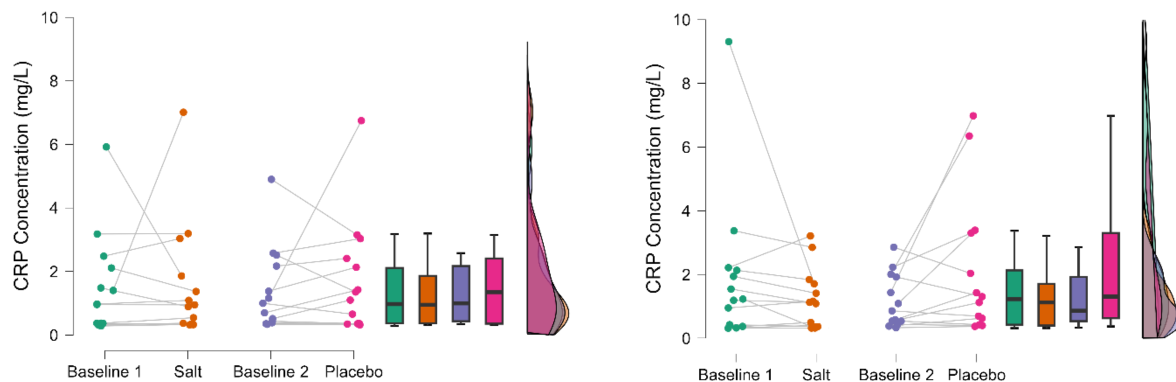

Figure 20. C-reactive protein concentration in plasma with 2 weeks of salt or placebo ingestion a) Pre-exercise or b) Post-exercise. No significant differences were found between conditions (p = 0.485) or following exercise (p = 0.859).

Repeated measures correlation (RMCORR) was performed between brachial FMD% and hyaluronan across all conditions and timepoints. No significant correlation was found (r = -0.041, p = 0.6959, 95% CI = [0.244, 0.165]) (Figure 21).

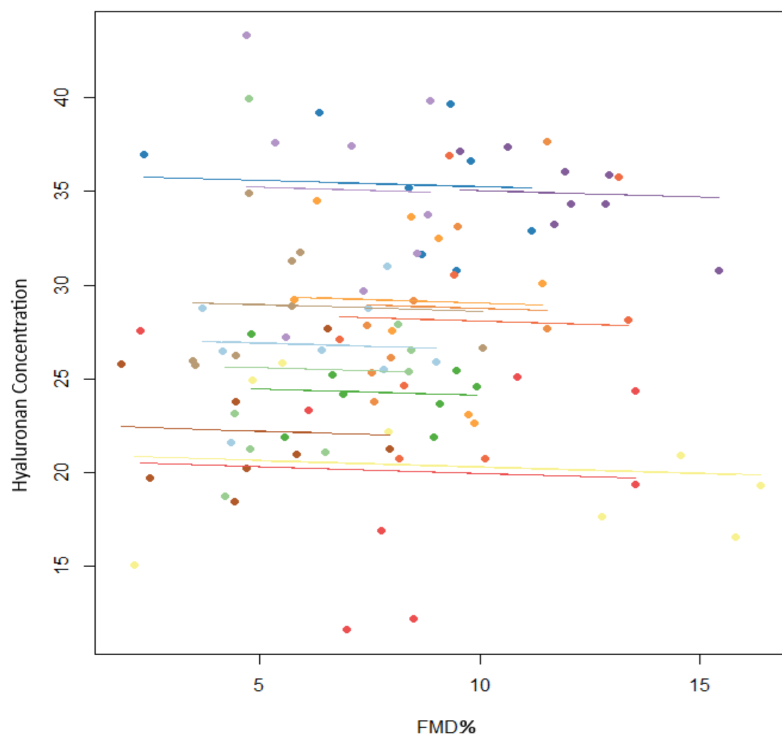

Figure 21. Repeated measures correlation of brachial FMD% and plasma hyaluronan concentration across all conditions and exercise. No significant correlation was found (r = -.041, p = 0.6959, 95% CI = [0.244, 0.165])

RMCORR between popliteal FMD% and hyaluronan concentration was also not significant (r = 0.138, p = 0.1907, 95% CI = [-0.331, 0.069]). (Figure 22).

