THE ROLE OF ANGIOTENSIN-(1-7) IN REGULATING VASCULAR ENDOTHELIAL FUNCTION IN POSTMENOPAUSAL WOMEN

by

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A dissertation submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Applied Physiology

Winter 2025

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ACKNOWLEDGMENTS

First, thank you to my mentor, Megan Wenner. Your guidance has helped shape me into the student, scientist, and person I am today. Your passion for science and women's health is truly contagious and has bled into those of us under your guidance. I am so grateful for your leadership over the past 4 years. Thank you to my committee members Drs. David Edwards, Drew Fancher, and Anna Stanhewicz. You have all played vital roles in my development as a researcher and a scientist. I am so grateful for your mentorship, guidance, and support throughout the scientific process.

Thank you to all past and current members of the WCRL, especially Ginny Nuckols and Kathy Masso. The long hours in the lab learning and working with you has been a pleasure. I am grateful to have met and worked with each of you and am very excited to see what is to come for all of you. Ally Schwab, I cannot thank you enough for everything you have done for me. You have crossed over from lab mate to travel partner to friend. I am so excited to see what you do as a scientist and teacher. I hope you know how valuable you are to the academic community. To the greater KAAP family – thank you for the memories in and outside the lab. I have learned so much from all of you and I wish you all the very best in your future endeavors. I hope our paths cross again.

Away from UD I have been blessed with some of the best friends anyone could ever ask for. Jess, Emilie, Marissa, Amanda, Gloria, Katie, Alex, and Shannon: I love you all and I am forever indebted to you for sticking by my side during this time. I have also been blessed with a seemingly endless network of support that is the Del Vecchio family which I am so grateful for. I can only pray that my grandparents in heaven can see me and be proud of their youngest/or only granddaughter. To my sweet and loving grandmother, Rosa, thank you for being my greatest and most genuinely loving supporter. Thank you, Anthony (Tony), my brother and my first best friend for everything. You inspire me every day with your tenacity and willingness to give your gifts to the world. I love you kid, and I am so excited to see what is ahead for you.

Finally, the great sacrifices and lengths that my parents have gone to provide my brother and I with lives full of opportunity, support, and ultimately, success has not been lost on me. Thank you, Dad, for being my first (and best) coach, for teaching me what is important in life and instilling values that have gotten me here and will continue to carry me through life. Thank you, Mom, for showing me the importance of hard work and resilience. You have been the best role model for me, and you have always been my hero. To both of you, thank you for loving me, and for giving me the opportunity to live, to learn, and to love. The only thing I have ever wanted was to make both of you proud. All of this is for you. I love you. Thank you.

Ad majorem Dei gloriam

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ABSTRACT

Postmenopausal women (PMW) exhibit reduced endothelial function compared to younger, premenopausal women (YW). This is mainly attributed to the loss of estrogen with menopause but the mechanisms underlying the decline in endothelial function remain unclear. Angiotensin-(1-7) [Ang-(1-7)] induces vasodilation through the Mas receptor (Mas_R) and has been shown to restore endothelium-dependent dilation in women with endothelial dysfunction. Animal studies suggest menopause and aging reduce vascular sensitivity to Ang-(1-7) which may cause a compensatory upregulation of Mas_R. However, the role of the Ang-(1-7)/Mas_R axis has not yet been studied in humans. The central hypothesis of this dissertation was that the Ang-(1-7)/Mas_R axis is a main regulator of vascular function in women and that there is dysfunction in this pathway that occurs during menopause which leads to the pathologies associated with the onset of CVD. We hypothesize that PMW would have decreased vascular sensitivity to Ang-(1-7), local administration of Ang-(1-7) would improve endothelial function, and PMW would show a compensatory-based upregulation of Mas_R on endothelial cells. **Methods**: Blood flow was measured using laser Doppler flowmetry. To assess vascular sensitivity, Ang-(1-7) was locally administered in escalating doses in the presence and absence of L-NAME via cutaneous microdialysis to elicit a dose-dependent response. Dose response curves were fit to a sigmoidal curve and the ED50, slope, area under the curve, and peak response were compared between groups. To assess endothelial function, local heating of the cutaneous circulation to 42°C – which elicits an endothelium-dependent dilation – was performed during microdialysis perfusions of lactated Ringers (control) or Ang-(1-7). All skin blood flow data are expressed as cutaneous vascular conductance as a percentage of the maximum dilation elicited by sodium nitroprusside perfusions with heating to 43°C (CVC%max). Separately, venous endothelial cells were collected from PMW and YW and stained for Mas_R using immunocytochemistry and are expressed as protein expression arbitrary units (A.U.) following normalization to a positive control. All data are presented as mean$\pm$SD and alpha was set to $P \leq 0.05$. **Results**: As expected PMW were significantly older than YW and showed elevated diastolic blood pressure, total cholesterol, LDL cholesterol, HDL cholesterol, and blood glucose that is characteristic with aging and menopause in this population. There were no differences in the dose-dependent response to Ang-(1-7) (Top: (YW: 99.93$\pm$17.32 vs. PMW: 97.58$\pm$31.32), LogED50 (YW: 9.34$\pm$10.5 vs PMW: 8.40$\pm$3.96), HillSlope (YW: 0.18$\pm$0.24 vs PMW: 0.25$\pm$0.21), or area under the curve (YW: 73.76$\pm$19.83, vs PMW: 67.35$\pm$12.01); $p>0.05$ for all variables). There was also no significant difference in NO-dependent dilation to Ang-(1-7) between YW and PMW (YW: 7.56$\pm$26.36 AUC vs. PMW: 17.40$\pm$25.75 AUC; $p=0.48$). PMW displayed a blunted endothelial function shown by a significantly attenuated response to local heating in the control site (YW: 91.26$\pm$4.87 $CVC_{\%max}$ vs. PMW: 85.97$\pm$5.63 $CVC_{\%max}$; $p=0.03$), however, there was no impact of Ang-(1-7) on the response to local heating in either group (YW: 89.23$\pm$10.35 $CVC_{\%max}$ vs. PMW 87.19$\pm$9.57 $CVC_{\%max}$; $p=0.88$). Lastly, there were no differences in endothelial Mas_R expression between groups (YW: 0.36$\pm$0.08 A.U. vs. PMW: 0.35$\pm$0.13 A.U.; $p=0.77$). **Conclusion**: The Ang-(1-7)/Mas_R axis does not appear to contribute to endothelial function in PMW as was hypothesized. These data are first to

examine the impact of Ang-(1-7)/Mas_R axis on endothelial function in PMW and provide an important first step for future research examining the relationship between this pathway and the physiological changes that occur with menopause.

Chapter 1

INTRODUCTION

1.1 Problem Statement

Following the onset of menopause, the risk of developing cardiovascular disease (CVD) dramatically increases, accounting for 1 in 3 deaths in women each year. A key mechanism contributing to the development of CVD is reduced vascular endothelial function, characterized by impaired nitric oxide (NO)-mediated vasodilation (Jensen-Urstad et al., 1997; Tousoulis et al., 2012). Postmenopausal women (PMW) exhibit lower endothelial function compared to younger, pre-menopausal women (YW) (Moreau et al., 2012). However, the specific mechanistic pathways affected by menopause and aging in women remain unclear, warranting further investigation.

Angiotensin-(1-7) [Ang-(1-7)] is a peptide that acts on the endothelial Mas receptor (Mas_R) to induce vasodilation (Santos et al., 2008). In YW, plasma concentrations of Ang-(1-7) are greater compared to aged-matched men and correlate with greater endothelial function (Sullivan et al., 2015). Experimental administration of Ang-(1-7) has been shown to restore impaired endothelial function in YW with elevated CVD risk. For example, Ang-(1–7) augmented endothelial vasodilation via NO-dependent mechanisms in women who had preeclampsia (Stanhewicz & Alexander, 2020) and in women with postural tachycardia syndrome (Stewart et al., 2009).

In animal models, Ang-(1-7)-induced vasodilation present in young female mice is absent in both older female mice (model for aging) and ovariectomized (OVX; model for menopause) female mice, suggesting vascular sensitivity to Ang-(1-7) is attenuated by menopause and biological aging (Costa-Fraga et al., 2018). Interestingly, this lack of Ang-(1-7)-induced vasodilation observed in older female mice is associated with an upregulation of Mas_R in endothelial cells presumably to compensate for the blunted vascular sensitivity. However, in animal models of hypertension and myocardial infarction, Mas_R expression is downregulated (Dias-Peixoto et al., 2012). In humans, Mas_R expression has yet to be examined in endothelial cells, and the Ang-(1-7)/Mas_R axis has not yet been studied in PMW.

1.2 Research Question

Given evidence of Ang-(1-7) related benefits in human vasculature and the recent mechanistic insight from animal models of aging and menopause, there is a critical need to determine the role of the Ang-(1-7)/Mas_R axis in PMW. Our long-term goal is to understand the mechanisms that contribute to declines in cardiovascular health in women as they age and experience menopause. The objective of this study is to examine the vascular sensitivity to Ang-(1-7), effects of Ang-(1-7) on endothelial function, and to examine expression of Mas_R in PMW.

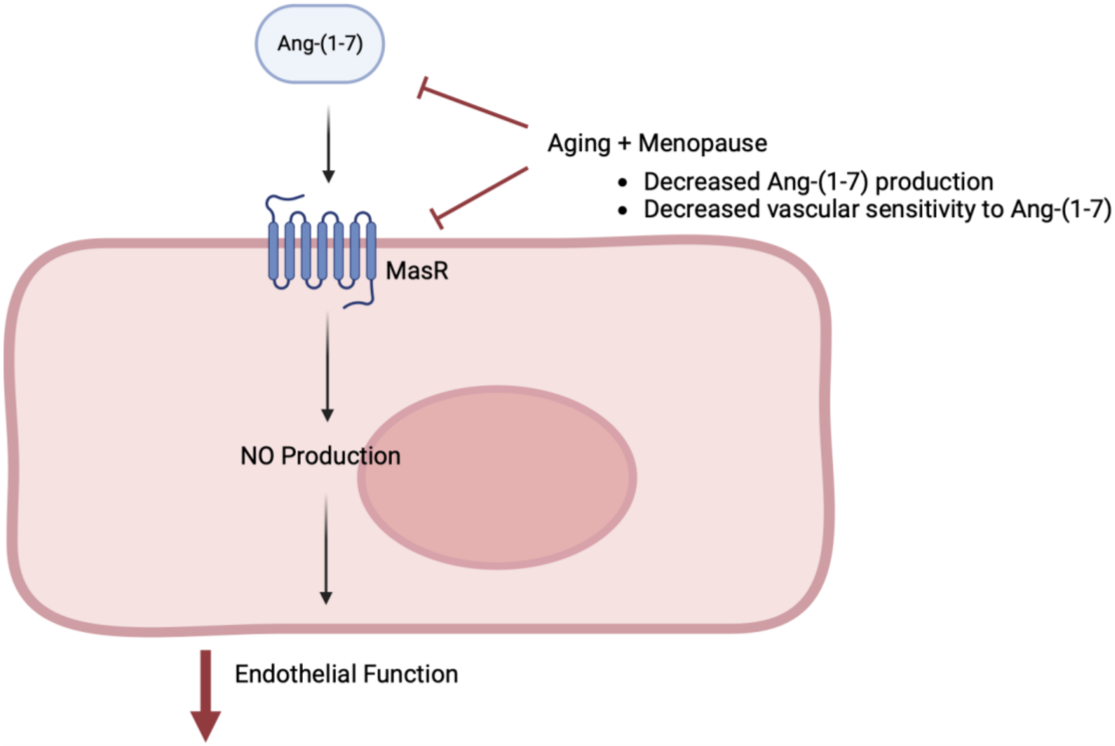


Figure 1.1 Proposed mechanisms of the role of the Ang-(1-7)/Mas_R axis on endothelial function in postmenopausal women.

Figure 1.1 provides a schematic of the proposed mechanisms of the role of the Ang-(1-7)/Mas_R axis on endothelial function PMW that was explored in this dissertation. The central hypothesis was PMW would display blunted vascular sensitivity to Ang-(1-7), Ang-(1-7) will improve endothelial function in PMW, and we examined Mas_R expression in endothelial cells from YW and PMW. We tested the central hypotheses and objectives through the following specific aims:

1.2.1 Aim 1: Examine the vascular sensitivity to Ang-(1-7)-induced dilation in YW and PMW.

We hypothesized Ang-(1-7) would induce dilation in both YW and PMW, but this response would be blunted in PMW indicating decreased vascular sensitivity to Ang-(1-7) in PMW.

1.2.2 Aim 2: Examine the effect of Ang-(1-7) on microvascular endothelial function in PMW.

We hypothesized that increasing the bioavailability of Ang-(1-7) would improve the impaired microvascular endothelial function in PMW to show more similar responses to YW. A secondary hypothesis is that Ang-(1-7) will improve the NO contribution to endothelium-dependent vasodilation in PMW.

1.2.3 Exploratory Aim 3: Quantify the presence of Mas_R in endothelial cells in YW and PMW.

We aimed to develop a protocol to assess endothelial Mas_R expression on venous endothelial cells harvested from women to explore whether endothelial Mas_R expression changes with menopause. The literature in animal models is conflicting, with some studies suggesting there is an upregulation that occurs in masR expression in diseased tissues, and other literature suggesting Mas_R is downregulated.

Chapter 2

LITERATURE REVIEW

2.1 Introduction and Overview

Cardiovascular disease (CVD) is the leading cause of death in women (El Khoudary et al., 2020). Women tend to develop CVD later in life compared to men (El Khoudary et al., 2020). When matched for age, the rate of CVD diagnoses is greater in postmenopausal women (PMW) than in premenopausal women (Kannel et al., 1976). One of the most notable physiological changes during menopause is a decline in estrogen production. Women have high endogenous estrogen from puberty and into most of their adult lives. Around midlife, when women reach menopause, estrogen production declines and stays very low throughout the rest of their lives (Harlow et al., 2012). This is important because estrogen has an established protective effect on cardiovascular function and is thus thought to prevent the development of CVD (Knowlton & Lee, 2012; Somani et al., 2019). So, this decline in circulating estrogen is thought to contribute to increased cardiovascular (CV) risk in postmenopausal women.

A nontraditional risk factor considered to be a reliable early predictor of CVD is endothelial dysfunction. Measurements of endothelial function correlate with coronary artery function and are predictive of CVD (Hansell et al., 2004), thus measurement of endothelial function can be a useful tool to establish CV risk throughout the lifespan. There are several physiological pathways that have been established to contribute to declines in endothelial function. Studying these pathways can provide insight into mitigating the risk of development of CVD.

One of the commonly targeted pathways related to CVD is the Renin-Angiotensin-System (RAS). Angiotensin-II (Ang II) is a common target of CVD treatments due to its vasoconstrictive nature driven by actions through the Angiotensin type 1 receptor (AT-1) (Medina & Arnold, 2019; Medina et al., 2020; Robles et al., 2014) and association with endothelial dysfunction. Angiotensin-(1-7) (Ang-(1-7)) acting through the Mas receptor (Mas_R) has gained recent attention as a mediator of vasodilation in what is considered the protective arm of the RAS (Ferreira & Santos, 2005; Medina & Arnold, 2019; Roks et al., 1999). The vasodilatory, anti-proliferative, and antioxidant nature of Ang-(1-7) has led to the belief of its potential to aid in the prevention and treatment of CVD. Furthermore, some evidence suggests a crosstalk between the Ang-(1-7)/Mas_R axis and estrogen that may further improve vascular endothelial function in women (Brosnihan et al., 2004; Costa-Fraga et al., 2018; Medina & Arnold, 2019; Neves et al., 2004; Sullivan et al., 2015). This review aims to discuss the current literature on endothelial function in women following menopause and to discuss rationale that the Ang-(1-7)/Mas_R axis is a novel pathway to explore in the regulation of vascular endothelial function in PMW.

2.1.1 Prevalence, Risk, and Pathology of Cardiovascular Disease in Women

CVD is among the leading causes of death in the United States and is the leading cause of death in PMW. CVD also impacts women differently than men. The American Heart Association (AHA) considers men aged >45 years at risk for CVD and women aged >55 years at risk for CVD (Virani et al., 2021). These guidelines consider that early in life, CV health is greater in women than it is in men but declines sharply around the time of the menopausal transition (Kannel et al., 1976; Virani et al., 2021). It is likely that the difference in the onset of CVD indicate sex differences in the pathology of CVD.

2.1.2 Traditional CVD Risk Factors

The American Heart Association (AHA) defines risk factors for CVD as modifiable or nonmodifiable. Nonmodifiable risk factors include age and sex and cannot be changed, whereas modifiable risk factors may improve with lifestyle changes. Modifiable risk factors include obesity, hypertension, dyslipidemia, smoking, physical inactivity, unhealthy diet, and diabetes. The AHA has defined "Life's Essential 8" as the determinants of health necessary to maintain CV health and lower the risk of developing CVD. These include: maintaining a healthy diet, engaging in physical activity, healthy sleep, maintaining a healthy weight, avoiding nicotine, maintaining blood pressure and healthy levels of blood lipids and blood glucose (Lloyd-Jones et al., 2022). These determinants of health address the modifiable risk factors of CVD, and following these guidelines, one can mitigate the risk of developing CVD by maintaining CV function and preventing the buildup of plaques in the circulation.

2.1.3 Nontraditional Risk Factor – Endothelial Function

Endothelial function refers to the function of the vascular endothelium, a singular layer of cells lining the inside of the vasculature that control the blood vessel's ability to respond to a stimulus. The endothelium acts as a mechanotransducer (Wilson et al., 2016) by detecting increases in shear stress within the vessels to cause a cascade of actions to regulate blood flow and vascular function. The endothelium also possesses receptors for many different hormones, proteins, compounds and other molecules that can trigger an endothelial response. When it receives a stimulus to vasodilate, the endothelium releases vasorelaxant factors that mainly act on the vascular smooth muscle, a layer of the blood vessel wall that contracts or relaxes to cause vasoconstriction or vasodilation. Some of these factors are nitric oxide (NO), prostaglandins (PGs), or endothelium-derived hyperpolarizing factor (EDHF). It is noted that bioavailability of vasorelaxant factors markedly declines with aging, coinciding with an increase in bioavailability of vasoconstricting factors. These physiological changes are associated with the onset of CVD, though these changes can take place years before CVD begins to manifest. The decreased vasoreactivity that occurs with endothelial dysfunction is a major cause of atherosclerotic buildup (Sitia et al., 2010). Therefore, the physiological changes that lead to declines in endothelial function far precede the onset of CVD and decreased endothelial function is predictive of future onset of CVD.

There is a steady decline in endothelial function throughout the lifespan in both men and women. However, the slope of this decline is steeper in men before midlife. Then, after midlife the slope of decline drastically increases in women and becomes steeper than seen in men. This evidence of a changepoint in the decline in endothelial function suggests that around midlife, physiological changes in women not present in men cause sharp declines in endothelial function (Celermajer et al., 1994). The marked cause of the decline in endothelial function during midlife is referring to menopause when sex hormone profiles change dramatically in a relatively short period of time. The rapid loss of estrogen in women following menopause leads to dramatic declines in endothelial function not seen in men. More recently, it has also been discovered that these declines in endothelial function are also associated with decreased progesterone and increases follicle stimulating hormone (FSH) (Wenner et al., 2024). The sex differences in the decline in endothelial function at midlife also coincide with sex differences in response to therapies that may improve endothelial function. For instance, men with endothelial dysfunction have seen improvements in many traditional therapies such as exercise or pharmacological agents while women have not seen these same improvements with the same therapies (Buchanan et al., 2022; Humphries et al., 2017; Santos-Parker et al., 2017; Seals et al., 2019). This is thought to be due in part to the absence of estrogen in PMW and differences in mechanisms that lead to endothelial dysfunction. Thus, it is important to study endothelial function and mechanisms that may cause declines in endothelial function in women.