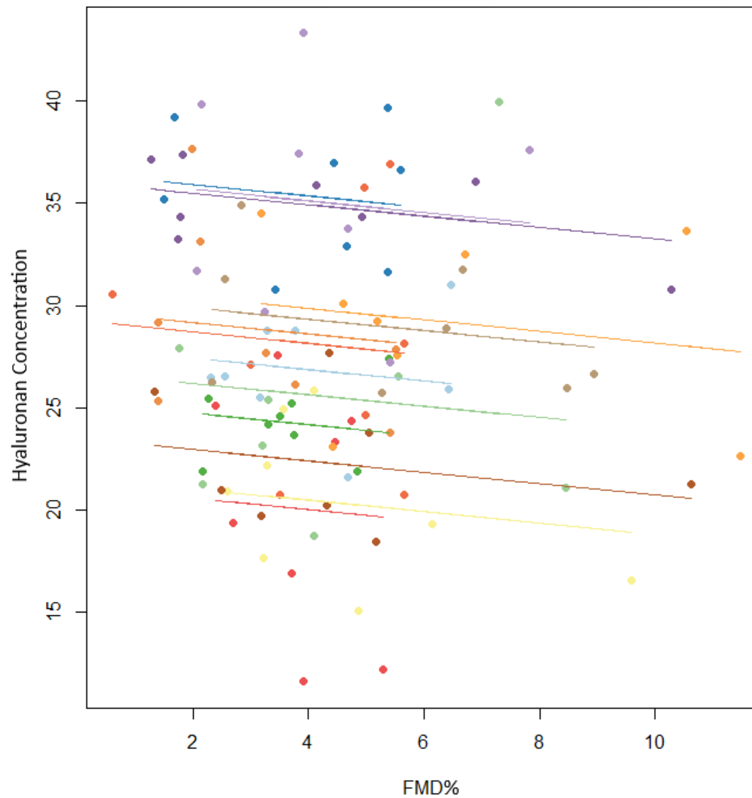

Figure 22. Repeated measures correlation of popliteal FMD% and plasma hyaluronan concentration across all conditions and exercise. No significant correlation was found (r = 0.138, p = 0.1907, 95% CI = [-0.331, 0.069])

Chapter 4: Discussion

The stated goal of this study was to determine the impact of high sodium intake on vascular health and if sodium loading had an impact on the integrity or function of the glycocalyx. An additional goal was to determine if the release of sodium stored in the glycocalyx was responsible for either a systemic or local effect on vascular function. This was accomplished using flow-mediated dilation as a metric of vascular health and related doppler ultrasound derived technique to determine hemodynamics. Biomarker analysis of hyaluronan and c-reactive protein served to quantify the levels of glycocalyx shedding and inflammation.

The findings of this study are as follows: A two-week high-sodium diet acutely reduces vascular function as measured by flow-mediated dilation when at rest, but this effect is not upheld post-exercise. However, a high-sodium diet does not change resting glycocalyx integrity or integrity in response to exercise over this period, suggesting that the vascular dysfunction occurs through a process unrelated to the glycocalyx.

Effects of Sodium Loading on Flow-Mediated Dilation

Effect on Brachial and Popliteal Artery Baseline Diameters

Baseline artery diameters in the brachial artery were not significantly different between conditions but were significantly elevated following exercise (Mean 3.698 ± 0.474 vs. 3.787 ± 0.422). This may be a result of increased systemic flow due to thermoregulation during exercise (Green et al., 2017). Baseline diameters of the popliteal artery were not significantly different between conditions or following exercise. The lack of significant difference between conditions indicates high intraparticipant variability. The lack of diameter change following exercise suggests that in this case, the observed decrease in popliteal artery FMD was not primarily a function of increased baseline diameter. Such results are not unheard of and could be a result of reactive oxygen species (ROS) scavenging of NO, or the artery partially recovering following the exercise, as well as increased tone of the artery limiting expansion relative to the brachial artery (Rush et al., 2005; Birk et al., 2012; Green et al., 2012)

Effect of Sodium Loading on Flow-Mediated Dilation of the Brachial Artery and the interaction with acute exercise

The high-sodium diet resulted in a significant decrease in absolute and relative FMD within the brachial artery of approximately 1.7%. This result supports existing research, which has found a similar magnitude of change in response to high sodium intake over comparable timeframes: 10.03±0.9% vs. 7.3±0.7% and 10.2±0.65 and 5.7±0.5 for Dupont et al., (2015) and Lennon-Edwards et al. (2015), respectively (Decker et al., 2023; Lennon-Edwards et al., 2015; Dupont et al., 2013; Matthews et al., 2015). This is likely due to decreased NO bioavailability due to superoxide production (Lenda et al., 2000; Nurkiewicz and Bogehold, 2007).

With the subsequent exercise bouts, there was no further exercise induced change in brachial absolute FMD or FMD% post-exercise when preceded by the sodium loading or placebo. As cycling is a lower-limb exercise, it may be expected that the brachial artery would not show a significant change post-exercise as it does not experience substantial changes in shear during the activity and requires less increase in blood flow or change in vascular activity that could alter FMD. Despite this, Jones et al. (2010) and Birk et al. (2013) have both found decreased brachial artery FMD following cycling (Birk et al., 2013; Jones et al., 2010).

When normalized to VTI, brachial artery FMD was not significantly different following sodium loading. However, it was significantly reduced pre- vs. post-exercise with this exercise effect being similar after sodium loading or placebo. As shear stress is the established stimulus for FMD, once this has been corrected for, some underlying changes to vascular reactivity may become apparent (Pyke and Tschakovsky, 2005). The observed decrease indicates that following exercise, the brachial artery expands less from the same shear stimulus (Padilla et al. 2008). As the initial shear-dependent FMD response is NO-mediated, the mechanism responsible likely surrounds decreased NO bioavailability. Increased local and systemic production of ROS from NADPH oxidase or xanthine oxidase enzymes during exercise has been implicated in reduced NO bioavailability due to its conversion to peroxynitrite (Rush et al., 2005) which may be the reason for this reduced FMD. Notably, this is also one mechanism through which sodium intake reduces vascular function (Lenda et al., 2000; Nurkiewicz and Bogehold, 2007; Rush et al. 2005). While FMD

normalized to VTI may have been significantly reduced, neither absolute nor relative FMD were significantly decreased post-exercise. Though statistically significant, the physiological relevance of normalized FMD would ideally be corroborated through additional testing of ROS or other measurements of the redox system.

Effect of Sodium Loading of Flow-Mediated Dilation of the Popliteal Artery and the interaction with acute exercise

Popliteal artery FMD was not significantly lowered following sodium loading. However, considering an η^2_p of 0.188, consistent with a large effect size and p value of 0.055 it is possible that significance could be reached with relatively few additional participants (Richardson, 2011). In relation to the acute exercise effect, popliteal artery FMD decreased immediately post-exercise and this is consistent with previously reported exercise responses (Birk et al., 2012; Dawson et al., 2013; Dawson et al., 2008). Normalizing popliteal FMD to VTI did not substantially change any conclusions since sodium loading remained non-significant whilst the exercise effect continued to be evident.

The lack of any significant Condition x Exercise interaction at the popliteal artery is noteworthy. This result suggests that the typical reduction in post exercise FMD was not further exacerbated by the sodium loading as was hypothesized. As flow mediated dilation vasodilation is shear induced but still may be controlled through multiple redundant biochemical pathways, it is reasonable that elevated sodium intake would not fully impair them all (Crecelius et al., 2015; Frangos et al., 1996). As detailed above, high sodium intake results in reduced NO bioavailability through increased ROS scavenging of NO. While the exercise response is partially NO-mediated, blockade of nitric oxide synthase leads to no significant change in exercise-induced hyperemia and likely the related shear-induced expansion (Frandsen et al. 2001). The release of adenosine and related compounds from skeletal muscle fibres and red blood cells which act as vasodilators at the resistance arterioles is a more significant pathway (Joyner and Casey, 2015; Marshall, 2007). An adenosine-dependent mechanism is triggered through the adenosine formed from the release of AMP from skeletal muscle and red blood cells. This adenosine then binds to the G-protein coupled receptors present on vascular smooth muscle cells, lowering cytosolic Ca^{2+} which triggers

subsequent relaxation of the smooth muscle (Atkinson et al., 2015; Crecelius et al., 2015; Frangos et al., 1996; Ralevic and Burnstock, 1998; Sato et al., 2005). These mechanisms at the resistance arterioles could impact FMD responses in the conduit artery and when the FMD is then assessed post-exercise, the artery is already in a dilated state and cannot further expand to the same extend which results in the appearance of endothelial dysfunction. Further statistical corrections for changes in baseline diameter are needed to truly assess whether the reduction in FMD is mediated by a physiological change in the milieu of the artery or whether it is simply a change in baseline diameter. This has been proposed by Batterham et al. and was investigated for use in this study. The common slope of log transformed baseline and peak diameter was 1.073 and $r^2 = 0.988$ (Figure A.1). As the slope is above unity (1.0) and r^2 is near 1, this study does not meet the requirements for allometric scaling when also considering there was no statistically significant change in baseline diameter post-exercise (Atkinson and Batterham, 2012).

Effect of Sodium Loading and acute exercise on the Hemodynamics of the Brachial and Popliteal Arteries

Brachial Artery Hemodynamics Following Sodium Loading and Exercise

The lack of a sodium-related change in resting reactive hyperemia related VTI, shear area-under-curve or mean blood flow is not unexpected. Sustained changes in BP or arterial stiffness have been observed following sodium loading but occur over longer durations than the two-week duration of this study, under higher levels of sodium loading or are found immediately following a high sodium meal. The post-prandial effects of sodium are less relevant for this study since at least 8 hours had passed since the final sodium intake before testing (Dickinson et al., 2014; Liu et al., 2013; Todd et al., 2010). In relation to acute exercise, the observed increase in brachial VTI, shear AUC and blood flow post-exercise was typical and may have been a result of increased cardiac output and shunting of blood to the skin and extremities to lower core body temperature, causing vasodilation (Atkinson et al., 2015; Green et al., 2017; Laughlin et al., 2012).

Popliteal Artery Hemodynamics Following Sodium Loading and Exercise

Similar to the brachial artery, popliteal artery reactive hyperemic VTI, shear AUC and mean blood flow were not different following sodium loading at rest. The distinct overall increase in these variables post-exercise is a direct result of the expected response to the exercise and the increased metabolic requirements of the leg tissues that followed. Like FMD, the lack of any difference in this post exercise response with the addition of sodium intake may be explained through the function of an alternative pathway for controlling hemodynamics not entirely dependent on a nitric oxide mediated pathway. Frandsen et al. (2001) showed that exercise-induced hyperemia in the leg is largely independent of NO bioavailability, and since a known mechanism for sodium-induced vascular dysfunction is reduced NO availability, the redundant mechanisms must be NO-independent. As detailed above, one mechanism involves adenosine as a vasodilator that acts directly on vascular smooth muscle. Additionally, Murrant et al. (2014) demonstrated that prostaglandins function in exercise-induced vasodilation independently of adenosine and NO as well (Murrant et al., 2014). Taken together, the reactive hyperemic responses did increase post-exercise to a similar extent with sodium or placebo likely induced by similar increases in tissue metabolic demands to 5 minutes of occlusion. Since dilatory mechanisms are redundant at the level of the arterioles, as described above, sodium ingestion was insufficient and no alterations in reactive hyperemic responses occurred.