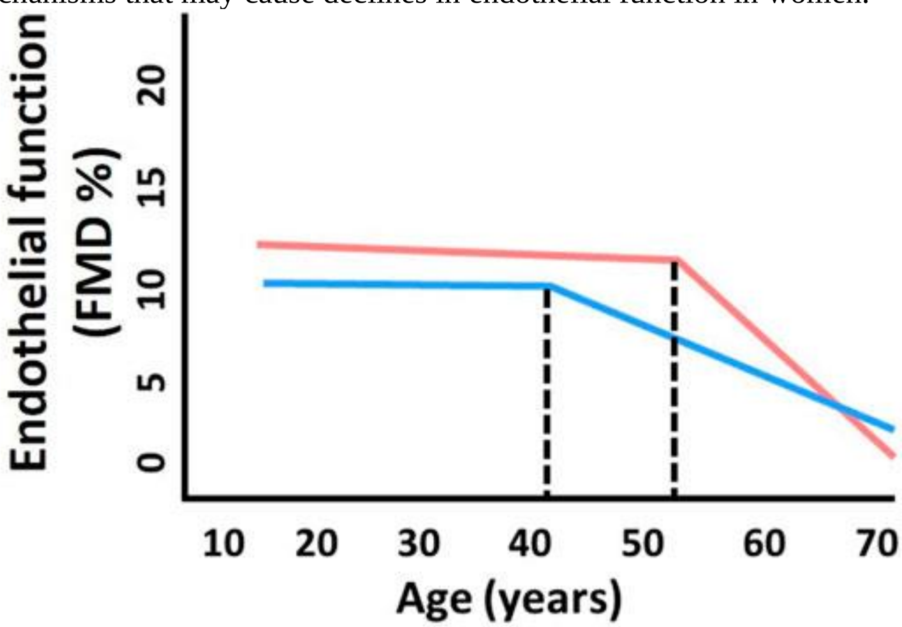


Figure 2.1 Evidence of sex differences in the decline in endothelial function (measured by brachial artery flow-mediated dilation; FMD) throughout the lifespan. Endothelial function in men (blue line) begins to decline within the 4th decade of life. Whereas the changepoint in women (red line) shows a later decline in endothelial function within the 5th decade of life, coinciding with the time of menopause. Following these changepoints, there is also a steeper slope in the decline in endothelial function in women compared to men, illustrating a greater rate of decline in women (Celermajer et al., 1994; Kuczmarski, 2021).

2.1.4 Traditional Pathology of CVD

Endothelial dysfunction is a predictor of CVD and leads to the pathogenesis of CVD. This mainly occurs by the loss of an overall vasodilatory state, when the endothelium has a diminished ability to release vasodilatory factors such as NO and there is an increase in vasoconstrictors (Brandes, 2014; Verma & Anderson, 2002). For instance, the RAS hormonally maintains vascular tone to prevent fluid loss (Su et al., 2021; Unger, 2002). Dysfunction and overactivation of this pathway, however, can lead to hypertension (Unger, 2002). RAS actions in the pathology of CVD are explained in greater depth in section 2.2.2.4. The vascular endothelium is also important in the active response to shear stress to mediate vascular tone. Endothelial dysfunction is a major step in the process of developing atherosclerosis (Mudau et al., 2012; Sitia et al., 2010) and in the development of hypertension (Brandes, 2014; Konukoglu & Uzun, 2017) as increased vasoconstriction increases vessel resistance.

Compounding the negative effects of hypertension, a buildup of atherosclerotic plaques can further lead to cardiovascular events (Ross, 1999). Atherosclerosis is generally caused by buildup of LDL-c usually caused by diet. Over years, fatty streak lesions are formed by oxidized LDL (oxLDL) in the intima space of the vessel wall of large arteries. Oxidation of droplets is facilitated by inducible nitric oxide synthase (iNOS). This process converts LDL and minimally modified (mmLDL) droplets into oxLDL and initiate the fatty streak formation. OxLDL are taken up into macrophages by scavenger receptors and stored in lipid droplets to form foam cells that gather in a fatty streak (Navab et al., 1996; Steinberg & Witztum, 1999; Yamada et al., 1998). OxLDL produces several effects including induction and activation of proinflammatory factors, inducing immune responses, increasing apoptosis and necrosis, enhancing coagulation, increasing cluster of differentiation 36 (CD36) expression, and increasing monocyte adhesion (Paulsson et al., 2000). Fatty streaks progress to more advanced lesions through an inflammatory process triggered by foam cells. Smooth muscle cells migrate further into the subendothelial space where they proliferate and synthesize extracellular matrix proteins that lead to formation of a fibrous plaque and contributes to the stiffening of the cell wall (Paulsson et al., 2000; Ross, 1999; Steinberg & Witztum, 1999).

Atherosclerotic plaques can lead to ischemia by narrowing the vessel cross-section (Glass & Witztum, 2001). While ischemic events can present an array of issues, the rupture of plaques leads to CV events such as stroke and MI (Davies et al., 1993; Glass & Witztum, 2001; Lee & Libby, 1997). If a plaque is not stable because of inflammatory immune responses or increased shear stress, it may rupture. Plaque rupture causes the contents in the plaque to be exposed to coagulants, resulting in further recruitment of platelets and finally forms a thrombus. The thrombus can migrate and cause blockages in other areas, especially in areas where the lumen is already narrowed by fibrous plaques or other fatty lesions (Dhawan et al., 2010; Glass & Witztum, 2001). These blockages can lead to a CV event such as myocardial infarction (MI) or stroke (Glass & Witztum, 2001; Scott, 2004).

One of the main causes of plaque rupture is shear stress (Cunningham & Gotlieb, 2005; Dhawan et al., 2010; Malek et al., 1999). Shear stress is the frictional force that is exerted on the wall of vessels by the blood moving through the vessel (Ballermann et al., 1998). If blood is moving through the vessel at increased force, this will increase the shear stress exerted on that vessel wall. This is worsened by stiffening of major arteries, hypertension, and endothelial dysfunction. In a healthy state, blood vessels maintain elasticity, known as the passive vasodilatory state, allowing them to account for increases in shear and to act as reservoirs for increasing blood pressure (Lu & Kassab, 2011; Ross, 1999).

2.2 Regulation of Endothelial Function

2.2.1 NO Signaling Pathway

NO production and release is triggered by mechanical stimuli such as shear stress, or by the binding of a ligand such as acetylcholine (Ach) or bradykinin (BK) (Hornig & Drexler, 1997) to its receptor located on the endothelial cell wall or within the endothelial cell. Following one of these stimuli, an influx of Ca^{2+} begins a signaling cascade begins protein kinase B (Akt)-mediated phosphorylation of different secondary mediators to either inhibit or activate them (Downward, 1998; Murga et al., 1998). A phosphatidylinositol-3-OH-kinase (PI3K)/Akt pathway is responsible for production of NO and subsequent release (Papapetropoulos et al., 1997; Zeng & Quon, 1996). NO is produced within endothelial cells when the nitrogen in L-arginine combines with O2 by the enzyme endothelial nitric oxide synthase (eNOS) (Fulton et al., 1999). eNOS can also be activated directly by vascular endothelial growth factor

(VEGF) and other signaling pathways such as the adenosine monophosphate-activated protein (AMPK) pathway brought on by other stimuli.

Once NO is released from the endothelial cell it can enter vascular smooth muscle cells (VSMCs). VSMCs are the main actors in regulating vascular tone as the vascular smooth muscle responds to stimuli to cause vasodilation and vasoconstriction. (Clark & Pyne-Geithman, 2005). NO enters the VSMC, stimulating the catalyzation of guanosine 5'-triphosphate (GTP) to cyclic GMP (cGMP) by the enzyme, soluble guanylate cyclase (sGC). cGMP, mediated by protein kinase G (PKG), causes a decrease in intracellular Ca2+ concentration, resulting in muscle relaxation and vasodilation (Llorens et al., 2002; Schulz & Triggle, 1994). Opposite of this, any stimulus causing an influx of extracellular Ca2+ would cause muscle contraction resulting in vasoconstriction. Ca2+ maintains vascular smooth muscle tone through the changes in Ca2+ concentrations that are regulated by ion channels (Clark & Pyne-Geithman, 2005; Collier et al., 2000).

2.2.2 Other Factors Impacting Endothelial Function

Besides NO, there are a myriad of other factors that play a significant role in regulating endothelial function. These include EDHF which can include any substance that directly hyperpolarizes VSMCs by activating ion channels and endothelium derived relaxing factors (EDRF) such as prostacyclin (PGI2). EDHF is most active during nitric oxide synthase (NOS) inhibition (Bauersachs et al., 1996; Luksha et al., 2009; Waldron et al., 1999). Therefore, it is thought that EDHF and NO work as complementary mechanisms for one another, and in some artery types, EDHF may act as a backup mechanism of endothelium-derived vasodilation when NO production is low.

Endothelium-dependent agonists such as Ach and BK cause an increase in intracellular $Ca2+$ (Luksha et al., 2009). This influx of $Ca2+$ triggers the synthesis of EDHF within the endothelial cell. Subsequently, endothelial cells hyperpolarize as $Ca2+$ activates $Ca2+$-dependent $K+$ channels (Luksha et al., 2009). EDHF then diffuses through myoendothelial (MEGJ) gap junctions to VSMCs. In the VSMCs, EDHF activates $K+$ channels to cause endothelium-dependent hyperpolarization followed by closing $Ca2+$ channels to cause relaxation (Busse et al., 2002; McGuire et al., 2001).

The prostaglandin, prostacyclin (PGI2) is considered a main mediator of vascular function as it has vasodilator and anti-thrombotic actions (Vane & Corin, 2003; Vane et al., 1990). PGI2 is an unsaturated carboxylic acid formed as a metabolite of arachidonic acid from phospholipids of endothelial cells (Ruan et al., 1997; Wright & Malik, 1996). PGI2 is released from endothelial cells as a result of induced shearing force from blood flow. Once released from the endothelial cells, PGI2 acts on the prostacyclin receptor located on platelets and VSMCs. PGI2 in the VSMC contributes to the conversion of ATP to cAMP thus contributing to muscle relaxation (Vane & Corin, 2003).

2.2.3 Mechanisms that Contribute to Endothelial Function

There are several mechanistic pathways that are known to interact with different aspects of the endothelium and the NO pathway which can impact endothelial function. Dysfunction in these pathways is associated with disease progression and greater incidence of CVD (Agapitov & Haynes, 2002; Elahi et al., 2009; Lakshmi et al., 2009; Unger, 2002). Therefore, it is important to consider these mechanisms when studying endothelial function. The Endothelin (ET-1) system, oxidative stress, and the Renin Angiotensin System (RAS) are among these mechanistic pathways which will be discussed in greater detail in the following sections.

2.2.3.1 The Endothelin System

Another mechanism responsible for endothelial dysfunction is the Endothelin System, led by ET-1. ET-1 is produced within endothelial cells and acts on ET_A and ET_B receptors to regulate vasomotor tone (Davenport et al., 2016; Horinouchi et al., 2013; Miyauchi & Masaki, 1999; Shihoya et al., 2016; Vierhapper et al., 1990). Due to the vasoconstrictive nature of ET-1, downstream, ET-1 causes increases in blood pressure, arterial stiffness, and an overall disease state (Ergul et al., 2006; McEniery et al., 2003; Schiffrin, 2001, 2005). ET-1 has also been known to lead to increases in oxidative stress that may further contribute to endothelial dysfunction (Callera et al., 2006; Pollock & Pollock, 2005). Increases in bioavailable ET-1 are associated with increased disease risk and the endothelin system has been established as a mechanism of endothelial dysfunction with aging, menopause, chronic kidney disease, type 2 diabetes, obesity, and markers of cardiovascular dysfunction such as hypertension and atherosclerosis (Babcock et al., 2023; Dhaun et al., 2006; Donato et al., 2009; Kuczmarski, 2021; Lerman et al., 1995; Wenner, Taylor, et al., 2011; Yanes et al., 2005; Ylikorkala et al., 1995).

2.2.3.2 Oxidative Stress

Oxidative stress is a leading cause of decreased NO in disease states. Oxidative stress is understood as an imbalance of pro-oxidants and antioxidants (Sies, 1986). Oxidants such as reactive oxygen species (ROS) accumulate due to different lifestyle factors and aging (Dawson et al., 2013). Previous literature has proposed that increases in oxidative stress can lead to a decrease in the production of NO due to a decrease in the bioavailability of substrates needed for NO production (Dawson et al., 2013). Furthermore, NO reacts with free radicals produced during exercise and can cause the formation of an oxidant that decouples eNOS further decreasing NO production and increases oxidative stress (Dawson et al., 2013). The increase in free radicals leads to scavenging of NO. Biproducts of the NO scavenging can also lead to the uncoupling of eNOS, further decreasing bioavailable NO.

2.2.3.3 RAS Actions in the Pathology of CVD

The RAS is a known regulator of the cardiovascular system. The starting points of the pathway come from 3 different organs: the liver, kidney, and lungs. Angiotensinogen is synthesized in the liver; renin is synthesized in the kidney and Angiotensin Converting Enzyme (ACE) is synthesized in the lungs. Renin facilitates the conversion of angiotensinogen to angiotensin I (Ang I). ACE facilitates the conversion of Ang I to Angiotensin II (Ang II). Ang II is considered the main effector of the RAS through its systemic effects on the vasculature, kidney, etc. Ang II also signals the release of aldosterone from the adrenal gland. Aldosterone is instrumental in the reabsorption of NaCl and water in the kidney.

In the classical interpretation of the RAS, Renin, an aspartyl protease released from renal juxtaglomerular cells in response to increased sympathetic activity or other stimuli cleaves Angiotensinogen to form Ang I (Fischer, 2002; Medina et al., 2020). Ang I is then hydrolyzed by ACE to form Ang II. Ang II can act as a systemic endocrine hormone or as a local paracrine or autocrine hormone (Unger, 2002). There are 2 types of Ang II receptors in humans: Angiotensin II receptor type 1 (AT1R) and

Angiotensin II receptor type 2 (AT2R) receptors. Both receptors mediate different actions of Ang II. AT1R, located in the VSMCs, kidney, heart, and adrenal gland, mainly regulates blood pressure, and is attributed to the pathology of CVD. The actions of Ang II through the AT1R played a major evolutionary role in maintaining blood volume and blood pressure. However, many of these actions are no longer needed in a modern lifestyle and generally cause more harm (Unger, 2002).

Ang II has many well-established actions on the vasculature that are associated with the development of CVD. AT1R is mostly known to mediate vasoconstriction via increased Ca2+ influx, causing a downstream VSMC contraction (Walkyria O. Sampaio et al., 2007). Ang II through the AT1R is also known to cause a pressor effect, cell proliferation, thrombosis, and oxidative stress. Ang II via AT1R contributes to phosphorylation of proto-oncogene tyrosine-protein kinase Src (c-Src) to activate Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, leading to formation of ROS. Ang II also leads to production of NFkB, a transcription factor in inflammation, and Mitogen-activated protein kinases (MAPKs) such as extracellular signal-regulated kinase ½ (ERK1/2) and p38MAPK, which can act as mediators in cell proliferation and fibrosis (Walkyria O. Sampaio et al., 2007; Santos et al., 2018).

Less is known about AT2R, and it is much less abundant in human tissues than AT1R. However, some evidence exists to suggest that this receptor may mediate vasodilation and be counteractive to the actions of the AT1R (Unger, 2002). Actions mediated by Ang II via AT2R have been reported as vasodilatory, anti-inflammatory, anti-prolific, anti-oxidative, although these pathways have not been fully elucidated. There is a nonclassical arm of the RAS considered to be vasoprotective in nature. This is led by the actions of Ang-(1-7) through the Mas receptor (Mas_R). The actions of this arm are described in depth in Section 2.5.

2.3 Measurements of Endothelial Function

Measurements of endothelial function correlate with coronary artery function and are predictive of CVD (Hansell et al., 2004). There are multiple pathways endothelial function can be measured *in vitro* and *in vivo*. *In vitro,* endothelial function can be measured in animal models or in isolated vessels. *In vivo* measurements of endothelial function in human subjects have evolved over the years. Previously, most of these measurements were more invasive, costly and time consuming. Though, more recently, these measurements have become more feasible. Flow-mediated Dilation (FMD) and Laser Doppler flowmetry with microdialysis are two methods commonly used to assess endothelial function in both the macrovasculature and the microvasculature respectively.

2.3.1 FMD

Brachial artery FMD has been established as a valid, reliable, and reproducible measure of endothelial function (Thijssen et al., 2011). This method relies on a reactive hyperemic response to cause vasodilation. A brief period of occlusion induces reactive hyperemia leading to an increase in shear stress. Shear stress is dependent on the release of NO and is thus indicative of endothelial function (Tagawa et al., 1994). Reactive hyperemia is impaired in many disease states including hypertension (Perticone et al., 2001), coronary artery disease (Chan et al., 2003; Schächinger et al., 2000), peripheral artery disease (Brevetti et al., 2003), and hypercholesteremia

(Mullen et al., 2001). Impaired reactive hyperemia is also strongly associated with greater CV risk (Mitchell et al., 2004).

The diameter of the brachial artery is examined before and after induction of a shear stress stimulus to determine the ability of the artery to vasodilate. The shear induced during FMD leads to signal transduction causing the production of vasodilator substances such as NO and EDHF. NO and EDHF diffuse from the endothelial cell to the smooth muscle cell and cause signal transduction that leads to a reduction in calcium concentration in the muscle cell and thus muscle relaxation. The relaxation of the smooth muscle leads to vasodilation. The magnitude of the vasodilatory response is key in the measurement of FMD, where a greater vasodilatory response shows a greater reactive hyperemic response. Thus, the mechanism induced by FMD is endothelium mediated and this measurement is considered a standard measurement of conduit artery endothelial function (Thijssen et al., 2011).

When performing FMD, ultrasonography is used to longitudinally image the brachial artery before and after occlusion of a cuff placed distal to the brachial artery. The cuff is inflated for 5 minutes to a pressure exceeding resting systolic blood pressure to serve as an occlusion to blood flow down the arm. The release of this cuff causes a rapid increase in shear stress that trigger the NO pathway, thus causing vasodilation in the brachial artery. The artery is continually imaged up to 3 minutes following cuff deflation to determine the increase in diameter because of shear. Results are calculated as FMD% (Baseline diameter/largest diameter following shear x 100) where a greater FMD% is indicative of greater endothelial function. This measure is considered the standard of measurement of endothelial function in the macrovasculature (Thijssen et al., 2011).

2.3.2 Microvascular Function

Laser Doppler flowmetry coupled with microdialysis perfusions is a method used to assess the function of the microvasculature *in vivo*. Lasers are placed on the skin and measure red blood cell flux in the cutaneous circulation by detecting shifts in near infrared (NIR) light caused by moving red blood cells (Bircher et al., 1994; Eun, 1995; Stern, 1975). These data, recorded as laser-Doppler flux (LDF) are typically normalized for mean arterial pressure and presented as cutaneous vascular conductance (CVC = LDF/MAP). Microdialysis perfusions of pharmacological agents may be utilized along LDF to manipulate blood flow within the capillary bed in the transdermal space (Kreilgaard, 2002). To perform these perfusions, a fiber with a porous membrane is inserted into the transdermal space of the skin to sit in the cutaneous capillary beds. The fiber allows drugs to be introduced to the cutaneous microcirculation of the skin. Lactated Ringers solution is widely used as a vehicle in microdialysis perfusions, and many different substances can be mixed with lactated Ringer's and perfused to cause vasodilation or vasoconstriction in the cutaneous circulation surrounding the membrane. Multiple fibers can be placed at once, at least 1 inch apart from another, to test vascular reactivity to substances, induce a specific pathway, or measure blood flow to introduction of a pharmacological agent. Endothelial function can be measured with LDF and local heating of the skin, a pharmacological stimulus, or reactive hyperemia. Treatments done using this methodology are all localized and acute.