Effect of Sodium Loading on Plasma Hyaluronan and C-Reactive Protein Concentrations

To potentially provide some insight into changes in vascular function with sodium intake and in response to acute exercise, we measured circulating concentrations of glycocalyx constituents. This was performed to assess whether sodium intake caused shedding and whether exercise induced shedding of this layer was further exacerbated under high sodium intake. Firstly, resting levels of plasma hyaluronan were unchanged following sodium loading or placebo which did not support our initial hypothesis. The most likely explanation is that the shear forces present at rest are not sufficient to cause shedding of the

glycocalyx, even if the integrity has been theoretically reduced by high sodium intake. The lack of an exercise induced change in this study can likely be attributed to a similar cause.

Hyaluronan concentrations were significantly elevated post-exercise, consistent with other literature (Kröpfl et al., 2021). While the magnitude was comparatively small, this may be due to exercise intensity or length at the selected intensity. Other studies using hyaluronan to determine glycocalyx integrity found both greater baseline quantity and changes post-exercise. Serum hyaluronan values pre- and post-exercise were 84.3 ± 21.8 to 121.4 ± 29.4 ng/mL and 126.7 ± 50.8 to 248.9 ± 135.6 for Kröpfl et al. (2021) and Steinach et al. (2022) respectively. (Kröpfl et al., 2021; Steinach et al., 2022). Differences in exercise intensity or duration may make these not directly comparable, though differences in peak or duration of sustained shear stress may explain some of the observed difference. Kröpfl et al., (2021) had a higher peak shear stress from increased intensity (85% VO_{2peak}) over the same 45-minute duration, though they used a decremental high intensity interval format (Kröpfl et al., 2021). Steinach et al., (2022) studied ultramarathon runners, where the exercise duration drastically exceeds that of the present study (Steinach et al., 2022). Samples were handled in a similar manner to previous studies, with plasma being isolated immediately following blood draw and stored at -80°C until analysis, removing handling as a factor. An alternate method for glycocalyx assessment could be to determine glycocalyx thickness, as a surrogate for glycocalyx health. Thickness can be measured through sidestream dark field imaging of the sublingual area. While this would give a direct measurement of the layer, it would not give any information about composition or which residues are shed into the blood (Goedhart et al., 2007; Nieuwdorp et al., 2008; Rovas et al., 2018; Kröpfl et al., 2021; Hahn et al. 2021).

The lack of a significant correlation between plasma glycocalyx levels and FMD results in a novel conclusion: Alterations to the glycocalyx are not responsible for the short-term decreases in FMD from sodium loading. It has been demonstrated that glycocalyx thickness is correlated with FMD (Smilowitz et al. 2021), and that sodium overload damages the glycocalyx (Oberleithner et al. 2011), but we found FMD was reduced following sodium intake with no commensurate increase in plasma glycocalyx products. A possible explanation put forward in Oberleithner et al. (2011) is that over the duration of high sodium intake, the

glycocalyx undergoes a structural reorganisation as opposed to an outright collapse. This is supported by Gao and Lipowsky (2010) who determined that removal of heparan sulfate residues results in compaction of the glycocalyx and some preservation of barrier function, with the remaining layer made up of predominantly hyaluronan and chondroitin sulfate residues. This may also partially explain the apparent lack of any baseline change in hyaluronan concentration we observed following sodium loading. However, due to the lack of a measure of direct glycocalyx height or density in our study, this idea of a reorganization remains speculative (Gao and Lipowsky, 2010; Oberleithner et al. 2011). Given the comparatively small amount of shedding induced through exercise in the present study, it is logical to conclude that the mechanosensory properties of the glycocalyx would still be mostly functional and that the observed decreases to FMD occur primarily through reactive oxygen species scavenging of NO or other neurohormonal changes to the regulation of vascular tone.

C-reactive protein concentrations were also not changed by sodium loading or exercise. These results are somewhat anticipated, as no conclusive relationship has been established between sodium loading and CRP concentration (Yilmaz et al., 2012; Gruppen et al., 2016) or following exercise at comparable intensities and durations (Markovitch et al., 2008; Murtagh et al., 2005; Sharhag et al., 2005). Variability in individual CRP concentrations may also be introduced through common illnesses such as upper respiratory infections, which can cause significant increases for several days after infection, even if the infection is unnoticeable by outside observers (Melbye et al., 2004; Whicher et al., 1985). If a participant was infected at any point during either the placebo or sodium loading periods it could mask an increase driven by sodium intake, especially if sodium only drove a modest increase compared to the values seen from a mild immune challenge. As participants were requested to maintain their existing diets and exercise routines, it is unlikely that any significant difference would be visible between conditions due to changes in diet or exercise.

Strengths and Limitations

The randomized cross-over design and two-week duration of this study represent significant strengths. The usage of a second baseline and placebo period allows for more robust comparisons of effects and determination of intraparticipant variability. The usage of

enterically coated capsules compared to drinks or meals helps to avoid the limitation of participants recognizing which group they are assigned to, while also improving reported participant comfort. The salt dose (12g per day NaCl, 4.8g Na), while higher than the Canadian average, approximates values that some populations consume, as opposed to some studies which overshoot even high values significantly (Liu et al., 2013). The assay techniques for ELISA is another strength. Re-assaying of all a participant's samples at the same dilution minimized variability that could be introduced through technical error or inter-plate variability. Each test being performed on a unique plasma aliquot also eliminated any changes caused by repeated freeze-thaw cycles.

This study does have notable limitations. The lack of a strictly controlled diet for participants could introduce significant variability between participants. While logistical reasons made supplying all participants with food infeasible, participants were asked to adhere to their typical diets. Despite this request, we did not verify if this was adhered to using dietary records. The demographic range of the participants is potentially a shortcoming as well. The relatively young ages of all participants and general physical fitness may ameliorate some level of induced vascular dysfunction relative to an untrained or older group. This may be a result of self-selection as those who are physically fit may be more willing to participate in a study involving strenuous exercise. As a result, the conclusions from this study may not be directly transferable across all demographics. Beyond participant limitations, there are technical limitations as well. The method for determining VTI and other hemodynamics involved tracing the outer envelope of the Doppler signal. This results in an overestimate of the absolute value compared to an intensity-weighted mean trace, but still preserves changes induced by conditions or the exercise stimulus. The analysis of all data and samples was done unblinded, which may introduce unconscious bias into the results. The use of only a single marker of glycocalyx degradation is a further limitation. Similar studies have used additional biomarkers such as syndecan-1, chondroitin sulfate and heparan sulfate (Hahn et al. 2021; Kröpfl et al. 2021; Majerczak et al. 2017). The measurement of plasma syndecan-1 was originally planned for this study, though for unknown reasons, the ELISA was nonfunctional, and the results produced were unusable.

Future Directions

This study is another step towards further understanding the mechanisms underlying sodium-induced vascular dysfunction. As a result, there are still many questions that require answers.

How Does Sodium Intake and Exercise Alter Other Biomarker Concentrations?

Beyond measuring additional glycocalyx biomarkers, there are many other related markers that may be useful. It has been established that glycocalyx integrity is affected by reactive oxygen species levels through increases in heparinase production (Rao et al., 2011). In addition, cytokines such as TNF-α have been associated with the degradation of the endothelial glycocalyx (Chappell et al., 2009). While glycocalyx integrity did not change in this study, given the known impact of sodium on the immune and redox systems, further investigation is warranted.

Does Sodium Loading Affect the Response to Different Exercise Intensities?

While there was no change to glycocalyx integrity resulting from simple salt intake in the present study, this does not suggest that it will not have an impact in all cases. The exercise modality of Kröpfl et al. (2021) implemented an average power of 85% of the maximum from an incremental test, in a decremental high intensity interval format. This contrasts with the lower-intensity steady-state exercise that was performed in this study in a few notable ways. While the overall duration of the exercise was set the same at 45 minutes, in Kröpfl et al., (2021) the peak shear stress on the arteries due to the increased maximum power may result in increased glycocalyx shedding, which may make a sodium-related change become apparent. In addition, exercise in Kröpfl et al. was specifically targeted to disrupt the redox system (Kröpfl et al., 2021). Under higher intensities, the metabolic demands of the muscle may also lower muscle and blood pH (Robergs et al., 2004), which could potentially alter glycocalyx dynamics. Longer duration exercise should also be further investigated, as was done by Steinach et al. (2022). Longer duration exercise presents additional challenges that aren’t present during shorter duration/higher intensity exercise bouts, which also may be helpful for further exploring sodium-induced dysfunction.

Increases in core temperature, dehydration and blood and muscle pH changes are all factors that could potentially impact the relationship between sodium and the glycocalyx (Steinach et al. 2022).

How Does Longer Term Sodium Loading Impact Vascular Health?

While practical and ethical considerations make studying time spans on the magnitude of months to years generally infeasible, shorter but still significant increases over the present study are possible. Todd et al. (2010) used a four-week duration of salt (NaCl) loading and found statistically significant increases in BP and arterial stiffness (Todd et al. 2010). While we observed no changes in BP over the two-week span of the current study, a four-week study duration may result in additional disruption in vascular function or glycocalyx integrity. Further cross-sectional studies studying the glycocalyx in individuals with chronically elevated sodium intake may allow for the investigation of non-acute changes. Examining larger populations in this manner may allow for grouping among factors like salt-sensitivity which would allow for additional population-scale responses to be observed, which is not possible in the present design.

Conclusions

In summary, this study represents a step forward in the assessment of sodium dependent changes to vascular health and the glycocalyx. While there were rapid negative changes to vascular function as measured by flow-mediated dilation, these effects were not further exacerbated post-exercise under high-sodium conditions. Furthermore, although acute exercise did induce glycocalyx shedding, there was no sodium related impact to glycocalyx integrity either at rest or post-exercise. This result was somewhat surprising, though not wholly unanticipated given the length of time that vascular dysfunction can take to present. Age and fitness level of participants may have been contributing factors to this, though analysis of different age or fitness level populations was beyond the scope of this study. Additional research into other mechanisms associated with glycocalyx shedding such as cytokines or inflammation and their relationship to sodium intake are still required to fully understand how the body responds. Furthermore, studies over longer durations are required to identify the role of chronic high sodium intake on glycocalyx health, as elevated sodium

intake levels are the norm for millions worldwide. This study begins to expand the understanding of dietary sodium intake and how it relates to the glycocalyx and overall vascular health.

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APPENDIX

Table A.1. Summary of two-way repeated measures ANOVA analysis of brachial artery baseline diameter.