2.3.2.1 Local Heating

Endothelial function in the cutaneous microvasculature is directly correlated with endothelial function in coronary arteries (Abularrage et al., 2005; Holowatz et al., 2008). Thus, methods to assess endothelial function in the cutaneous microvasculature can predict coronary artery dysfunction. Local heating in the skin with microdialysis perfusions is an established method to assess microvascular endothelial function. Heating to 42°C causes a biphasic response in skin blood flow (Minson et al., 2001; Minson et al., 2002). This biphasic response includes an initial peak, followed by a nadir and a gradual plateau. The initial peak is considered 80% mediated by the axon reflex on sensory nerves and 20% is mediated by NO. It remains unclear what mediates the nadir. The plateau phase is mainly mediated by NO and this response is attributed to endothelial function, where a greater plateau is indicative of greater endothelium-mediated dilation (Holowatz et al., 2003; Minson et al., 2002; Wong, 2013). Following the plateau, the eNOS inhibitor L-NG-Nitro arginine methyl ester (L-NAME) is often used to block endothelial NO production and quantify the NO contribution to the plateau by finding the difference between the local heating plateau and the L-NAME-induced plateau. At the end of the experiment, the NO donor sodium nitroprusside (SNP) is perfused to induce maximal vasodilation. This allows normalization of data at all timepoints to maximal dilation by presenting data as a percentage of maximal dilation (CVC%max). Heating to 39°C has also been shown to cause a similar local heating response, though this temperature has not been studied as in depth. Past studies have also used 35°C and 44°C, but these temperatures have not been used as widely (Evans et al., 1993).

Minson et al. (Minson et al., 2002) was among the first to report decreased microvascular endothelial function in older adults. They also confirmed this response was NO-mediated via eNOS by perfusing L-NAME (a nonspecific NOS inhibitor). When L-NAME was perfused, the plateau was significantly blunted in both younger and older adults. The initial peak, though not as commonly studied, also echoes the evidence that the local heating response is blunted with aging. Minson et al. (Minson et al., 2002) found a significantly blunted initial peak in older adults compared to younger adults.

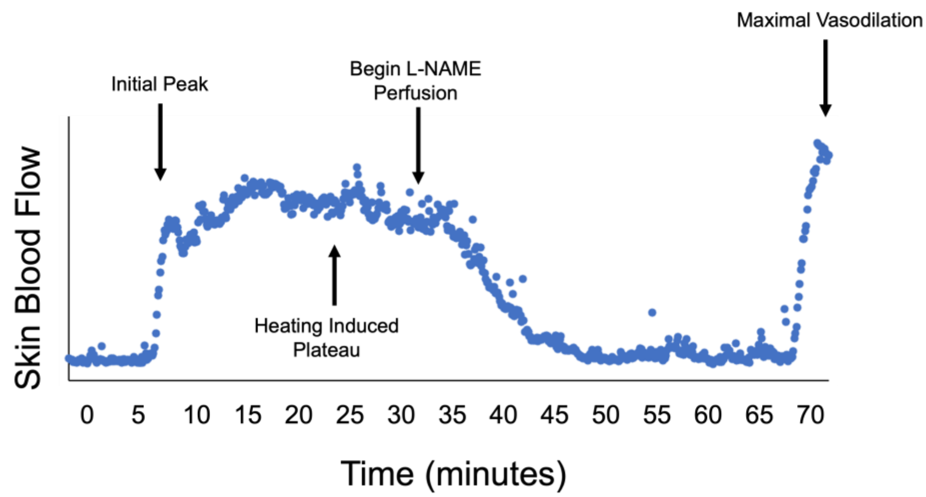


Figure 2.2 Representative tracing of a local heating response in the cutaneous microcirculation. When heating to 42°C begins there is an initial peak mediated mainly by the axon reflex. Then skin blood flow plateaus. The plateau is mainly NO mediated and indicative of endothelial function. At the end of the plateau, L-NAME is perfused to block eNOS production of NO. The L-NAME-induced plateau is used to calculate the NO contribution to the local heating plateau. Finally, skin is heated to 43°C and SNP is perfused to induced maximal dilation to normalize data.

2.3.2.2 Acetylcholine Perfusions

Though local heating is commonly used to study endothelial function in the cutaneous microcirculation, there are other methods that may induce an endothelium-mediated response. The perfusion of acetylcholine (Ach) in increasing doses induces a dose-dependent endothelial response. This is considered a pharmacological stimulus rather than a physiological stimulus provided by local heating (Agarwal et al., 2009; Holowatz et al., 2005; Kellogg Jr et al., 2005; Wilson et al., 2016).

Ach-mediated dilation is endothelium-dependent and thus microdialysis perfusions of Ach are used increase bioavailable Ach to test endothelium-dependent dilation. Though it is not fully NO-mediated, Ach can act as a stimulus for NO release. In this way, Ach can induce the release of NO. In a healthy state, Ach is released by endothelial organic cation transporters (OCTs) on endothelial cells as a response to the mechanical stimulus of shear stress (Fukaya & Ohhashi, 1996; Milner et al., 1990; Wilson et al., 2016). This Ach then helps to produce NO via eNOS to work downstream to cause a reduction in Ca2+ in smooth muscle that leads to relaxation of the vascular smooth muscle. Thus, local administration of Ach can be used to induce a pharmacological endothelial response.

Ach is used to induce vasodilation during many different types of experiments. Ex vivo, vessels can be treated with Ach to induce dilation. In human research Ach has been infused intravenously or intraarterially, usually coupled with measurements of forearm blood flow. Ach has also been perfused through microdialysis fibers during laser doppler flowmetry. This allows researchers to study endothelial function in the microvasculature in response to this pharmacological stimulus. These Ach perfusions can be mixed with other substances to target different pathways that may further influence Ach-induced dilation. These methods employing Ach have been proven to be endothelium-dependent and driven by the actions of NO (Holowatz et al., 2005; Kellogg Jr et al., 2005; Wilson et al., 2016). Thus, Ach perfusions with LDF is one of the many methods useful in the assessment of endothelial function.

2.4 Sex Hormones

Sex hormones are present throughout the body. Their main function is to regulate reproduction; however, they are systemic and thus have greater systemic effects independent of reproduction. The pathways that regulate production of sex hormones originate in the brain in both biological men and women, but the result of the pathway differs between sexes. In biological women the main biologically active sex hormone is estradiol- 17β (E2) (Wise et al., 2009), with progesterone (P4) being a secondary actor. In biological men, testosterone is the dominant hormone. Follicle stimulating hormone (FSH), luteinizing hormone (LH) and Anti-Mullerian Hormone (AMH) all act prominently in both female and male reproduction.

Sex hormones have been studied extensively for their effects outside the reproductive system. This is due in part to the endocrine nature of these hormones, that they circulate in the blood and receptors exist outside the reproductive system. The receptors for E2, especially, have been known to mediate many different effects on other body systems. Specifically with regard to the CV system, E2 via estrogen receptors (ERs) located on the endothelium is known to have a protective effect on the vasculature that has been well documented in the literature (Arora et al., 1998; Baker et al., 2003; Best et al., 1998; Brosnihan et al., 1997; Chakrabarti et al., 2008; Gavin et al., 2009; Hashimoto et al., 1995; Knowlton & Lee, 2012; LeBlanc et al., 2009; McNeill et al., 1999; Meendering et al., 2008; Mendelsohn & Karas, 1999; Miller & Duckles, 2008; Moreau et al., 2013; Shaul, 1999; Somani et al., 2019). This phenomenon has led to the hypothesis that premenopausal women are protected from CV dysfunction and has been the focus of much research on CV function in women.

2.4.1 Hormonal Regulation of the Female Reproductive System

Female reproduction is regulated by a hormone pathway referred to as the hypothalamic-pituitary-ovarian (HPO) axis (Figure 2.3). The pathway begins in the hypothalamus, where gonadotropin-releasing hormone (GnRH) is synthesized and released in a pulsatile manner into the hypothalamic-hypophysial-portal vessel system. This triggers the synthesis and release of the gonadotropins FSH and LH from the anterior pituitary gland. FSH stimulates E2 production and ovarian follicles to mature, and LH stimulates ovulation and formation of the corpus luteum. Once an ovum is released, P4 is produced by the corpus luteum, and P4 and E2 provide negative feedback to the hypothalamus and pituitary gland to inhibit production of GnRH. If there is no pregnancy, E2 and P4 levels drop and the HPO axis restarts. If there is a pregnancy, the placenta begins to produce P4, halting any further ovulation (Dwyer & Quinton, 2019; Janes, 2020; Popat et al., 2008).

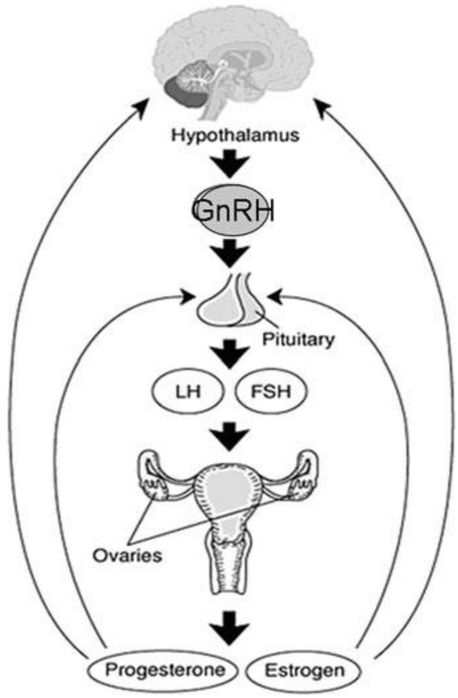


Figure 2.3 The hypothalamic-pituitary-ovarian axis. Gonadotropin releasing hormone (GnRH) release from the hypothalamus is sensed by the anterior pituitary gland to release Luteinizing hormone (LH) and follicle stimulating hormone (FSH) to act on the ovaries. Figure from Popat et al. (Popat et al., 2008).

2.4.2 Changes in Ovarian Hormones During Menopause

Throughout the lifespan, women experience different phases of reproductive function. These reproductive phases can be classified as premenses, premenopause, perimenopause, menopause and then women live the rest of their lives in postmenopause. During premenopause, women have regular fluctuations in hormones known as the menstrual cycle. Women generally have regular menstrual cycles of about 25-35 days. During this time, reproductive function is at its greatest and pregnancy is possible. However, as women approach menopause, the regular hormonal fluctuations driving the menstrual cycle become irregular.

As women age, they progressively lose follicles, the portion of the ovary containing oocytes. As these follicles are lost, menstrual cycles become irregular. Around this time, the neural production of LH also becomes more irregular due to changes in the GnRH pulsatility (Weiss et al., 2004), making ovulation difficult. The decline in LH has downstream effects on female reproductive hormone production. E2 and P4 release both decline due to a lack of production from the ovaries, and decreased signaling from LH, triggering a concurrent rise in FSH due to a change in the negative feedback loop where E2 and P4 would normally regulate the release of GnRH but can no longer inhibit the release of FSH by the hypothalamus and pituitary gland (Burger et al., 2002; Randolph Jr et al., 2003). This increase in FSH is most associated with the menopausal transition (Randolph Jr et al., 2003; Randolph Jr et al., 2011; Santoro & Randolph, 2011). FSH concentration directly correlates with the inability to conceive and is used as an indicator of the number of oocytes (Randolph Jr et al., 2003; Randolph Jr et al., 2011; Santoro & Randolph, 2011). This increase in FSH is maintained throughout the menopausal transition where FSH continues to stimulate E2 release in an attempt to maintain the regular menstrual cycle, but decline in the amount and responsiveness of ovarian follicles leads to fluctuations in E2 release that eventually diminish (Davis et al., 2015; Weiss et al., 2004). This also causes infrequent ovulation, and results in a decline in P4 (Davis et al., 2015). The changes in E2, P4, and FSH are depicted in Figure 2.4. These changes in sex hormones with menopause have impacts throughout the body as these hormones are regularly circulating. E2 has been studied in depth for its impact on the vasculature and the decline in E2 with menopause is associated with decreased CV function.

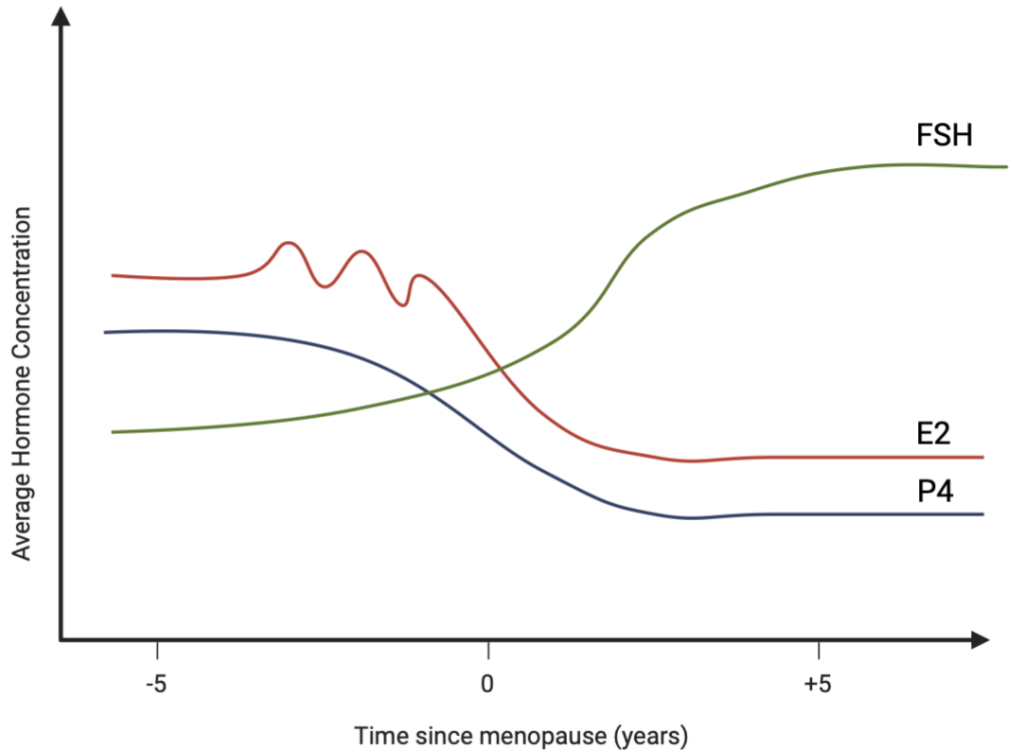


Figure 2.4 Changes in hormonal profiles. Estradiol (E2; red line) concentration becomes irregular in the time leading up to menopause and rapidly declines at menopause, then stays low throughout later life. Progesterone (P4; blue line) levels also decrease dramatically around the time of menopause and remain low. Follicle stimulating hormone (FSH; green line) increases around the time of menopause and stays elevated throughout life following menopause. Adapted from Davis et al. 2015 (Davis et al., 2015).

2.4.3 Estrogen Actions on the Vasculature

Impacts of E2 on the vasculature can be classified as genomic and nongenomic (Mendelsohn, 2002). Genomic impacts refer to the ability of estrogen to impact the DNA in a cell, while non-genomic refers to the ability of E2 to cause immediate changes in a cell via signaling cascades. To exert its genomic effects on the cell, E2 can enter a cell via passive diffusion. When E2 enters a vascular cell, it binds with the intracellular estrogen receptor α (ERα) and estrogen receptor β (ERβ). This forms estrogen-ER complexes form dimers (Cowley et al., 1997) to bind to DNA sequences of target genes to upregulate transcription of these genes. Some of these genes forms proteins that act as coactivator proteins, integrators of transcription (Shibata et al., 1997) or may act as enzymes. There are also some that interact with corepressors to suppress transcription of other target genes (Mendelsohn & Karas, 1999) these genomic actions of E2 cause long-term effects on the vasculature (Mendelsohn, 2002). E2 has also been found to induce expression and production of eNOS (Chambliss & Shaul, 2002). Previous studies have shown eNOS-dependent measurements of endothelial function are significantly blunted in women following menopause (Sorensen et al., 1998), suggesting a downregulation in eNOS following menopause that is hypothesized to be due to long term reduction in estrogen exposure (Chambliss & Shaul, 2002).

The nongenomic effects of E2 can increase the amount of bioavailable NO through several different mechanisms to cause immediate vasodilation (Mendelsohn, 2002). E2 activates eNOS in what is termed the estrogen-ER-eNOS rapid activation pathway. This pathway works through ERα in 2 different mechanisms that lead to direct activation of eNOS. ERα interacts directly with the p85 subunit of PI 3-kinase, activating the PI 3 kinase-Akt-eNOS pathway (Haynes et al., 2000; Simoncini et al., 2000). E2 through ERα has also been associated with the activation of MAP kinase and HSP90 to activate eNOS (Chen et al., 1999) and activates the PI3k/Akt pathway to activate eNOS and facilitate NO production (Hafezi-Moghadam et al., 2002; Simoncini et al., 2000). E2 has also been known to activate BK channels (De Wet et al., 2006) and is associated with increased BK-mediated vasodilation (Barton et al., 1998).

Intracellular G protein estrogen receptors (GPERs) located on endoplasmic reticulum are also important in mediating the nongenomic signaling pathways of E2 (Alexander et al., 2011; Haas et al., 2009; Miller & Duckles, 2008; Revankar et al., 2005). GPERs have been reported to contribute to regulation of vascular tone and blood pressure where activation of these receptors can have a vasoprotective effect (Revankar et al., 2005). GPER decreases vascular tone via activation of phospholipase C, leading to an increase in Ca2+ movement. It is hypothesized that GPERs impact phosphorylation of ERK-1/2, a secondary messenger involved in the NO vasodilatory pathway (Nadal et al., 2000).

2.4.4 Sex Hormones and Endothelial Function in Women

E2 in women is known to have a significant impact on endothelial function by increasing NO production by contributing to the activation and expression of eNOS (Chen et al., 1999; MacRitchie et al., 1997; Shaul, 1999; Yang et al., 2000). E2 plays a role in cardio protection (Rosenthal & Oparil, 2000). ERα and ERβ receptors can be found on endothelial cells and VSMCs (Mendelsohn & Karas, 1999), leading to the hypothesis that E2 plays a role in endothelial function. E2 via the ERα and ERβ receptors contributes directly to the production of NO by activating kinase c-Src (Filardo et al., 2000; Li et al., 2007), ERK (Kim et al., 2018; Scaling et al., 2014), PI3K (Revankar et al., 2005), and Akt (Sharma & Prossnitz, 2011) and by increasing eNOS mRNA expression in endothelial cells (Hishikawa et al., 1995; MacRitchie et al., 1997). Further, E2 binding in endothelial cells activates eNOS to increase production of NO (Kim & Bender, 2005).

Premenopausal women are at a lower risk of developing atherosclerosis (Stanhewicz et al., 2018) and they display fewer incidences of hypertension (Khalil, 2005). This is due in part to greater endothelial function in premenopausal women (Celermajer et al., 1994; Moreau et al., 2012; Stanhewicz et al., 2018). Sex hormones influence vasodilatory and vasoconstrictive factors such as NO, PGI2, EDHF, and ET-1 and most likely contribute to endothelial function in this way (Khalil, 2005). E2 has an established antioxidant, vasodilatory and anti-inflammatory effect in women (Knowlton & Lee, 2012). These actions of E2 lead to the idea that E2 has a protective effect on the cardiovascular system in young women.

2.4.4.1 Endothelial Function throughout the Menstrual Cycle

Considering the changes in hormone levels throughout the menstrual cycle, and the impacts hormones can have on endothelial function, there has been much interest in detecting potential changes to endothelial function throughout the menstrual cycle. Hashimoto et al. (Hashimoto et al., 1995) measured FMD during menses, the follicular phase, and the luteal phase of the menstrual cycle. They concluded there is variation in endothelial function throughout the menstrual cycle. FMD was lowest and comparable to the value in men when estradiol was lowest but was significantly higher during the follicular and luteal phases when E2 was highest. In contrast to these findings, Williams et al. (Williams et al., 2001) measured FMD in women at the late and early follicular phase and early and late luteal phase. They found a significant drop in FMD% (about 6% decrease) during the early luteal phase. FMD was not different between any other phases. These results suggest endothelial function is lowest during ovulation. However, Williams et al. did not measure sex hormones and only counted days to estimate menstrual cycle phase. So, based on these results, we cannot classify the exact menstrual cycle phase at each timepoint.