Cases	Sum of Squares	df	Mean Square	F	p	η^2_p
Condition	0.063	3	0.021	0.675	0.573	0.053
Residuals	1.113	36	0.031			
Exercise	0.206	1	0.206	7.633	0.017	0.389
Residuals	0.324	12	0.027			
Condition ✻ Exercise	0.041	3	0.014	1.713	0.182	0.125
Residuals	0.290	36	0.008			

Note. Type III Sum of Squares

Table A.2. Summary of two-way repeated measures ANOVA analysis of brachial artery absolute FMD.

Cases	Sum of Squares	df	Mean Square	F	p	η^2_p
Condition	0.077	3	0.026	4.663	0.007	0.280
Residuals	0.199	36	0.006			
Exercise	0.045	1	0.045	2.679	0.128	0.183
Residuals	0.202	12	0.017			
Condition ✻ Exercise	0.014	3	0.005	1.078	0.371	0.082
Residuals	0.161	36	0.004			

Table A.3. Summary of Post-hoc Analysis of Brachial absolute FMD. Results are averaged over exercise.

		Mean Difference	SE	df	t	p_{holm}
Baseline 1	Salt	0.066	0.016	12	4.145	0.008
	Baseline 2	0.013	0.026	12	0.485	1.000
	Placebo	-8.462×10^{-4}	0.019	12	-0.044	1.000
Salt	Baseline 2	-0.053	0.020	12	-2.604	0.092
	Placebo	-0.067	0.017	12	-3.875	0.011
Baseline 2	Placebo	-0.014	0.023	12	-0.602	1.000

Note. P-value adjusted for comparing a family of 6 estimates.

Table A.4. Summary of two-way repeated measures ANOVA analysis of brachial artery FMD%.

Cases	Sum of Squares	df	Mean Square	F	p
Condition	50.011	3	16.670	3.319	0.031
Residuals	180.808	36	5.022		
Exercise	52.165	1	52.165	3.066	0.105
Residuals	204.202	12	17.017		
Condition ∗ Exercise	11.188	3	3.729	0.993	0.407
Residuals	135.223	36	3.756		

Table A.5. Post-hoc analysis of brachial artery FMD%. Results are averaged over exercise.

Hoc Comparisons - Condition

		Mean Difference	SE	df	t	Cohen's d	p_{holm}
seline 1	Salt	1.675	0.435	12	3.845	0.541	0.014 *
	Baseline 2	0.411	0.802	12	0.512	0.133	1.000
	Placebo	-0.035	0.587	12	-0.060	-0.011	1.000
lt	Baseline 2	-1.264	0.624	12	-2.026	-0.408	0.262
	Placebo	-1.710	0.495	12	-3.454	-0.553	0.024 *
seline 2	Placebo	-0.446	0.712	12	-0.626	-0.144	1.000

< .05

Table A.6. Summary of two-way repeated measures ANOVA analysis of brachial artery FMD% normalized to VTI.

Cases	Sum of Squares	df	Mean Square	F	p
Condition	2.519×10^{-5}	3	8.398×10^{-6}	4.399	0.010
Residuals	6.872×10^{-5}	36	1.909×10^{-6}		
Exercise	5.211×10^{-5}	1	5.211×10^{-5}	8.941	0.011
Residuals	6.993×10^{-5}	12	5.828×10^{-6}		
Condition ✻ Exercise Condition	6.753×10^{-6}	3	2.251×10^{-6}	1.091	0.365
Residuals	7.425×10^{-5}	36	2.062×10^{-6}		

Table A.7. Post-hoc analysis of brachial artery FMD% normalized to VTI. Results are averaged over exercise.

		Mean Difference	SE	df	t	Cohen's d	p_{holm}
Baseline 1	Salt	0.001	3.583×10^{-4}	12	3.647	0.638	
	Baseline 2	5.978×10^{-4}	4.519×10^{-4}	12	1.323	0.292	
	Placebo	2.461×10^{-4}	3.519×10^{-4}	12	0.699	0.120	
Salt	Baseline 2	-7.089×10^{-4}	4.034×10^{-4}	12	-1.757	-0.346	
	Placebo	-0.001	2.613×10^{-4}	12	-4.058	-0.518	
Baseline 2	Placebo	-3.516×10^{-4}	4.401×10^{-4}	12	-0.799	-0.172	

* $p < .05$, ** $p < .01$

Note. P-value adjusted for comparing a family of 6 estimates.

Table A.8. Summary of two-way repeated measures ANOVA analysis of popliteal artery baseline diameter.

Cases	Sum of Squares	df	Mean Square	F	p	η^2_p
Condition	0.451	3	0.150	1.048	0.383	0.080
Residuals	5.161	36	0.143			
Exercise	1.060×10^{-4}	1	1.060×10^{-4}	0.002	0.961	2.074×10^{-4}
Residuals	0.511	12	0.043			
Condition ∗ Exercise	0.041	3	0.014	0.374	0.773	0.030
Residuals	1.302	36	0.036			

Table A.9. Summary of two-way repeated measures ANOVA analysis of popliteal artery absolute FMD.