However, there are differing conclusions in the literature on endothelial function throughout the menstrual cycle. Arora et al. (Arora et al., 1998) measured endothelial function in response to iontophoresis of Ach in the forearm during both the follicular and luteal phases of the menstrual cycle. They concluded endothelial function was impaired during menses compared to the luteal phase. This contrasts with the results published by Williams et at. (Williams et al., 2001), though these were different methods of assessing endothelial function and it is possible that the pharmacological stimulus of Ach impacts a different pathway affected by female sex hormones than the mechanical stimulus of FMD. Considering the literature, hormone levels can impact the mechanisms being studied, so when the impact of changing levels of hormones throughout the menstrual cycle is not the focus of the study, it is necessary to standardize menstrual cycle phase to mitigate any discrepancies that may arise between phases.

2.4.4.2 The Impact of Menopause on Endothelial Function in Women

The combination of chronological aging with a lack of E2 production leads to several deleterious pathways in PMW that lead to endothelial dysfunction (Somani et al., 2019). E2 directly impacts the production of NO via the ERα receptor. ERα and eNOS endothelial cell expression is significantly lower in PMW than in YW (Gavin et al., 2009). There is a decline in ERα expression along with decreased NO production that is rescued by E2 stimulation of eNOS *in vitro* (McNeill et al., 1999) and *in vivo* (Adler et al., 2018; Hashimoto et al., 1995; Kauser & Rubanyi, 2008; Meendering et al., 2008; Miner et al., 2011). Animal models of aging and menopause have confirmed these findings (Kang et al., 2011; LeBlanc et al., 2009). They compared vasodilatory responses in young female rats, old female rats, young and old ovariectomized female rats (OVX) and young and old OVX female rats treated with E2. They found significantly impaired vasodilation in old female rats compared to young female rats. Old female rats and young OVX female rats showed similar vasodilation, echoing the interaction between ovarian hormones and aging on vasodilation.

In humans, endothelial function declines with aging, but there is a clear impact of menopause on endothelial function, where women show more exaggerated declines in endothelial function following menopause compared to age-matched men. Celermajer et al. (1994) provided evidence of a decline in FMD occurring later in life in women than in men and at a sharper decrease (Figure 2.1; (Celermajer et al., 1994)). They concluded that women had a sharper decline in endothelial function than men starting in midlife that corresponds to the decline in estrogen that occurs with menopause. Taddei et al. (1996) found evidence of a decline in endothelial function prior to menopause assessing forearm blood flow in response to Ach perfusions (Taddei et al., 1996). However, Taddei et al. (1996) depicted a changepoint in endothelial function, or a change in slope, at the 4^{th} decade in life, earlier than Celermajer et al. (1994). This is likely because Taddei et al. (1996) were studying microvascular function compared to macrovascular function and impairments in endothelial function can be seen earlier in the microvasculature than in the macrovasculature. The group studied by Taddei et al. (1996) still had estrogen circulating and therefore the decline in endothelial function could not be attributed to estrogen. Wenner et al. (2024) confirmed the findings of Taddei et al. (1996) in that they found a decline or changepoint in endothelial function measured by FMD arterial stiffness measured by carotid-femoral pulse wave velocity during the 4^{th} decade of life. This was also associated with an increase in FSH and a decline in P4 rather than a decline in E2, thus suggesting that the increase in FSH and decrease in P4 may be moreso accountable for early changes in endothelial function in women during premenopause (Wenner et al., 2024). Despite much evidence that the decline in E2 is considered the driving factor for endothelial dysfunction in PMW, this recent literature provides rationale to study the changes that occur in other hormones and how those might impact endothelial function in women.

2.4.4.3 Potential Mechanisms Impacting Endothelial Function in PMW

The decline in endothelial function in PMW has been attributed to decreases in E2 (Harlow et al., 2012) which has been linked to endothelial function through NO production (Somani et al., 2019; Stanhewicz et al., 2018). However, it remains unclear which mechanistic pathways are impacted by this decline in endogenous E2. There is evidence that ET-1 plays a significant role in endothelial function in PMW (Kuczmarski et al., 2020; Kuczmarski et al., 2021; Sebzda et al., 2018; Shoemaker et al., 2021; Wenner & Moreau, 2019; Wenner et al., 2017). It has also been shown that the endothelin B (ETB) receptor may mediate vasodilation in young, healthy women but not in PMW with endothelial dysfunction (Shoemaker et al., 2021; Wenner et al., 2017). Further, it has been concluded that ETB receptor function is regulated by E2 (Shoemaker et al., 2021). It is likely that the function or abundance of the ETB receptor is a driving force for endothelial dysfunction in PMW with diminished E2 especially considering PMW show lower endothelial cell expression of ETB (Kuczmarski et al., 2020). Greater circulating E2 (Polderman et al., 1993) and hormone replacement in PMW have also been shown to decrease plasma levels of ET-

1 (Best et al., 1998; Wilcox et al., 1997). While these results are promising, this is one of many different pathways that is impacted by changing levels of E2 with menopause.

Oxidative stress is also implicated in declines in endothelial dysfunction in postmenopausal women. It has been hypothesized that the loss of E2 during menopause impairs the redox balance, leading to an overall increase in oxidative stress that can cause vascular endothelial dysfunction (Hildreth et al., 2014; Moreau et al., 2020). This is due to the ability of E2 to act as a scavenger of ROS (Keaney Jr et al., 1994; Wagner et al., 2001) and to increase mitochondrial production of antioxidants (Bellanti et al., 2013; Stirone et al., 2005). Endurance exercise training is often proposed as a method of mitigating oxidative stress (Man et al., 2020). This is mainly attributed to the change in redox balance that occurs with regular exercise (Dawson et al., 2013; McArdle & Jackson, 2000). As would be expected, regular physical activity in this population might protect from some vascular dysfunction caused by oxidative stress (Moreau et al., 2007; Moreau et al., 2006). However, the protection from oxidative stress from exercise occurs in women differently than in men (Moreau & Ozemek, 2017; Seals et al., 2019). In PMW completing an exercise training program, improvements in endothelial function are only present in women treated with E2, likely through an oxidative stress-related mechanism (Moreau et al., 2013). Thus, any changes in endothelial function that occur because of oxidative stress in PMW are likely attributed to the loss of E2, adding redox balance as a potential mechanism that impacts endothelial function in PMW.

The RAS is another system that has been extensively researched due to the effects of the vasculature as they pertain to sex hormones. E2 has been known to inhibit Ang II, while testosterone is associated with increased circulating Ang II and renin (necessary to form Ang II) in animal models (Chen et al., 1992; Ellison et al., 1989). Vascular responsiveness to Ang II is less sensitive in female animal models and women. Brosnihan et al. (1997) found that E2 replacement in female rat models of hypertension attenuated the pressor response, decreased ACE activity, and decreased circulating Ang II while increasing Ang-(1-7) concentration in the plasma (Brosnihan et al., 1997). These results suggest E2 can cause a shift in the RAS by downregulating the side that increased CV dysfunction and increasing activity in the cardioprotective side of the RAS. The following section of this review will provide greater detail on this arm of the RAS.

2.5 Vasoprotective Arm of the RAS

There is a lesser-known side of the RAS that has gained attention in the past 20 years due to its therapeutic potential as a vasodilator. This dilator effect is mainly due to the actions of Angiotensin-(1-7) (Ang-(1-7)) (Ferreira & Santos, 2005; Walkyria O. Sampaio et al., 2007; Walkyria Oliveira Sampaio et al., 2007; Santos, 2014; R. A. Santos et al., 1992; Silveira et al., 2013). Ang-(1-7) is known to counter regulate the deleterious effects of Ang II such as inflammation, vasoconstriction, proliferation, and increased oxidative stress. Ang-(1-7) also acts independently to induce vasodilation and improve endothelial function via NO-mediated mechanisms. Figure 2.4 depicts the full RAS with an emphasis of the balance between the vasoprotective arm of the RAS with the classical view of the RAS.

2.5.1 Angiotensin-(1-7)

Ang-(1-7) can be formed through two different pathways. Most commonly, Ang-(1-7) is formed through the conversion of Angiotensin-II through the ACE-2 enzyme and the conversion from Ang I through the enzymes neprilysin (NEP), thimet oligopeptidase (TOP), and prolyl oligopeptidase (POP) (Ferreira & Santos, 2005; Fischer, 2002). Ang-(1-7) acts through the Mas receptor and leads to vasodilatory and anti-inflammatory pathways. Ang-(1-7) has been interpreted as having opposite effects of Ang-II (Roks et al., 1999) though newer research has suggested that Ang-(1-7) may also work through pathways independent of Ang-II (Brosnihan et al., 1996; Fernandes et al., 2001; Heitsch et al., 2001; Medina & Arnold, 2019; Walkyria Oliveira Sampaio et al., 2007; Yang et al., 2018).

2.5.1.1 Receptors for Ang-(1-7)

The Mas receptor has been isolated as the G-protein coupled receptor (GCPR) that mediates Ang-(1-7)-induced dilation (Walkyria Oliveira Sampaio et al., 2007). Treatment with Ang-(1-7) combined with A-779 (a Mas receptor blocker) has blunted vasodilation in rat hearts (Giani et al., 2007) and Mas-knockout mice are nonresponsive to Ang-(1-7)-induced dilation (Botelho-Santos et al., 2012; Pinheiro et al., 2009). While Ang-(1-7) mainly acts through Mas_R, blockade of AT-1 and AT-2 has blocked some vasodilatory actions of Ang-(1-7) (Albrecht, 2007; Almeida-Santos et al., 2016; Bürgelová et al., 2002). Ang-(1-7) has also been known to interact with ACE, given that some studies have found increased levels of Ang-(1-7) following ACE inhibition, though it is still unclear exactly what this interaction does (e Silva & Teixeira, 2016; Tom et al., 2003). Further, blockade of the bradykinin (B2) receptor blocks the vasodilatory actions of Ang-(1-7) (Albrecht, 2007; Brosnihan et al., 1996; Li et al., 1997).

There is evidence of a second GPCR compatible with Ang-(1-7), called Mas-related G protein-coupled receptor (MrgD). Studies using MrgD animal knockout models have concluded MrgD also plays a role in mediating the vasoprotective actions of Ang-(1-7) (Tetzner et al., 2016). It has also been suggested Ang-(1-7) may also interact with AT2 (Mansoori et al., 2016). However, Tetzner et al. (2016) using a model of Mas_R and MrgD transfected cells with AT2 receptor blocker, PD123319, found that Ang-(1-7) did not interact with AT2, but that PD123319 can also block Mas_R and MrgD by blocking vasodilatory actions of Ang-(1-7).

2.5.1.2 Vascular Actions of Ang-(1-7)

There are several intracellular signaling pathways by which Ang-(1-7) impacts vascular function. These can be classified as actions on the endothelium or as actions on the VSMCs. There is evidence that Ang-(1-7) production is highly dependent on the endothelium (R. A. Santos et al., 1992). Ang-(1-7) mediates vasodilation via NO-dependent pathways. Through the Mas receptor located on endothelial cells, Ang-(1-7) contributes to the activation of eNOS via phosphorylation of Ser1177 (Walkyria Oliveira Sampaio et al., 2007). MAPKs such as ERK-1/2, p38, and JNK act as mediators in cell proliferation, fibrosis, and cell remodeling. Ang-(1-7) inhibits these MAPKs, thus decreasing their harmful effects (Tallant & Clark, 2003). Antioxidative effects of Ang-(1-7) in the vasculature have also been well documented. Ang-(1-7) is key in blocking the formation of ROS in the endothelium. Ang II contributes to the formation of ROS via NADPH and the phosphorylation of c-Src (Walkyria O. Sampaio et al., 2007). Ang-(1-7) through Mas_R blunts this response through the phosphorylation of SHP2. SHP2 then blocks c-Src from contributing to the formation of ROS.

Animal models have detailed many of the effects and actions of Ang-(1-7) (Botelho-Santos et al., 2012; Brosnihan et al., 1996; Giani et al., 2007; Ren et al., 2002; Sobrino et al., 2017; Zhang et al., 2019). Ang-(1-7) improves endothelial-mediated vasodilation in many animal studies that have detailed the ability of Ang-(1-7) to contribute to signaling pathways for bradykinin (Fernandes et al., 2001), prostaglandins (Andreatta-van Leyen et al., 1993), and the NO pathway (Walkyria Oliveira Sampaio et al., 2007; Santos et al., 2018). Ang-(1-7) induces NO-mediated dilation in canine coronary artery rings (Brosnihan et al., 1996). This dilation is most likely driven by NO in the endothelium as indicated by a lack of Ang-(1-7)-induced dilation in rings with denuded endothelium or that have been treated with the eNOS inhibitor, L-NAME, thus blocking endothelial NO production (Brosnihan et al., 1996). Zhang et al. (2019) has more recently used an animal model to determine the utility of Ang-(1-7) in hypertension. Using Spontaneously hypertensive rats (SHR) and Wistar Kyoto rats (WKR) as a control group, they intravenously infused Ang-(1-7) into the rats and showed increased arterial dilation in the SHR similar to that of WKR. These results suggest the utility of Ang-(1-7) in a hypertensive model (Zhang et al., 2019).

While human studies involving Ang-(1-7) are less extensive than those in animal models, it has been confirmed that Ang-(1-7) causes vasodilation in humans, especially those in a diseased state. Schinzari et al. (Schinzari et al., 2018) found increased forearm blood flow in obese subjects following intra-arterial perfusions of Ang-(1-7). Sasaki et al. (Sasaki et al., 2001) was the only study to find Ang-(1-7)-mediated vasodilation in healthy subjects, though while this dilation was significant from baseline, it was not as great as that in hypertensive men in that study. This was also the only study performed in humans that infused multiple doses of Ang-(1-7). $1x10^{-10}$ mol/min did not cause significant dilation in either normotensive or hypertensive men, $1x10^{-9}$ mol/min caused significant dilation in both groups, though greater in hypertensive men. $1x10^{-8}$ mol/min caused significant dilation, similar in both groups. This finding of differences in the magnitude of dilation leads to further questions about the dose necessary to cause dilation. If it is true that different doses of Ang-(1-7) cause dilation at different magnitudes, it is important to test a range of doses to find those that are most effective to determine the underlying differences in magnitude of dilation between subject groups. Finally, this study only included men (Sasaki et al., 2001) and thus we cannot use these results to make conclusions on the actions of Ang-(1-7) on the vasculature within the general population. Further, the evidence that Ang-(1-7) interacts with estrogen provides further rationale that this pathway should be studied in women. These mechanisms are explained in greater detail in section 2.5.1.3.

While there is a consensus that Ang-(1-7) is a vasodilator, there have been some studies that have not seen this vasodilation in humans. Davie & McMurray et al. (Davie & McMurray, 1999) did not see any vasodilation in heart failure patients being treated with an ACE inhibitor. However, given the actions of ACE inhibitors and their effect on the conversion of Ang-II to Ang-(1-7) it is possible that the ACE inhibitors blocked the Ang-(1-7) production. Both Ueda et al. (Ueda et al., 2000) and Wilsdorf et al. (Wilsdorf et al., 2001) found no change in forearm blood flow with intra-arterial perfusions of Ang-(1-7) in healthy men. This is of interest and in line with other literature on Ang-(1-7) microdialysis perfusions of Ang-(1-7) in women (Stanhewicz

& Alexander, 2020; Stewart et al., 2009) who found increased vasodilation in diseased women, but not in healthy controls. It is likely that Ang-(1-7) may only improve dilation in diseased populations.

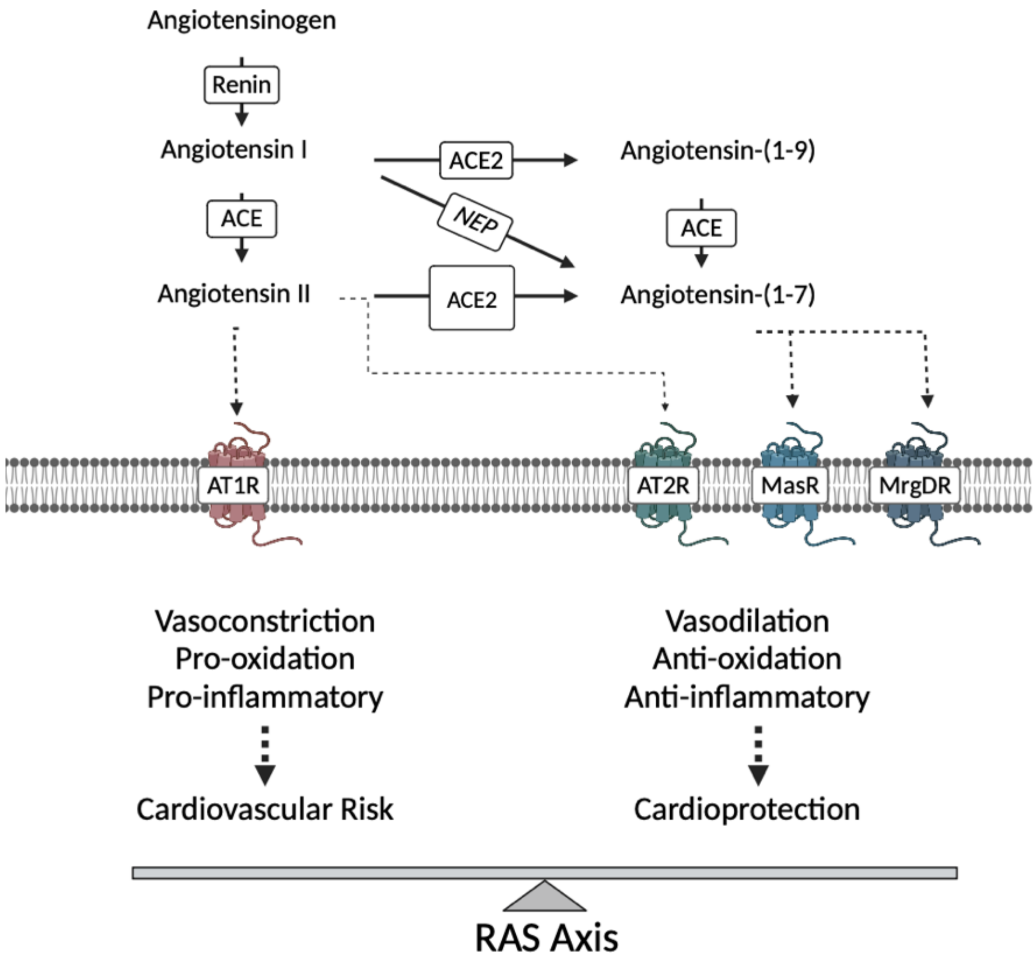


Figure 2.5 Signaling Pathway of the Renin Angiotensin System. Ang II through the AT1 receptor causes actions that increase cardiovascular risk. Ang-(1-7) through the MasR contributes to cardioprotection. Actions of Ang II through the AT2 receptor and Ang-(1-7) through the MrgD also cause cardioprotective effects. (Adapted from Medina et al. (Medina et al., 2020).

2.5.1.3 Ang-(1-7) Interactions with Estrogen

Currently, there is a lack of knowledge surrounding menopause and the mechanisms contributing to the decline in endothelial function seen during this time. It has been suggested that the RAS may play a role in this decline. Most research has focused on the actions of Ang II on endothelial function. Data from animal models have renewed interest in Ang-(1-7), especially for its potential to improve CVD health in women. Within the current literature studying animal models of menopause, there is evidence of a crosstalk between Ang-(1-7) and E2 that may have implications in menopause.