Cases	Sum of Squares	df	Mean Square	F	p	η^2_p
Condition	0.143	3	0.048	2.935	0.046	0.197
Residuals	0.584	36	0.016			
Exercise	0.219	1	0.219	24.399	< .001	0.670
Residuals	0.108	12	0.009			
Condition ✻ Exercise	0.021	3	0.007	1.271	0.299	0.096
Residuals	0.196	36	0.005			

Note. Type III Sum of Squares

Table A.10. Post-hoc analysis of popliteal artery FMD. Results are averaged over exercise conditions.

		Mean Difference	SE	df	t	p_{holm}
Baseline 1	Salt	0.082	0.032	12	2.538	0.104
	Baseline 2	-0.005	0.030	12	-0.157	1.000
	Placebo	-0.007	0.049	12	-0.135	1.000
Salt	Baseline 2	-0.086	0.030	12	-2.830	0.091
	Placebo	-0.088	0.032	12	-2.750	0.091
Baseline 2	Placebo	-0.002	0.035	12	-0.057	1.000

Table A.11. Summary of two-way repeated measures ANOVA analysis of popliteal artery FMD%.

Cases	Sum of Squares	df	Mean Square	F	p	η^2_p
Condition	45.323	3	15.108	2.787	0.055	0.188
Residuals	195.154	36	5.421			
Exercise	61.711	1	61.711	21.196	< .001	0.639
Residuals	34.938	12	2.911			
Condition ✻ Exercise	1.663	3	0.554	0.215	0.886	0.018
Residuals	92.897	36	2.580			

Note. Type III Sum of Squares

Table A.12. Summary of two-way repeated measures ANOVA analysis of popliteal artery FMD% normalized to VTI.

Cases	Sum of Squares	df	Mean Square	F	p
Condition	3.430×10^{-5} [a]	3 [a]	1.143×10^{-5} [a]	1.838 [a]	0.158 [a]
Residuals	2.240×10^{-4}	36	6.223×10^{-6}		
Exercise	1.073×10^{-4}	1	1.073×10^{-4}	19.688	< .001
Residuals	6.542×10^{-5}	12	5.451×10^{-6}		
Condition ✻ Exercise	7.158×10^{-6}	3	2.386×10^{-6}	0.635	0.597
Residuals	1.353×10^{-4}	36	3.758×10^{-6}		

Note. Type III Sum of Squares

Table A.13. Summary of two-way repeated measures ANOVA analysis of brachial artery velocity-time integral (VTI).

Cases	Sum of Squares	df	Mean Square	F	p
Condition	63646.920	3	21215.640	0.510	0.678
Residuals	$1.498 \times 10^{+6}$	36	41606.429		
Exercise	935391.716	1	935391.716	15.687	0.002
Residuals	715556.111	12	59629.676		
	180103.048	3	60034.349	1.196	0.325
Residuals	$1.807 \times 10^{+6}$	36	50197.071		

Note. Type III Sum of Squares

Table A.14. Summary of two-way repeated measures ANOVA analysis of popliteal artery velocity-time integral (VTI).

Cases	Sum of Squares	df	Mean Square	F	p	η^2
Condition	254115.664	3	84705.221	1.350	0.274	0.046
Residuals	$2.259 \times 10^{+6}$	36	62747.708			
Exercise	494312.402	1	494312.402	7.546	0.018	0.089
Residuals	786104.213	12	65508.684			
Condition ∗ Exercise	18482.643	3	6160.881	0.126	0.944	0.003
Residuals	$1.763 \times 10^{+6}$	36	48973.312			

Note. Type III Sum of Squares

Table A.15. Summary of two-way repeated measures ANOVA analysis of brachial artery shear area-under-curve (AUC) within the fifteen seconds following cuff release.

Cases	Sum of Squares	df	Mean Square	F	p
Condition	$3.092\times10^{+7}$	3	$1.031\times10^{+7}$	0.401	0.753
Residuals	$9.255\times10^{+8}$	36	$2.571\times10^{+7}$		
Exercise	$2.572\times10^{+8}$	1	$2.572\times10^{+8}$	8.882	0.011
Residuals	$3.475\times10^{+8}$	12	$2.896\times10^{+7}$		
Condition ∗ Exercise	$7.121\times10^{+7}$	3	$2.374\times10^{+7}$	0.973	0.416
Residuals	$8.779\times10^{+8}$	36	$2.439\times10^{+7}$		

Note. Type III Sum of Squares

Table A.16. Summary of two-way repeated measures ANOVA analysis of popliteal artery shear area-under-curve (AUC) within the fifteen seconds following cuff release.

Cases	Sum of Squares	df	Mean Square	F	p	η^2
Condition	$7.500\times10^{+7}$	3	$2.500\times10^{+7}$	1.757	0.173	0.061
Residuals	$5.123\times10^{+8}$	36	$1.423\times10^{+7}$			
Exercise	$9.992\times10^{+7}$	1	$9.992\times10^{+7}$	7.390	0.019	0.081
Residuals	$1.623\times10^{+8}$	12	$1.352\times10^{+7}$			
Condition ∗Exercise	$7.069\times10^{+6}$	3	$2.356\times10^{+6}$	0.227	0.877	0.006
Residuals	$3.733\times10^{+8}$	36	$1.037\times10^{+7}$			

Table A.17. Summary of two-way repeated measures ANOVA analysis of blood flow within the brachial artery during the 15 seconds following cuff release.