Neves et al. (Neves et al., 2004) was among the first to establish a relationship between Ang-(1-7) and E2 in animal models. Isolated mesenteric arteries from female rats with and without E2 replacement, and OVX rats (serving as the model for menopause) and control rats were treated with Ang-(1-7) during different phases of the estrous cycle. OVX rats had significantly blunted dilation in response to Ang-(1-7) that was restored with E2 replacement. Also, during the high estrogen phase, there was much greater dilation in the rats than during the low estrogen phase. Further, Mompeon et al. (Mompeón et al., 2016) showed that exposure to E2 increased Ang-(1-7) production in cultured human umbilical vein endothelial cells (HUVECs). This increased production was found to be through the ERα receptor. Saberi et al. (Saberi et al., 2016) found increased serum NO metabolites (nitrite) in E2 treated OVX rats following Ang-(1-7) administration that was reversed in the presence of A779. Similarly, bradykinin administration with Ang-(1-7) increased serum nitrite in E2 treated OVX rats and was again reversed in the presence of A779. Renal blood flow (RBF) is increased in response to Ang-(1-7) infusion in OVX female rats. This was confirmed to be through an NO-dependent mechanism because when L-NAME was also infused, this effect was blunted (Dehghani et al., 2015).

More recently, Costa-Fraga et al. (Costa-Fraga et al., 2018) used animal models of both menopause and aging and compared sex differences in vascular function in response to Ang-(1-7). Aortic rings were taken from younger and older female mice, younger OVX mice, and younger and older male mice to be treated with Ang-(1-7). Older female mice showed decreased vasoreactivity to Ang-(1-7) compared to young female mice. This effect was not seen in male mice as the vasodilatory response to Ang-(1-7) was minimal in male mice and there were no differences in Ang-(1-7) induced dilation between young and old male mice. It is unclear as to why the male mice did not respond to Ang-(1-7) in this study, given that in human studies, men do show a vasodilatory response to Ang-(1-7) (Sasaki et al., 2001). However, when the aortic rings of the older female mice were also treated with E2, vasodilation was the decreased vasodilation was reversed in the older female mice beyond that of the young female mice, suggesting the aging effect of Ang-(1-7) on the vasculature is through an E2-specific mechanism. To further test this, the same experiments were performed on young female mice models of menopause. Aortic rings from OVX female mice showed no vasodilation, like that of the older female mice. However, when treated with E2, the OVX female mice showed vasodilation similar to the healthy young female mice (Costa-Fraga et al., 2018). These results further suggest that E2 plays a key role in Ang-(1-7)-induced vasodilation.

Costa Fraga et al. (Costa-Fraga et al., 2018) performed western blots of Mas_R expression in samples taken from the abdominal aorta in young female mice, old female mice, and old female mice treated with E2. The old female mice had a significantly higher expression of Mas_R than the young female mice which was rescued in the old female mice treated with E2. The authors indicated that this could be a failed compensation due to a decreased vascular sensitivity to Ang-(1-7) with aging. Other research also indicates that plasma levels of Ang-(1-7) decrease with age (Miller & Arnold, 2023), which could further lead to an upregulation of receptors to compensate for the lack of bioavailable substrate.

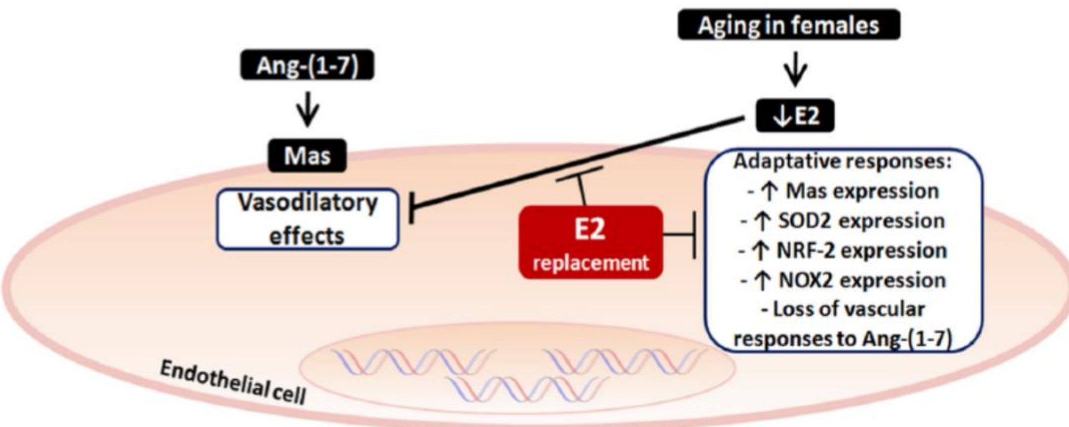


Figure 2.6 Proposed mechanism of the impact of aging and menopause on the vasodilatory effects of Ang-(1-7) on the vasculature. The Ang-(1-7). Mas_R axis promotes NO-mediated vasodilatory effects that can be cardioprotective. However, with aging and menopause there is a decline in bioavailable E2 that has been attributed with a loss of the vasodilatory effects of Ang-(1-7). (Costa Fraga et al. 2018).

Two studies have detailed Ang-(1-7)-mediated dilation in women. However, they were both in diseased populations. Stewart et al. (Stewart et al., 2009) found that microdialysis perfusions of 100 umol/min of Ang-(1-7) improved vasodilation in the microvasculature of women with Postural Tachycardia Syndrome (POTS) but had no effect on the microvasculature in healthy controls. Stanhewicz & Alexander

(Stanhewicz & Alexander, 2020) found increased vasodilation during Ach perfusion in the microvasculature of women with a history of preeclampsia but no differences in the dilation in controls who had a healthy pregnancy. These results together suggest Ang-(1-7) can cause improvements in endothelial function in women with preclinical CVD but does not impact endothelial function in healthy young women.

Descriptive research has also suggested Ang-(1-7) might aid in the protective effect on the vasculature in young women (Sullivan et al., 2015). Sullivan et al. (Sullivan et al., 2015) measured plasma levels of Ang-(1-7) in young men and young women and found that the group of young women (49.5$\pm$4.4 fmol/mL) had greater concentration of plasma Ang-(1-7) than the men (35.9$\pm$3.1 fmol/mL). Furthermore, plasma Ang-(1-7) concentration was associated with greater FMD %, a measure of macrovascular endothelial function, indicating a potential sex difference and relationship between circulating Ang-(1-7) and endothelial function (Sullivan et al., 2015). Interestingly, there were no sex differences in plasma Ang II in this study, and Ang II did not display a relationship with endothelial function in either young men or women this study. These results further suggest that endothelial function may be driven by the ratio of Ang-(1-7) to Ang II, rather than Ang II expression as has previously been believed (Sullivan et al., 2015). They hypothesized that E2 may lead to an increase in NEP within the vasculature, thus increasing Ang-(1-7) formation. This has not yet been confirmed as the mechanism for this association but should be further examined.

2.5.1.4 $\mathbf{Mas_R}$ Interactions with Estrogen

There are currently disagreements within the literature as to how Mas_R responds to states of disease and dysfunction. Costa-Fraga et al. (2018) found Mas_R

expression is greater in older female mice due to a hypothesized failed upregulation to compensate for dysfunction within the Ang-(1-7)/Mas_R pathway. This upregulation hypothesis is confirmed in models of cardiomyocyte apoptosis (Chang et al., 2018) and cerebral ischemic stroke (Lu et al., 2013) in the diseased tissue. However, some other research has suggested there is decreased Mas_R expression in animal models with increased CVD risk and vascular dysfunction. In models of left ventricular hypertrophy and myocardial infarction in WKR, Dias-Piexoto et al. (Dias-Peixoto et al., 2012) found that western blots of Mas_R in the animal hearts showed decreased Mas_R expression compared to the control. research is necessary to elucidate the presence of Mas_R with aging, and whether an up or downregulation exists with increasing cardiovascular risk. Furthermore, it remains unknown what mechanisms would be driving the over or under expression of Mas_R.

Interestingly, using cell culture and *ex vivo* models, Sobrino et al. (Sobrino et al., 2017) confirmed the role of Mas_R in NO-mediated vasorelaxation influenced by E2. Blockade of Mas_R and ER together was successful in blocking the ability of E2 to cause vasorelaxation. They exposed HUVECs to E2 in the presence of Mas_R blocker & estrogen receptor antagonist. E2 was shown to increase in eNOS mRNA and eNOS activity (via phosphorylation of Ser1177). This was not seen in the presence of Mas_R antagonist or an estrogen receptor antagonist. This study also included *ex vivo* experiments to test vasorelaxation in preconstricted (noradrenaline) mesenteric vessels in response to E2. Vasorelaxation was blocked similarly in wild type vessels treated with estrogen receptor blockade alone, Mas_R blockade alone, estrogen receptor blockade + Mas_R blockade combined, and in Mas_R deficient mice. (Sobrino et al., 2017), suggesting blockade of estrogen receptor or Mas_R elicits the same blunting of the vasodilatory effects of E2. Furthermore, Costa Fraga et al. (Costa-Fraga et al., 2018) found that E2 treatment in older mice reversed the upregulation of Mas_R seen with aging. This suggests E2 as well as aging plays a role in expression of Mas_R (Figure 2.6. More work is necessary to determine if E2 impacts the function of the receptor as well as the expression, and whether this is due to a loss of vascular sensitivity to Mas_R, or a decrease in bioavailable Ang-(1-7).

2.5.2 Ang-(1-7) Outside the Vasculature

Outside the vasculature, Ang-(1-7) acts within several different organ systems. The primary organ Ang-(1-7) impacts is the kidney. Though the actions of Ang-(1-7) in the kidney are highly variable and mainly depend on the specific mechanism, disease state, or subject model. For instance, Ang-(1-7) has an antidiuretic effect in water loaded rats (Baracho et al., 1998; Santos & Baracho, 1992) but a natriuretic effect in isolated rat kidneys (DelliPizzi et al., 1994; Hilchey & Bell-Quilley, 1995), hypertensive rats (Benter et al., 1995), anesthetized rats (Handa et al., 1996; Vallon et al., 1998) anesthetized dogs (Heller et al., 2000). Interestingly, Ang-(1-7) opposes the actions of Ang II in the kidney by decreasing Ang II-mediated vasoconstriction of renal arteries in rat models (van der Wouden et al., 2006), decreasing Ang II mediated phosphorylation of p38, ERK ½, and JNK (Su et al., 2006), inhibits Ang II stimulated release of NE in rat models (Stegbauer et al., 2004; Stegbauer et al., 2003), slows the Ang II decrease in Na+ excretion (Bürgelová et al., 2002). In human *in vitro* studies measuring renal blood flow via angiography, Ang-(1-7) causes vasodilation within the kidneys (Twist et al., 2013; van Twist et al., 2016; Van Twist et al., 2014), decreases LDL-induced lipid deposition (Huang et al., 2014), and decreases podocyte injury (Tian et al., 2014), and *in vivo*, increases renal blood flow in hypertensive subjects (van Twist et al., 2016). Generally, however, most literature agrees that Ang-(1-7) increases renal blood flow (Maleki & Nematbakhsh, 2016; Mori et al., 2014; Saberi et al., 2016; Sampaio et al., 2003; Wang et al., 2012), decreases oxidative stress in the kidney (Mori et al., 2014; Papinska et al., 2015; Shi et al., 2015; Zheng et al., 2015), decreases proteinuria (Becker et al., 2007; Benter et al., 1995; Benter et al., 2006; Giani et al., 2007; Giani et al., 2011; Singh et al., 2010), and increases sodium excretion and urine osmolality (Becker et al., 2007; Bürgelová et al., 2002; Ferreira et al., 2006) further leading to a hypotensive effect.

Ang-(1-7) is also present in the lungs, liver, brain, and ovary. Within the lung, Ang-(1-7) is anti-inflammatory, anti-fibrotic, and hypotensive (Chen et al., 2013; Li et al., 2015; Ma et al., 2016; Magalhães et al., 2015; Meng et al., 2014; Rodrigues-Machado et al., 2013; Wagenaar et al., 2013). A large role of Ang-(1-7) within the endocrine system is in the pathology of diabetes and insulin resistance. Ang-(1-7) increases phosphorylation of AKT which is known to improve insulin signaling by leading to GLUT translocation (Cao et al., 2014; Giani et al., 2009). In the brain, Ang-(1-7) is important in maintaining the baroreflex and the maintenance of blood pressure in specific brain regions. Ang-(1-7) plays a role in maintaining bradycardia in baroreflex control (Bomtempo et al., 1998; Campagnole-Santos et al., 1992). Finally, Ang-(1-7) can be found in the ovary (Costa et al., 2003; Pereira et al., 2009) and plays a role in stimulating E2 production to improve the efficiency of ovulation (Viana et al., 2011). Ang-(1-7) is also present during pregnancy. In pregnant women, there are higher levels of Ang-(1-7) in the plasma and urine (Chen et al., 2014; Merrill et al., 2002) and low Ang-(1-7) concentrations are associated with preterm births (Chen et al., 2014), preeclampsia (Merrill et al., 2002; Velloso et al., 2007) and gestational diabetes (Nogueira et al., 2007).

2.5.3 ACE2

ACE2 is instrumental in the production of Ang-(1-7), thus a decrease in ACE2 would lead to decreased Ang-(1-7) production. Iwai & Horiuchi (Iwai & Horiuchi, 2009) detailed the balance between ACE and ACE2 activity. ACE leads directly to the formation of Ang II while ACE2 leads to the formation of Ang-(1-7). Therefore, a greater ratio of ACE to ACE2 would lead to greater production of Ang II than Ang-(1-7) and is associated with increased cell proliferation, oxidative stress, and hypertension (Iwai & Horiuchi, 2009).

Ji et al. 2008 studied animal models of hypertension and menopause in OVX female rats. These animals showed a reduction in ACE2 activity and protein expression. However, when treated with E2, this decreased expression and activity is rescued, suggesting menopause and E2 plays a role in the expression and activity of ACE2, and that menopause may be associated with decreased ACE2, leading to decreased Ang-(1-7) production (Ji et al., 2008). To further these findings, Mompeon et al. (Mompeón et al., 2016) showed that when HUVECs were treated with E2 they had an increased ACE2 mRNA expression. The same dose of E2 also caused increased Ang-(1-7) production. The authors hypothesized these results suggest there may be an interplay between E2 and ACE2 with Ang-(1-7) (Mompeón et al., 2016). Though these results have not been confirmed in humans the relationship between E2 and ACE2 provides a potential pathway for study in the changes in Ang-(1-7) production in women following menopause.

2.6 Conclusion

PMW have an increased risk in CVD attributed to the decline in circulating E2 following menopause. While this CVD risk in PMW is well established, the mechanisms contributing to this decline remain unknown. Impaired endothelial function leads to the increased risk of CVD and commonly precede CVD. However, the decline in endothelial function in PMW occurs differently than it does in men, leading to a discrepancy in the literature in the pathology of endothelial function and CVD in women. It is important to identify pathways that might impact endothelial function in women and might be useful targets in the treatment or prevention of CVD in PMW. The RAS has been studied in the past for its effects on the vasculature, but the more recently discovered vasoprotective arm of the RAS led by Ang-(1-7) has the potential to provide a novel target of CVD and endothelial dysfunction in women.

Presented in this review is strong evidence that Ang-(1-7) as a key factor impacting endothelial function in women due to its interactions with E2. Given this evidence and the increased risk of endothelial dysfunction and CVD in PMW, there is a need to elucidate the role of Ang-(1-7) in endothelial function in this population. This pathway may provide a useful target in the prevention and treatment of endothelial dysfunction in PMW. The overall aim of this project was to increase the understanding of the implications of Ang-(1-7) on endothelial function in women and can provide more evidence for this potential therapeutic target in CVD in PMW. Thus, the current project studies the vascular sensitivity to Ang-(1-7), the role of Ang-(1-7) on endothelial function, and the presence of Mas_R in postmenopausal women. Chapters 3-5 detail the results of these experiments.

Chapter 3

VASCULAR SENSITIVITY TO ANGIOTENSIN-(1-7) IN PREMENOPAUSAL AND POSTMENOPAUSAL WOMEN

3.1 Introduction

Postmenopausal women (PMW) experience an increased risk of cardiovascular disease (CVD) around the time of menopause and is more severe than that in men of similar age (El Khoudary et al., 2020). This increase in risk of CVD is largely attributed to the decline in estrogen production following menopause (Jensen-Urstad et al., 1997; Mosca et al., 2011; White, 2002). Estrogen is known to be vasoprotective and have beneficial effects on the endothelium (Somani et al., 2019). However, the specific molecular signaling pathways that are affected by the loss of estrogen remain unclear.

Ang-(1-7) is a vasodilator in the protective arm of the RAS. Ang-(1-7) mainly acts by counter regulating the vasoconstrictive arm of the RAS led by the actions of Ang II (Ferreira & Santos, 2005; Medina & Arnold, 2019; Walkyria O. Sampaio et al., 2007; Ueda et al., 2000). There are also mechanisms where Ang-(1-7) can independently cause vasodilation through pathways related to bradykinin and nitric oxide (NO) (Brosnihan et al., 1996; Fernandes et al., 2001; Heitsch et al., 2001; Walkyria Oliveira Sampaio et al., 2007). Importantly, there is also evidence to suggest that Ang-(1-7) elicits vasodilation in humans in a dose-dependent manner (Sasaki et al., 2001).

Literature suggests Ang-(1-7) is a female-specific mechanism that offers protective effects on the vasculature in young, premenopausal women (YW). It has also been suggested that Ang-(1-7) may interact with estrogen (Costa-Fraga et al., 2018; Neves et al., 2004). Importantly, Costa Fraga and colleagues (2018) conducted a study in animal models that suggests the vasodilatory response caused by Ang-(1-7) is impacted by menopause and aging in females (Costa-Fraga et al., 2018). In a mouse model of menopause and aging, it was found that both ovariectomized female mice, with induced menopause, and older female mice showed decreased vascular sensitivity to Ang-(1-7) compared with healthy, young female mice. However, the vasodilatory response to Ang-(1-7) was enhanced in the presence of estrogen, suggesting estrogen may be necessary to induce the vasodilatory actions of Ang-(1-7). Notably, both young and old male mice did not show any differences in Ang-(1-7)-dependent dilation, further suggesting this is a female-specific phenomenon (Costa-Fraga et al., 2018).

Thus, it is possible that the loss of estrogen with menopause causes a blunted vascular sensitivity to Ang-(1-7). This would cause diminished ability of the vasculature to respond to Ang-(1-7) and could thus lead to some of the pathologies that lead to CVD. Further, considering Ang-(1-7)-induced dilation is NO-dependent as Ang-(1-7) does not induce dilation during NO blockade (Brosnihan et al., 1996), reduced vascular sensitivity to Ang-(1-7) would likely reduce this NO-dependent dilation. Accordingly, the objective of this study was to determine whether Ang-(1-7) can induce dilation in the microcirculation of YW and PMW, and to determine if the vascular sensitivity to Ang-(1-7) is impacted by aging and menopause in women. We tested the working hypothesis that Ang-(1-7) would induce vasodilation in both YW and PMW, but that this dilation would be attenuated in PMW, suggesting reduced vascular sensitivity to Ang-(1-7) in women after menopause.

3.2 Methods

3.2.1 Participants

10 YW (22±3 yrs) and 9 PMW (55±4 yrs) completed the protocol. YW were all premenopausal and tested during the early follicular phase of the menstrual cycle or during the placebo week if using OCPs (n=3). PMW were all between ages 50-65 and were at least 1 year after their final menstrual period (self-report). PMW were also excluded if they were using hormone replacement therapy. Institutional Review Board approval was obtained from the University of Delaware and all protocols adhered to the standards of the Declaration of Helsinki. All participants gave verbal and written informed consent prior to any participation in the study. A screening visit was then completed after obtaining consent for all participants. Screening included measurement of resting BP (Dinamap DASH 2000; GE Medical, Chicago, IL), height and weight to determine BMI, and a 12h fasted blood draw for clinical labs. Self-reported physical activity data were also collected by having all participants complete a Modified Activity Questionnaire (MAQ) (Pereira et al., 1997). All study visits occurred in the same laboratory setting to provide a controlled environment for data collection.

3.2.2 Microdialysis and Laser Doppler Flowmetry

The cutaneous microcirculation has been used as a model to study mechanistic changes in vascular function in humans (Debbabi et al., 2010; Holowatz, 2010). Microdialysis perfusions to the cutaneous circulation are also less invasive than intravenous infusions and provide a more isolated response to a stimulus (Holowatz et al., 2008). Previous studies in humans have used microdialysis perfusions coupled with laser Doppler flowmetry in the cutaneous circulation to study Ang-(1-7) (Stanhewicz & Alexander, 2020; Stewart et al., 2008).

To prepare the skin for microdialysis fiber insertion, the skin on the dorsal side of the forearm was examined to mark needle insert and exit points for each site. Sites were away from visible veins and were at least 1 inch apart from each other with the insert and exit for each site measured to be 2cm apart. The skin was then sterilized, and ice was placed on the skin for 10 min to decrease pain. Two needles were inserted into the intradermal layer of the skin about 2-5mm below the surface of the skin within the cutaneous microcirculation. Microdialysis fibers (CMA 31 Linear, Harvard Apparatus, Holliston, MA) were threaded through the needles. Needles were removed, leaving the porous membrane of the fiber in the skin. The porous membrane has a molecular weight cutoff at 55-kDa to allow smaller substances to pass through the pores and act within the local microcirculation. Heaters and laser Doppler probes (MoorLab, Temperature monitor SH02; Moor Instruments, Devon, UK) were placed on top of each site. Skin temperature was maintained at 32ºC and lactated Ringers was perfused through each fiber at a rate of 2 µl/min using a syringe pump (Bee Hive controller and Baby Bee microinfusion pumps, Bioanalytical Systems, West Lafayette, IN) for 1 hour to allow for recovery from a hyperemic response.

3.2.3 Ang-(1-7) dose-response protocol

Doses used during this study were chosen based on pilot testing in our laboratory and on previous studies (Stanhewicz & Alexander, 2020; Stewart et al., 2009). The doses used for the current study were $1x10^{-2}$ M, $1x10^{-2.125}$ M, $1x10^{-2.25}$ M,

$1x10^{-2.5}$ M, $1x10^{-2.625}$M, $1x10^{-2.75}$M, $1x10^{-3}$ M, $1x10^{-4}$ M, $1x10^{-5}$ M. Ang-(1-7) (Tocris Biosciences, Bristol, UK) was dissolved in lactated Ringer's solution in each concentration as serial dilutions. For a subset (8 YW, 7 PMW), the same doses were dissolved in lactated Ringer's containing 15mM L-NAME. All drugs were sterilized through a 0.2µm syringe microfilter (Whatman Puradisc 13 mm Syringe filters, Florham Park, NJ).

Following recovery from the hyperemic response, there was a 10-min long baseline period while perfusion of lactated Ringers continued, and blood pressures were taken at the final minute. Each dose was perfused in the order of increasing concentration for 5 minutes each at 2 µL/min while skin temperature was maintained at 32ºC. Following perfusion of all doses, lactated Ringers was perfused in both sites to flush remaining drug in the fiber, then skin temperature was increased to 43ºC and the NO donor, sodium nitroprusside (SNP) was perfused until maximal dilation was reached. Brachial artery blood pressures were measured at the final minute of each dose perfusion and each minute during SNP perfusion. Representative tracings from this protocol can be found in Figures 3.1 and 3.4.

3.2.4 Data Analysis

Physical activity data were calculated as moderate-to-vigorous intensity physical activity (MVPA) in hours/week from the results of the MAQ. Cutaneous blood flow data were taken as an average from the final minute of perfusion of each dose. Brachial artery blood pressures from the corresponding time were used to calculate cutaneous vascular conductance (CVC) which was determined as red blood cell flux/mean arterial pressure. All data was normalized to maximal vasodilation during SNP perfusion and expressed as CVC%max by calculating (CVC/CVCmax)*100.

3.2.5 Statistical Analysis

A power analysis was performed based on pilot data from our lab, suggesting a difference of 15 $\%CVC_{max}$ in YW vs PMW, and previous data analyzing dose response curves in PMW vs YW (Kuczmarski, 2021) and determined sample size of 8 per group was needed to detect large effect of 1.75, with power of 80% and alpha set to $P < 0.05$. Participant characteristics were compared between groups using unpaired t tests. A two-way repeated measures ANOVA with Sidak multiple comparisons test was used to compare differences in dilation between groups at all doses. Using CVC%max of each data point, pharmacological curves were fit for YW and PMW (Wenner, Wilson, et al., 2011). Curves were fit as a sigmoidal curve with a variable slope represented by the equation: Y = Bottom + (Top-Bottom)/1+10(LogED50-X)Hill Slope. Top (maximum) represent y at the top of the plateau, or maximal dilation. Bottom (minimum) represent y at the bottom of the plateau or the minimal dilation. LogED50 is the x value when the response is halfway between the bottom and top. Hillslope is the slope or steepness of the curve that will represent the robustness of the vasodilatory response to Ang-(1-7). To calculate NO contribution, area under the curve (AUC) was determined for each individual participant in both Ang-(1-7) and Ang-(1-7)+L-NAME sites. NO contribution was determined by using AUC for each subject for Ang-(1-7) and L-NAME+Ang-(1-7). NO contribution was then calculated as Ang-(1-7) AUC – Ang-(1-7)+L-NAME AUC and was compared between groups using an unpaired t test. Finally, we performed Pearsons Correlations between maximal vasodilation (a measure of the robustness of the vasodilatory response to Ang-(1-7)) and AUC (a measurement of the full vasodilatory response to Ang-(1-7)) with total cholesterol, HDL cholesterol, LDL cholesterol, glucose, BMI, SBP, DBP, and MVPA. Statistics were completed using Graphpad Prism.

3.3 Results

As expected, PMW were significantly older than YW. They also had greater DBP, MAP, total cholesterol, HDL cholesterol, and glucose (Table 3.1).

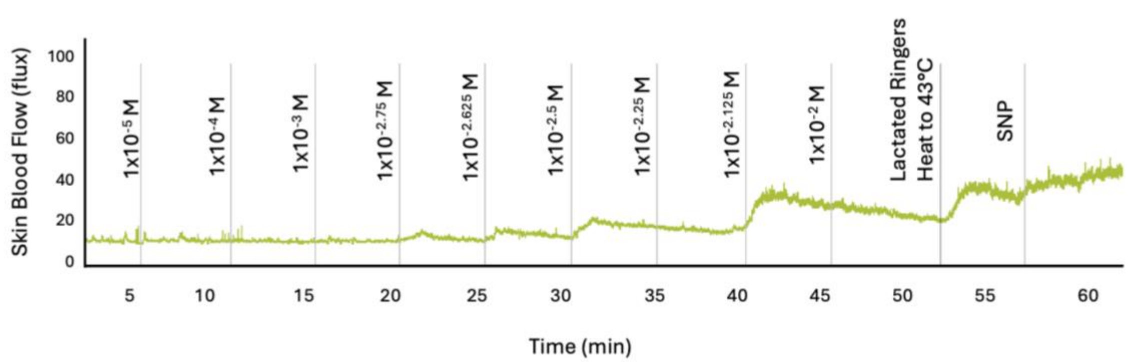
	YW (n = 10)	**PMW** (n = 9)
Age, yrs	22 ± 3	55 ± 4 *
BMI, kg/m^2	23 ± 3	25 ± 3
SBP, mmHg	108 ± 7	113 ± 10
DBP, mmHg	65 ± 5	72 ± 7 *
MAP. mmHg	80 ± 5	86 ± 8 *
Total Cholesterol, mg/dL	170 ± 31	199 ± 35 *
HDL, mg/dL	61 ± 11	73 ± 8 *
LDL, mg/dL	94 ± 26	112 ± 34
Glucose, mg/dL	87 ±6	92 ±10 *
MVPA (hrs/week)	4.85 ± 2.34	4.10 ± 4.43

Table 3.1 Participant characteristics. Values are mean±SD. YW, young women; PMW, postmenopausal women; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; HDL, high-density lipoprotein; LDL, low-density lipoprotein; MVPA, moderate-to-vigorous physical activity. Groups were compared using unpaired t-tests. **P*<0.05 vs. YW.

Ang-(1-7) induced dilation in both groups starting at $1x10^{-2.5}$ M (main effect for dose, P< 0.001). Post hoc analysis showed significant vasodilation compared to baseline at $1x10^{-2.5}$ M (P=0.03), $1x10^{-2.25}$ M (P<0.001), $1x10^{-2.125}$ M (P<0.001), and

1x10^{-2} M (P<0.001). Dilation remained significantly elevated from the first dose (1x10^{-5} M) at 1x10$^{-2.5}$ M (P=0.03), 1x10$^{-2.25}$ M (P<0.001), 1x10$^{-2.125}$ M (P<0.001) and 1x10^{-2} M (P<0.001). Compared to the second dose (1x10^{-4} M), dilation was significantly elevated at 1x10$^{-2.25}$ M (P=0.01), 1x10$^{-2.125}$ M (P=0.01), and 1x10^{-2} M (P<0.001). Compared to the third dose (1x10^{-3} M) dilation was significantly elevated at 1x10$^{-2.25}$ M (P=0.03), 1x10$^{-2.125}$ M (P=0.02), and 1x10^{-2} M (P<0.001). Compared to the fourth dose (1x10$^{-2.75}$ M), only 1x10^{-2} M showed significant dilation (P<0.001). Compared to the fifth dose (1x10$^{-2.65}$ M) dilation was significantly elevated at 1x10$^{-2.25}$ M (P=0.03), 1x10$^{-2.125}$ M (P=0.01), and 1x10^{-2} M (P<0.001). The final dose was also significantly different compared to 1x10$^{-2.5}$ M (P<0.001), 1x10-$^{2.25}$ (P=0.03), and 1x10$^{-2.125}$ M (P=0.04). However, there was no main effect for group (P=0.94) or an interaction effect (P=0.89). Dose response data in the Ang-(1-7) site was fit to a sigmoidal curve as previously described (Figure 3.2 (Wenner, Wilson, et al., 2011). Parameters of the curve for each group are found in Table 3.2. There were no differences between groups in any parameters of the curves (P= 0.614). Individual responses to Ang-(1-7) perfusions can be found in figure 3.3.

A.



B.

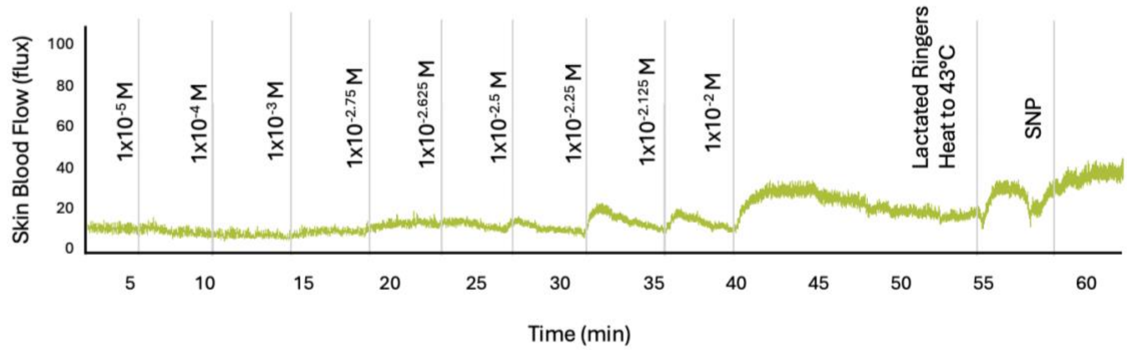


Figure 3.1 Representative Tracing of Ang-(1-7) Dose Response protocol in (A) 1 young woman and (B) 1 postmenopausal woman. 0-5 minutes baseline measurement (perfusion of lactated Ringers at 32°C). Gray lines represent perfusion of new dose. All doses were perfused at 32°C. Following perfusion of all doses lactated Ringers was perfused, and skin temperature was set to 43°C. To achieve maximal dilation sodium nitroprusside (SNP) was perfused at 43°C.

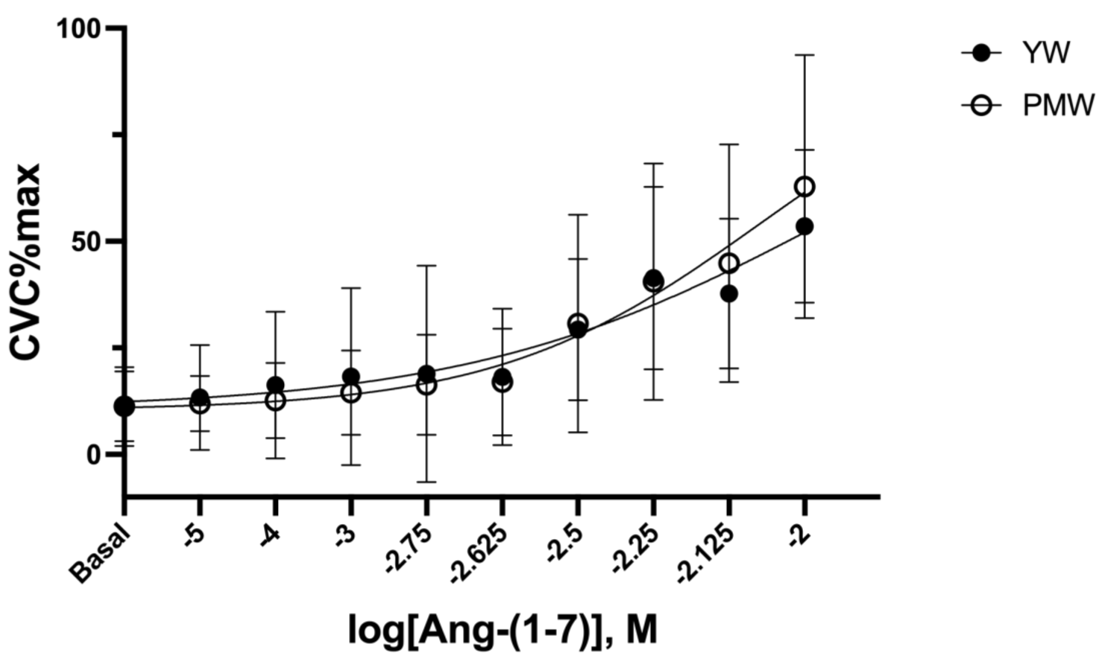
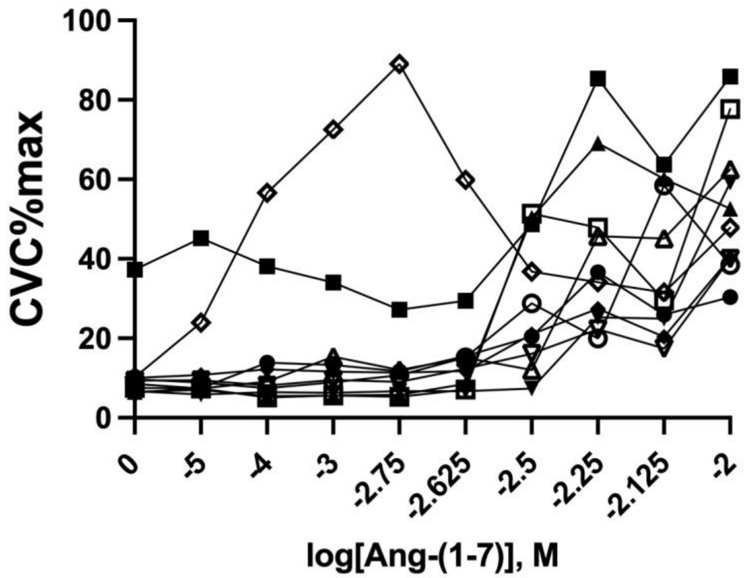


Figure 3.2 Ang-(1-7) Dose response curves in young women (YW; n=10; closed circles) and postmenopausal women (PMW; n=9; open circles). Data are means+SD of vasodilation (cutaneous conductance, %max dilation) responses to Ang-(1-7) perfusions in YW and PMW.

	YW (n=10)	**PMW (n=9)**
Top	99.93 ± 214.56	97.58 ± 100.10
Bottom	10.57 ± 9.36	10.33 ± 5.75
LogED50	9.339 ± 10.5	8.394 ± 3.96
HillSlope	0.181 ± 0.237	0.251 ± 0.207
AUC	73.76 ± 19.83	67.35 ± 12.01

Table 3.2 Parameters of Ang-(1-7) dose response curves in YW and PMW determined from fitting to sigmoidal curve with variable slope. Top: y value at top of plateau; Bottom: y value at bottom of plateau; LogED50: x value when response is halfway between bottom and top; Hillslope: slope of curve; AUC: area under the curve. Slopes P>0.05 for all parameters. Values are mean for each respective curve + SE.

A.



B.

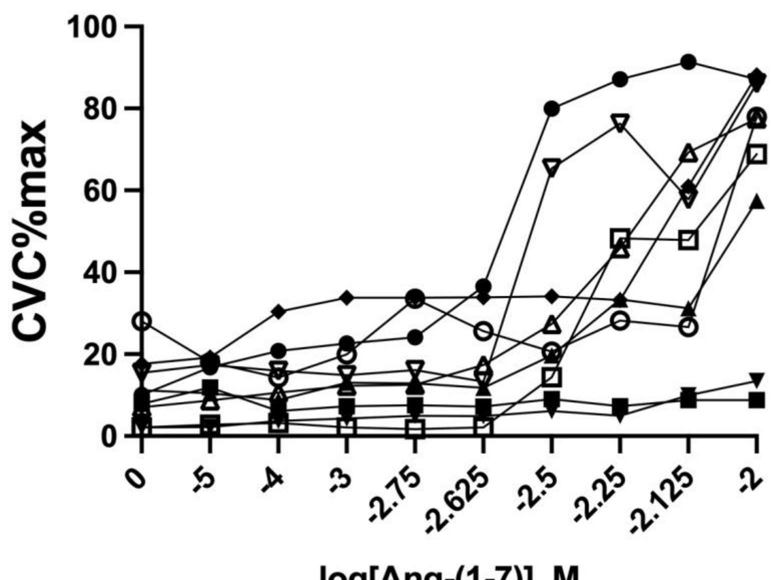


Figure 3.3 Individual responses to Ang-(1-7) perfusion in A. young women (YW; n=10) and B. postmenopausal women (PMW; n=9).

A.

B.

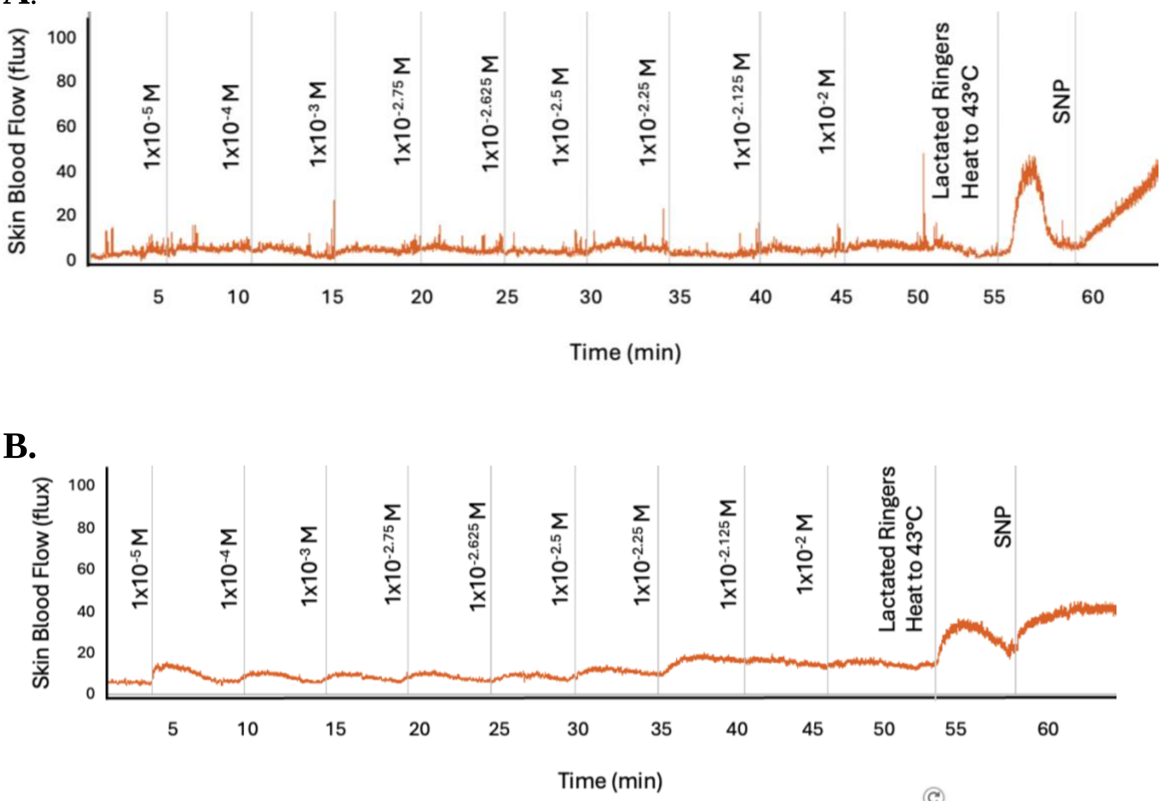


Figure 3.4 Representative Tracing of Ang-(1-7)+L-NAME Dose Response protocol in (A) 1 young woman and (B) 1 postmenopausal woman. 0-5 minutes baseline measurement (perfusion of lactated Ringers at 32°C). Gray lines represent perfusion of new dose. All doses of Ang-(1-7) were dissolved in 15 mM L-NAME. All doses were perfused at 32°C. Following perfusion of all doses lactated Ringers was perfused, and skin temperature was set to 43°C. To achieve maximal dilation sodium nitroprusside (SNP) was perfused at 43°C.