Cases	Sum of Squares	df	Mean Square	F	p
Condition	24054.109	3	8018.036	0.711	0.552
Residuals	405716.399	36	11269.900		
Exercise	349023.661	1	349023.661	29.699	< .001
Residuals	141026.730	12	11752.228		
Condition ∗ Exercise	69299.095	3	23099.698	1.854	0.155
Residuals	448607.695	36	12461.325		

Note. Type III Sum of Squares

Table A.18. Summary of two-way repeated measures ANOVA analysis of blood flow within the popliteal artery during the 15 seconds following cuff release.

Cases	Sum of Squares	df	Mean Square	F	p
Condition	170711.667	3	56903.889	0.614	0.610
Residuals	$3.337 \times 10^{+6}$	36	92693.459		
Exercise	477086.415	1	477086.415	6.756	0.023
Residuals	847442.959	12	70620.247		
Condition ∗ Exercise	15666.616	3	5222.205	0.099	0.960
Residuals	$1.902 \times 10^{+6}$	36	52836.034		

Note. Type III Sum of Squares

Table A.19. Summary of two-way repeated measures ANOVA analysis of plasma hyaluronan concentration.

Cases	Sum of Squares	df	Mean Square	F	p	η^2_p
Condition	37.355	3	12.452	0.570	0.639	0.045
Residuals	787.100	36	21.864			
Exercise	72.987	1	72.987	6.014	0.030	0.334
Residuals	145.635	12	12.136			
Condition ✻ Exercise	33.559	3	11.186	0.583	0.630	0.046
Residuals	690.386	36	19.177			

Note. Type III Sum of Squares

Table A.20. Summary of two-way repeated measures ANOVA analysis of plasma C-reactive protein concentration.

Cases	Sum of Squares	df	Mean Square	F	p
Condition	7.636 [a]	3 [a]	2.545 [a]	0.833 [a]	0.485 [a]
Residuals	110.010	36	3.056		
Exercise	0.033	1	0.033	0.033	0.859
Residuals	12.115	12	1.010		
Condition ✻ Exercise	3.312 [a]	3 [a]	1.104 [a]	1.577 [a]	0.212 [a]
Residuals	25.201	36	0.700		

Note. Type III Sum of Squares

[a] Mauchly's test of sphericity indicates that the assumption of sphericity is violated ($p < .05$).

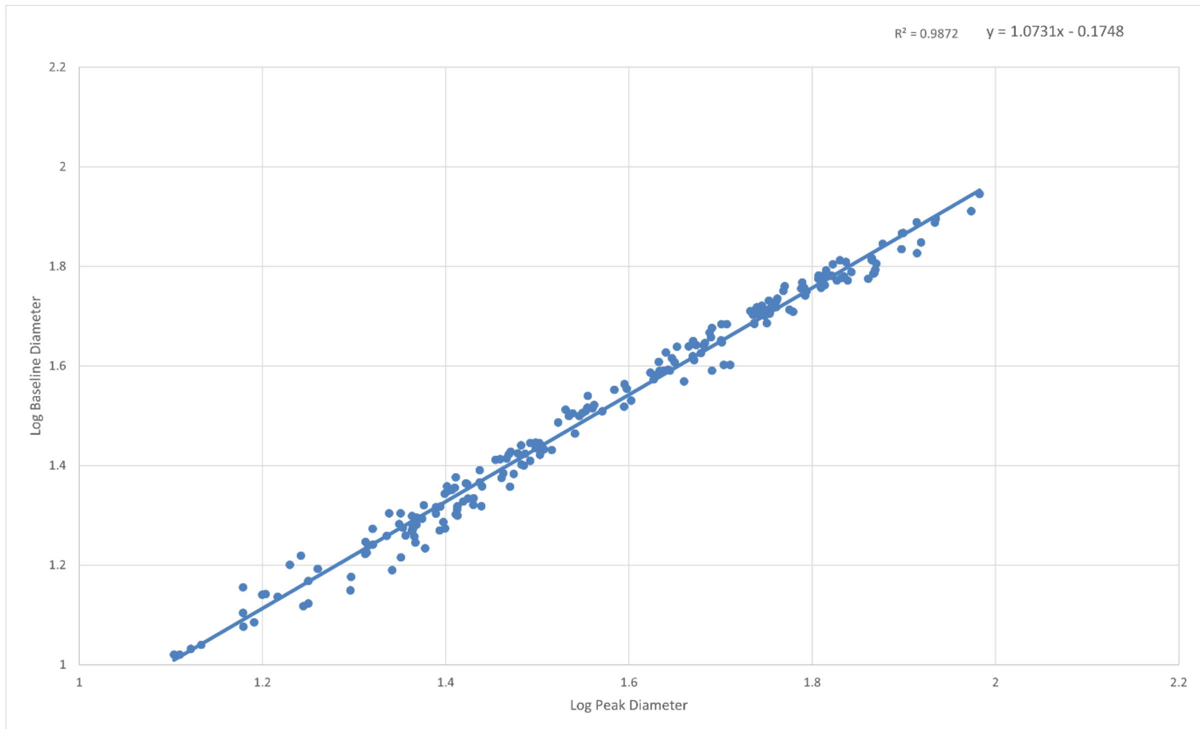

Figure A.1. Log-log comparison of baseline and peak artery diameters for allometric scaling. $R^2 = 0.9872$