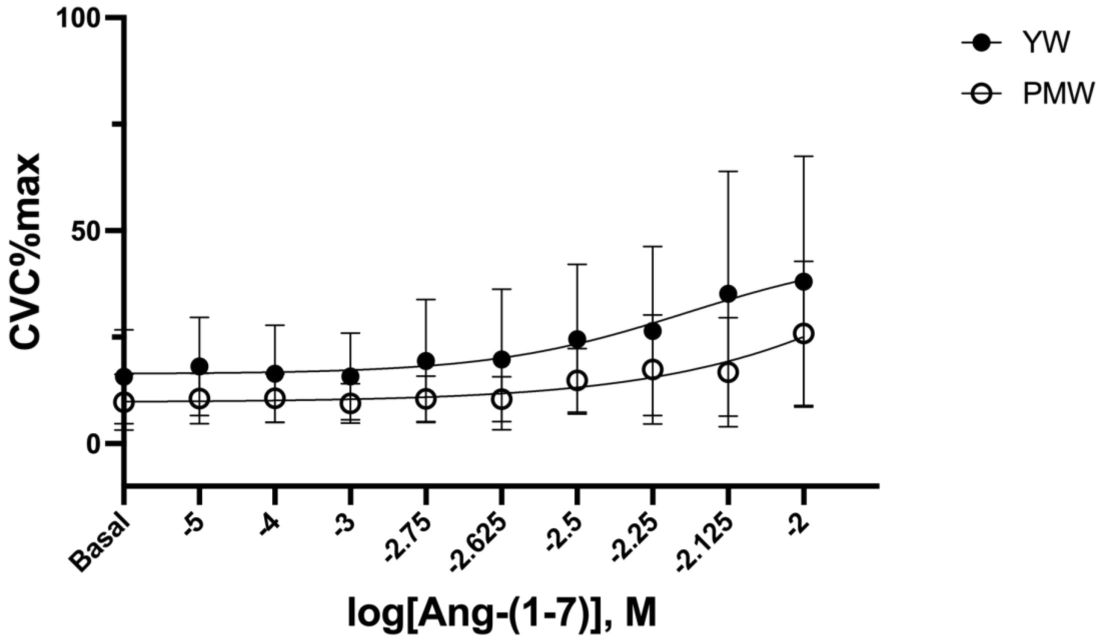


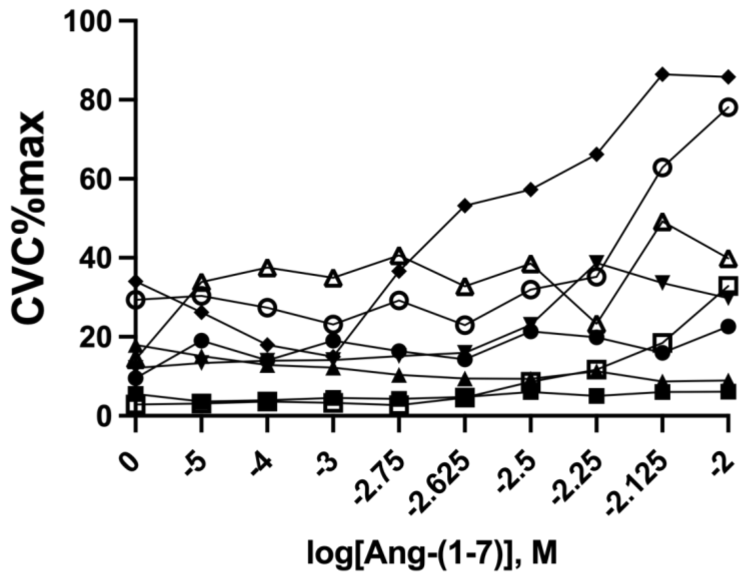
Figure 3.5 Ang-(1-7) Dose response curves in young women in the presence of L-NAME (YW; n=8; closed circles) and postmenopausal women (PMW; n=5; open circles). Data are mean+SD of vasodilation (cutaneous conductance, %max dilation) responses to Ang-(1-7) perfusions in YW and PMW.

	YW (n=8)	**PMW (n=7)**
Top	45.09 ± 33.78	26.63 ± 27.06
Bottom	16.37 ± 4.15	9.72 ± 2.9
LogED50	7.45 ± 3.49	11.9 ± 38.18
HillSlope	0.336 ± 0.488	0.237 ± 0.468
AUC	74.68 ± 14.82	45.04 ± 7.59

Table 3.3 Parameters of Ang-(1-7) + dose response curves with L-NAME in YW and PMW determined from fitting to sigmoidal curve with variable slope. Top: y value at top of plateau; Bottom: y value at bottom of plateau; LogED50: x value when response is halfway between bottom and top; Hillslope: slope of curve; AUC: area under the curve. Slopes P>0.05 for all parameters. Values are mean for each respective curve ± SE.

Of those who completed the protocol, Ang-(1-7)+L-NAME was perfused in a subset of 8 YW and 7 PMW. L-NAME was only perfused in a subset. In cases where there were issues with fibers or perfusion, only Ang-(1-7) was perfused. There were no differences between groups in NO contribution (P=0.44; Figure 3.3).

A.



B.

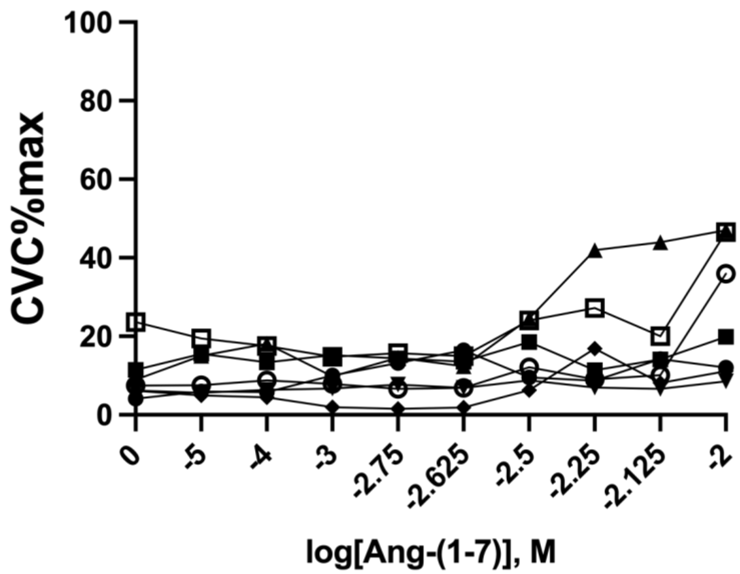


Figure 3.6 Individual responses to Ang-(1-7) perfusion in the presence of L-NAME A. young women (YW; n=8) and B. postmenopausal women (PMW; n=7).

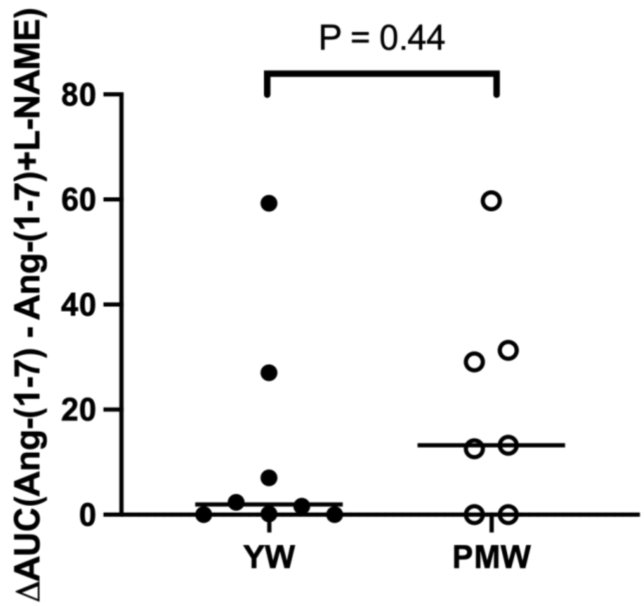


Figure 3.7 NO contribution to Ang-(1-7) dependent dilation in a subset of 8 young women (YW; closed circles) and 7 postmenopausal women (PMW; open circles). NO contribution calculated as AUC(Ang-(1-7))-AUC(Ang-(1-7)+L-NAME). Line is representative of means with individual data points.

We found no significant correlation between total cholesterol and max dilation (r=0.13; P=0.62) or total cholesterol and AUC (r= 0.14; P=0.57), HDL cholesterol and max dilation (r= 0.45; P= 0.06), HDL and AUC (r= 0.37; P= 0.14), LDL cholesterol and max dilation (r= 0.04; P= 0.88), LDL and AUC (r= 0.004; P= 0.99), glucose and max dilation (r= -0.46; P= 0.06), glucose and AUC (r= -0.09; P= 0.73), BMI and max dilation (r= -0.43; P= 0.08), BMI and AUC (r= -0.36; P= 0.14), SBP and max dilation (r=0.10; P= 0.70), SBP and AUC (r= -0.17; P= 0.51), DBP with max dilation (r=0.32; P= 0.20), DBP and AUC (r= -0.01; P= 0.98), MVPA and max dilation (r=0.14; P=0.58), or MVPA and AUC (r=0.27; P=0.27).

3.4 Discussion

This was the first study to determine if Ang-(1-7) could induce dilation in the microcirculation of humans and to test whether the vasculature of YW and PMW respond differently to Ang-(1-7). The primary findings of the current study are that Ang-(1-7) induces vasodilation in the microcirculation of women in a dose-dependent manner. The vasodilatory response to Ang-(1-7), indicative of vascular sensitivity to Ang-(1-7), was similar in young, premenopausal women and PMW. The NO contribution to vasodilation was not different between YW and PMW. Lastly, we did not find any association between the individual vasodilatory responses to Ang-(1-7) with cholesterol, BMI, blood pressure, blood glucose, or physical activity. These findings indicate that vascular sensitivity to Ang-(1-7) may be preserved in healthy PMW.

3.4.1 Ang-(1-7) as a Vasodilator

Ang-(1-7) is a known vasodilator in the vascular system that acts on several different pathways to cause vasodilation. The results of this study suggest there is a vasodilatory effect of Ang-(1-7) in women, which agrees with the current literature in animal models and cell studies examining the vasodilatory effect of Ang-(1-7) (Alenina et al., 2008; Brosnihan et al., 1996; Ferrario et al., 2005; Ferreira & Santos, 2005; Zhang et al., 2019). However, previous research regarding vasodilation in response to Ang-(1-7) in humans showed vasodilation at different doses (Sasaki et al., 2001). Sasaki et al. (2001) measured changes in forearm blood flow using venous occlusion plethysmography as a method to test vasodilation in response to intra-arterial infusions of $1x10^{-10}$ M, $1x10^{-9}$ M, and $1x10^{-8}$ M and found significant vasodilation at $1x10^{-9}$ M and $1x10^{-8}$ M. These doses are much smaller than the doses used in the current study and we did not see dilation at the smallest doses which are more concentrated than those used by Sasaki et al. (2001). However, the methodology differs between these studies, in that Sasaki et al. (2001) used intra-arterial infusions whereas the current study used cutaneous microdialysis and it is possible that these methods of administration are to account for these differences. Further, this study included men and examined a hypertensive population while the current study focused on women and the impact of menopause on vasodilatory responses to Ang-(1-7). The results of this study add to those previously reported by Sasaki et al. (2001) by extending the finding of vasodilation to Ang-(1-7) to a different population and by using a different method.

With regard to the impact of menopause on the vasodilatory effect of Ang-(1-7), the lack of differences between YW and PMW contrast with the findings in animal models of menopause (Costa-Fraga et al., 2018). Costa Fraga et al. showed a significant dose-dependent vasodilatory response to Ang-(1-7) in arterial rings of young mice (Costa-Fraga et al., 2018). This response was absent in old mice and OVX mice, suggesting that both age and menopause may significantly impact the vascular sensitivity to Ang-(1-7). Regardless of the lack of differences between groups, the results of the current investigation provide evidence that the vasculature in both younger women and postmenopausal women can similarly respond to exposure of Ang-(1-7) in a dose-dependent manner. Further, the results of the current study provide more evidence to add to the body of literature that Ang-(1-7) causes vasodilation in the microvasculature in humans by providing dose-response curves as an illustration of Ang-(1-7) as a vasodilator. These curves may provide valuable information for future investigations that aim to further elucidate the role of Ang-(1-7) as a vasodilator.

3.4.2 Estrogen

Current literature is largely in agreement that Ang-(1-7) likely interacts with estrogen, leading to the rationale that a loss of estrogen following menopause might cause dysfunction in the Ang-(1-7) pathway. When examining the results of this investigation in the context of menopause, it is important to note that the most robust differences in vascular sensitivity found by Costa Fraga existed when the blood vessels were also treated with E2 (Costa-Fraga et al., 2018). Based on the literature in these animal models, it is possible that there might be a greater response to Ang-(1-7) in PMW in the presence of E2. This is likely due to the interactions between Ang-(1-7) and the estrogen-dependent vasodilatory pathways. It has been suggested that Ang-(1-7) administration combined with E2 increase production of NO, likely leading to a greater vasodilatory response (Saberi et al., 2016). It has also been suggested that E2 may further increase Ang-(1-7) production through the ERα receptor, further increasing the robustness of the vascular response to Ang-(1-7) (Mompeón et al., 2016). In the current study we did not measure circulating E2, so while the PMW included in this study should have very low levels of E2, this cannot be confirmed. We also did not treat with E2 as has been shown in animal studies to increase the robustness of the response to Ang-(1-7). Therefore, we also cannot confirm whether E2 would change the response to Ang-(1-7) in these women.

3.4.3 The Role of Nitric Oxide in Ang-(1-7)-Mediated Vasodilation

Previous studies have suggested that Ang-(1-7) causes vasodilation through an NO-dependent mechanism by increasing NO production and upregulating NO-dependent pathways. Thus, we hypothesized that the dilation caused by Ang-(1-7) in the current study would be through an NO-dependent mechanism. We tested this hypothesis by perfusing L-NAME, a nonspecific nitric oxide synthase blocker along with all tested doses of Ang-(1-7). There were no significant differences in NO contribution between groups. This is surprising given the evidence that Ang-(1-7) mainly causes dilation via NO-dependent pathways (Brosnihan et al., 1996; Fernandes et al., 2001; Heitsch et al., 2001; Walkyria Oliveira Sampaio et al., 2007). However, Ang-(1-7) is also known to act on other systems within the vasculature that also elicit vasodilation. Ang-(1-7) through Mas_R increases cAMP via prostaglandins and increases transcription of actors in vasodilatory pathways. Further, Ang-(1-7) also inhibits Ang II-mediated production of markers of oxidative stress that are known to cause vasoconstriction such as reactive oxygen species (ROS), nuclear factor kappa B (NFkB), and mitogen-activated protein kinases (MAPKs) (Walkyria O. Sampaio et al., 2007; Santos et al., 2018). So, by counteracting these pathways alone, Ang-(1-7) promotes a vasodilatory state and it is possible that dilation caused by Ang-(1-7) is less reliant on NO than previously hypothesized.

Given the evidence from the current study along with the literature that Ang-(1-7) can act on systems independent of NO to cause vasodilation, further investigation should examine other pathways that may be involved in Ang-(1-7) dependent dilation. For instance, the role of Ang-(1-7) as a counter regulator of Ang II should be further explored especially as it pertains to menopause. This is important considering Ang II leads to many of the pathologies that cause CVD in PMW (Javeshghani et al., 2006;

Mirza et al., 2008; Yung et al., 2011). Finally, it should be noted that the mechanisms by which Ang-(1-7) causes NO-dependent vasodilation are largely through Mas_R (Fernandes et al., 2001; Walkyria Oliveira Sampaio et al., 2007; Sh et al., 2018; Yang et al., 2018). There are other possible receptors for Ang-(1-7) such as MrgD (Tetzner et al., 2016), of which the roles and pathways through this receptor have not been fully elucidated. There is also evidence that Ang-(1-7) may interact with the Angiotensin type 2 receptor (AT2) (Mansoori et al., 2016). Though the exact mechanisms by which Ang-(1-7) might cause vasodilation through these receptors have not been fully elucidated, it is possible that those pathways are independent of NO.

3.4.4 Limitations

There are several limitations with the current study design. We presently do not have data on circulating ovarian hormones in these women. Considering the current literature suggesting estrogen plays a role in the vasodilatory effect of Ang-(1-7), it was hypothesized that higher estrogen might impact the vascular sensitivity to Ang-(1-7) and the ability of Ang-(1-7) to cause vasodilation (Costa-Fraga et al., 2018; Mompeón et al., 2016; Saberi et al., 2016; Sobrino et al., 2017). Regarding mechanisms and pathways through which Ang-(1-7) induces vasodilation, only the NO pathway was tested as a potential mechanism through which Ang-(1-7) impacts vascular function. However, many other pathways were discussed as potential targets by which Ang-(1-7) causes vasodilation such as by counter acting Ang II.

Finally, there is a high degree of variability in this current dataset. This is especially observed in the SE of the top values of the curves fit to the vasodilatory response to Ang-(1-7). There are also 2 PMW who do not appear to respond to Ang-(1-7), and 1 YW shows maximal dilation at an early dose before declining. Though the doses chosen for this study were based on pilot testing in which we observed a plateau at higher doses, it is possible that some participants did not show a true plateau. We did not find any impact of any other baseline characteristic variable (cholesterol, BMI, blood glucose, or blood pressure) on the vasodilatory responses in the women in this study. It is unclear as to what is driving the variability in these data. It is possible that a greater sample size is needed to better depict the response to Ang-(1-7) in these populations.

3.4.5 Conclusion

In conclusion, Ang-(1-7) causes vasodilation in the microvasculature in women in a dose-dependent manner. However, when the vasodilatory response to Ang-(1-7) is not different between young women and postmenopausal women. The lack of differences in vascular sensitivity between YW and PMW may be because the PMW in this study were all generally healthy and did not have elevated BP or cholesterol and thus may have preserved vascular sensitivity to Ang-(1-7). Further, NO does not seem to play a significant role in this vasodilatory response in these women, suggesting other mechanistic pathways through which Ang-(1-7) causes vasodilation may be responsible for the actions of Ang-(1-7) in women. The dose-dependent vasodilatory response shown in these data provide an important step in elucidating the role of Ang-(1-7) in the microvasculature and the role it plays in vasodilatory responses in women. Future studies should further test the vascular sensitivity to Ang-(1-7) in the microvasculature of PMW in the presence of estrogen. Given the current evidence in the literature, estrogen may have an impact on the vasodilatory response to Ang-(1-7) in this group. Future studies may also include women with less favorable lipid profiles, hypertension, or lower levels of physical activity to determine if any of these variables may be related to the vascular sensitivity to Ang-(1-7). Finally, future studies should also isolate which receptor is mediating this vasodilatory response to further identify the specific pathway through which Ang-(1-7) works in women.

Chapter 4

THE IMPACT OF ANGIOTENSIN-(1-7) ON ENDOTHELIAL FUNCTION IN POSTMENOPAUSAL WOMEN

4.1 Introduction

Following menopause, women have an elevated risk of cardiovascular disease (CVD) that is thought to be caused by different mechanisms than those in men. Endothelial function, or the ability of the vascular endothelium to respond to a stimulus, declines with aging and is a precursor to the development of CVD. Previous research indicated that endothelial function is preserved in women until the 5th decade, after which the declines in endothelial function are accelerated (Celermajer et al., 1994). However, recent findings from our laboratory suggest that the decline in endothelial function may begin earlier than previously noted (Wenner et al., 2024). Fluctuations in reproductive hormones contribute to the decline in endothelial function with menopause, especially the loss of estrogen which is known to have protective effects on the vasculature (Somani et al., 2019). Despite the consensus that the decline in endothelial function following menopause occurs in part due to loss of estrogen, the exact mechanisms that play a role in this change remain unknown. This gap in knowledge has prompted the study of different mechanistic pathways that may impact endothelial function of postmenopausal women (PMW) specifically.

Angiotensin-(1-7) (Ang-(1-7)) has recently gained attention in the literature for its impact on endothelial function. It is known to cause vasodilation and act through endothelium-specific mechanisms (Brosnihan et al., 1996; Ferreira & Santos, 2005;

Medina & Arnold, 2019; Walkyria O. Sampaio et al., 2007; Walkyria Oliveira Sampaio et al., 2007; R. A. Santos et al., 1992). Specifically, it has been found that Ang-(1-7) contributes to activation of endothelial nitric oxide synthase (eNOS), essential for the production of nitric oxide (NO), a main actor of endothelium-dependent vasodilation (Brosnihan et al., 1996; Walkyria Oliveira Sampaio et al., 2007).

Additional research suggests that Ang-(1-7) may be a female specific mechanism that impacts endothelial function. Sullivan et al. (2015) found that women have a greater concentration of plasma Ang-(1-7) than young men and that this is associated with greater endothelial function. Cell studies show that exposure to estradiol (E2) increases Ang-(1-7) production in human umbilical veinous cells (HUVECs) (Mompeón et al., 2016). Further, animal models of menopause have demonstrated that Ang-(1-7) is only able to improve endothelium dependent dilation in aortic rings in the presence of E2 (Costa-Fraga et al., 2018). Given the vast evidence that Ang-(1-7) interacts with estrogen to contribute to the regulation of endothelial function, it is likely that the loss of estrogen in PMW leads to a dysfunction in the Ang-(1-7) pathway, thus resulting in endothelial dysfunction. Therefore, it is possible that increasing the bioavailability of Ang-(1-7) in these women might improve the ability of this pathway to impact endothelial function.

It has been shown that local Ang-(1-7) administration potentiates endothelium-dependent dilation in the microvasculature in women with impaired endothelial function (POTS patients and women who have had preeclampsia) (Stanhewicz & Alexander, 2020; Stewart et al., 2009). Considering these female populations have an increased risk of CVD associated with impaired endothelial function, it is likely these results can be found in other female populations with impaired endothelial function.

Despite the evidence Ang-(1-7) likely interacts with estrogen to regulate endothelial function in women, whether increasing the bioavailability of Ang-(1-7) can enhance endothelial function in PMW has not yet been studied. Therefore, the purpose of the current investigation was to test the hypothesis that administration of Ang-(1-7) will restore endothelium-dependent vasodilation in PMW. Further, we hypothesize that Ang-(1-7) would improve the NO contribution to endothelium-dependent vasodilation in PMW.

4.2 Methods

4.2.1 Participants

15 YW (23 $\pm$ 3 yrs) and 13 PMW (56 $\pm$ 4 yrs) completed the protocol. YW were all premenopausal and tested during the early follicular phase of the menstrual cycle or during the placebo week if using oral contraceptives (OCPs; n=6). PMW were all between ages 50-65, went through natural menopause, and had been at least 1 year since their final menstrual period (self-report) and were not using any form of hormone therapy. Institutional Review Board approval was obtained from the University of Delaware and all protocols adhered to the standards of the Declaration of Helsinki. All participants gave verbal and written informed consent prior to any participation in the study. A screening visit was completed after obtaining consent for all participants. Screening included measurement of resting BP (Dinamap DASH 2000; GE Medical, Chicago, IL), height and weight to determine body mass index (BMI), and a 12h fasted blood draw for clinical labs. Self-reported physical activity

data were also collected by having all participants complete a Modified Activity Questionnaire (MAQ) (Pereira et al., 1997). All study visits occurred in the same laboratory setting to provide a controlled environment for data collection.

4.2.2 Microdialysis and Laser Doppler Flowmetry

To prepare the skin for microdialysis fiber insertion, the skin on the dorsal side of the forearm was examined to mark needle insert and exit points for each site. Sites were away from visible veins and were at least 1 inch apart from each other with the insert and exit for each site measured to be 2cm apart. The skin was then sterilized, and ice was placed on the skin for 10 min to decrease pain. Two needles were inserted into the intradermal layer of the skin about 2-5mm below the surface of the skin within the cutaneous microcirculation. Microdialysis fibers (CMA 31 Linear, Harvard Apparatus, Holliston, MA) were threaded through the needles. Needles were removed, leaving the porous membrane of the fiber in the skin. The porous membrane has a molecular weight cutoff at 55-kDa to allow smaller substances to pass through the pores and act within the local microcirculation.

Heaters and laser Doppler probes (MoorLab, Temperature monitor SH02; Moor Instruments, Devon, UK) were placed on top of each site. Skin temperature was maintained at 32°C and lactated Ringers was perfused through each fiber at a rate of 2 ul/min using a syringe pump (Bee Hive controller and Baby Bee microinfusion pumps, Bioanalytical Systems, West Lafayette, IN) for 1 hour to allow for recovery from a hyperemic response.

4.2.3 Local Heating Protocol

Local heating of the skin induces an NO-dependent response that is known to be endothelium mediated (Holowatz et al., 2003; Minson et al., 2001; Minson et al., 2002). $1x10^{-4}$ M Ang-(1-7) was dissolved in lactated Ringers as previously described (Stanhewicz & Alexander, 2020; Stewart et al., 2009) and shown to improve endothelial function in premenopausal women with preclinical CVD. Following hyperemia, either Ang-(1-7) or lactated Ringers was perfused in each site for 45 minutes while maintaining the skin temperature at 32ºC. Local heating of each site to 42ºC was done for ~30min, a stable plateau was reached, followed by perfusion of 15mM L-NAME until a nadir plateau was maintained for calculation of NO-dependent dilation. Lactated Ringers was then perfused in both fibers and skin temperature was turned to 43ºC. Finally, SNP was perfused until maximal dilation was reached. Brachial artery blood pressure was measured throughout (Dinamap DASH 2000; GE Medical, Chicago, IL). A representative tracing can be found in Figure 4.1.

4.2.4 Data Analysis

Physical activity was calculated as moderate-to-vigorous physical activity (MVPA) in hours/week. Cutaneous vascular conductance (CVC) is determined as red blood cell flux/mean arterial pressure. All data are normalized to maximal vasodilation during SNP perfusion and expressed as CVC%max by calculating (CVC/CVCmax)*100. Data points were taken as an average of 2 min from baseline (before heating), a stable plateau during local heating, and a stable plateau during L-NAME perfusion. The NO contribution was calculated as (CVC%max heating - CVC%max heating L-NAME)/100.

4.2.5 Statistical Analysis

A power analysis based on previous studies using the same dose of Ang-(1-7) in the microcirculation (Stanhewicz & Alexander, 2020; Stewart et al., 2009) and for similar studies examining differences in microvascular endothelial function in YW vs PMW (Wenner et al., 2017) suggests a sample of 15 women per group would yield a power of 80%, using an effect size of 1.25, and alpha set to $P < 0.05$,. Participant characteristics were compared between groups using student's t tests. A 2-way ANOVA was used to compare differences in the impact of Ang-(1-7) on endothelial function in PMW compared with YW and on the differences in the NO contribution to the local heating plateau. Fisher's LSD post hoc test was run to compare differences between groups. Lastly, Pearson's correlations were run to assess whether any differences in baseline characteristics were related to differences in endothelial function. Correlations were run between the CVC%max at the plateau during local heating in the control site and in the Ang-(1-7) site with total cholesterol, HDL cholesterol, LDL cholesterol, glucose, BMI, SBP, DBP, and MVPA. The same correlations were run with the NO contribution to determine if these variables had any impact on the NO contribution to local heating in either site.

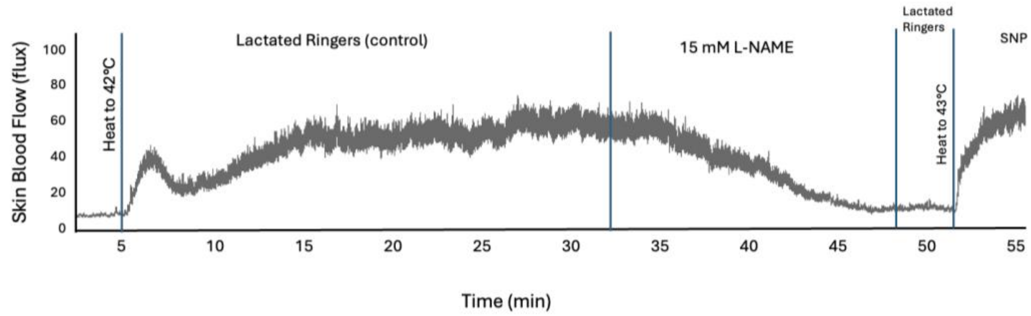
4.3 Results

As expected, PMW were older than YW, and had greater SBP, DBP, and MAP than YW. PMW also showed elevated total cholesterol, LDL cholesterol and blood glucose compared to YW.

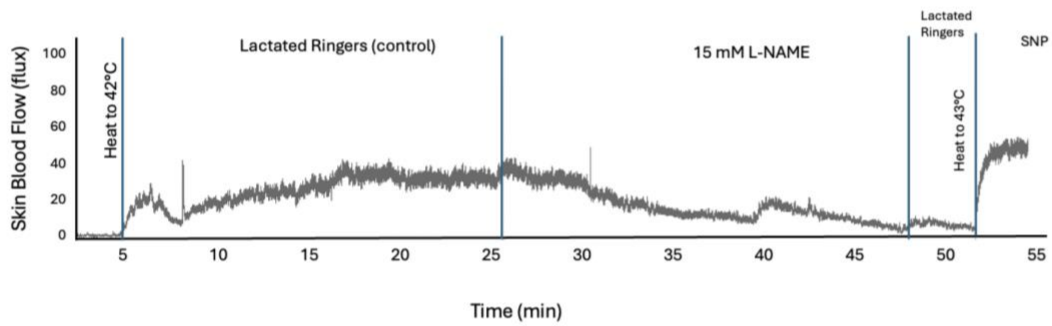
	YW (n = 15)	**PMW** (n = 13)
Age, yrs	23 ± 3	56 ± 4 *
BMI, kg/m^2	24 ± 3	25 ± 3
SBP, mmHg	108 ± 7	115 ± 9 *
DBP, mmHg	66 ± 5	72 ± 6 *
MAP. mmHg	80 ± 4	86 ± 7 *
Total Cholesterol, mg/dL	170 ± 26	204 ± 36 *
HDL, mg/dL	63 ± 13	69 ± 10
LDL, mg/dL	91 ± 23	119 ± 34 *
Glucose, mg/dL	85 ± 5	92 ±11 *
MVPA (hrs/wk)	4.47 ± 2.51	4.64 ± 5.55

Table 4.1 Participant Characteristics. Values are mean±SD. PRE, premenopausal women; PMW, postmenopausal women; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; HDL, high-density lipoprotein; LDL, low-density lipoprotein; MVPA, moderate-to-vigorous physical activity. Groups were compared using unpaired t-tests. *P<0.05 vs. YW.

A.


Lactated Ringers (control)
15 mM L-NAME
Lactated Ringers
SNP
Heat to 42°C
Heat to 43°C
Skin Blood Flow (flux)
0
20
40
60
80
100
5
10
15
20
25
30
35
40
45
50
55
Time (min)

B.


Lactated Ringers (control)
15 mM L-NAME
Lactated Ringers
SNP
Heat to 42°C
Heat to 43°C
Skin Blood Flow (flux)
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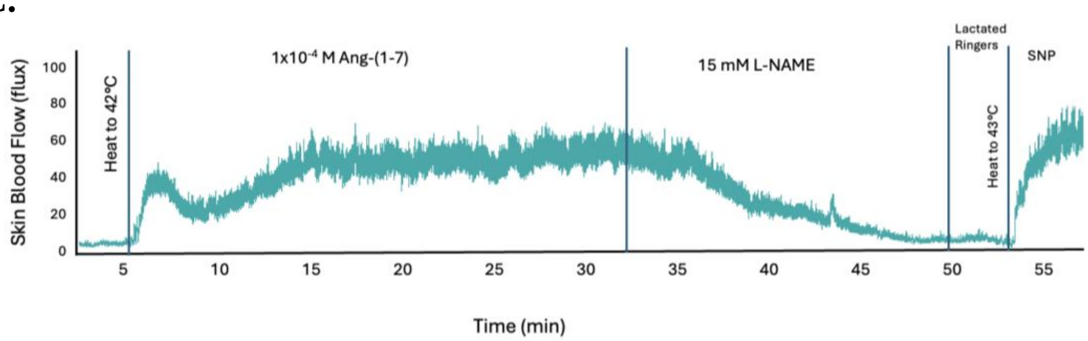
Time (min)

C.


1x10^{-4} M Ang-(1-7)
15 mM L-NAME
Lactated Ringers
SNP
Heat to 42°C
Heat to 43°C
Skin Blood Flow (flux)
0
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60
80
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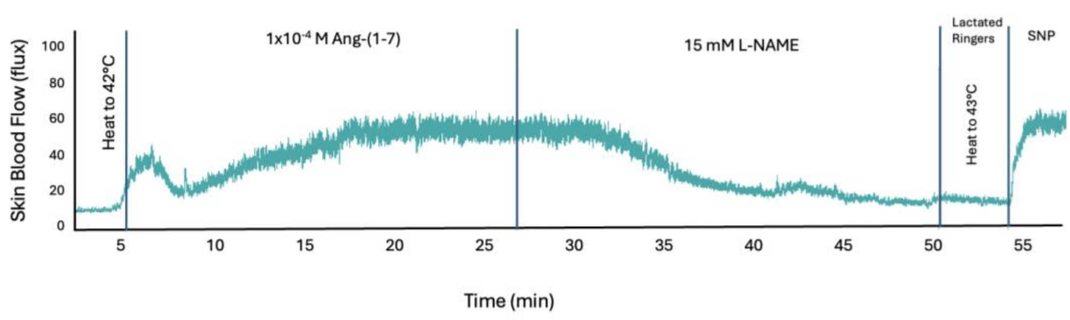
Time (min)

D.


1x10^{-4} M Ang-(1-7)
15 mM L-NAME
Lactated Ringers
SNP
Heat to 42°C
Heat to 43°C
Skin Blood Flow (flux)
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Time (min)

Figure 4.1 Representative tracings of local heating response in the control site in (A) 1 young woman (YW) and (B) 1 postmenopausal woman (PMW) and Ang-(1-7) site in (C) the same YW and (D) the same PMW. Five min of baseline at 32°C was followed by local heating to 42°C to a stable plateau for ~30 min while perfusing either lactated Ringers (A., control) or 1x10-4 M Ang-(1-7) (B.). After a stable plateau was reached, 15 mM L-NAME was perfused until reaching a stable nadir plateau. Lactated Ringers was perfused again before heating to 43°C and then perfusing sodium nitroprusside (SNP) to reach maximal vasodilation.

Endothelium-dependent dilation is shown in Figure 4.2. In a 2-way ANOVA analyzing the local heating response for PMW and YW in the control and Ang-(1-7) sites, there was a main effect of group ($P = 0.03$), but not drug ($P = 0.88$), or an interaction ($P = 0.32$). Post hoc analysis showed that PMW have lower endothelial function (control site, $P = 0.03$). However, the local heating response to Ang-(1-7) was similar between groups ($P = 0.39$) and was not different from the control site within either group (YW $P = 0.49$; PMW $P = 0.69$). The reduction in blood flow in response to L-NAME was similar between groups ($P = 0.64$). The % NO contribution calculated as (local heating response – L-NAME-induced plateau) was also not different between groups (Main effect for Group: $P=0.50$; Drug: $P=0.16$; Interaction: $P=0.34$) and is shown in Figure 4.3.

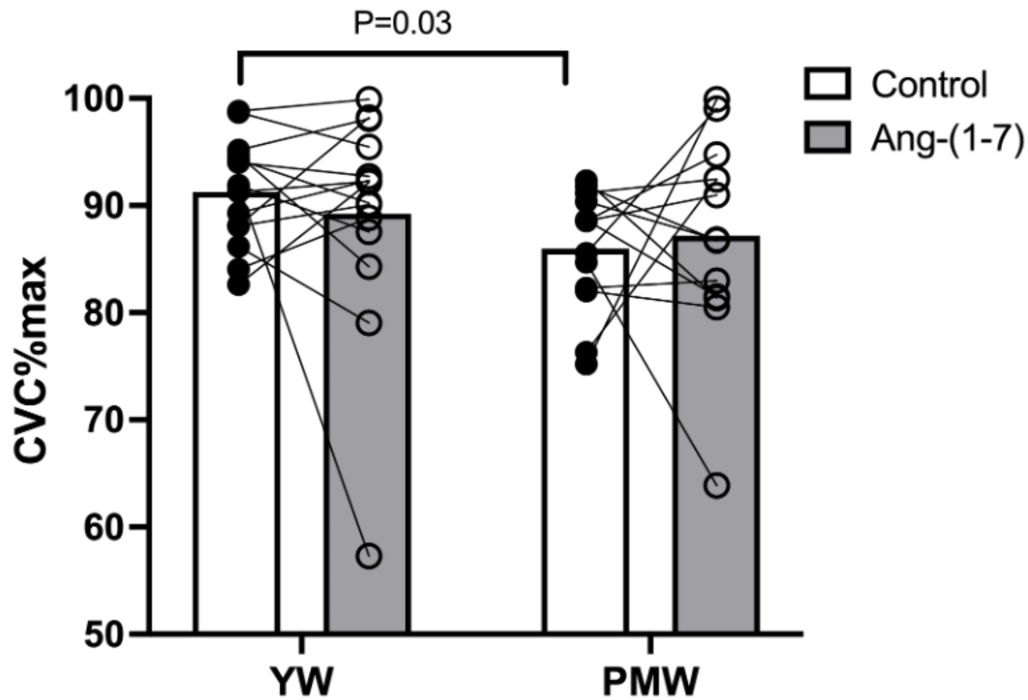

Figure 4.2 Local Heating response to Ang-(1-7) in young women (YW; n=15) and postmenopausal women (PMW; n=13) in control site (lactated Ringers) and experimental site (Ang-(1-7)). Bars show means with individual values for control site (white bar/closed circles) and means with individual values for Ang-(1-7) site (gray bars/open circles) in both groups. Each participant's' individual values are connected between sites. 2-way ANOVA determined a main effect for group (P = 0.03), but not drug (P = 0.88), or an interaction (P = 0.32). Post hoc analysis showed that PMW have lower endothelial function (control site, P = 0.03).

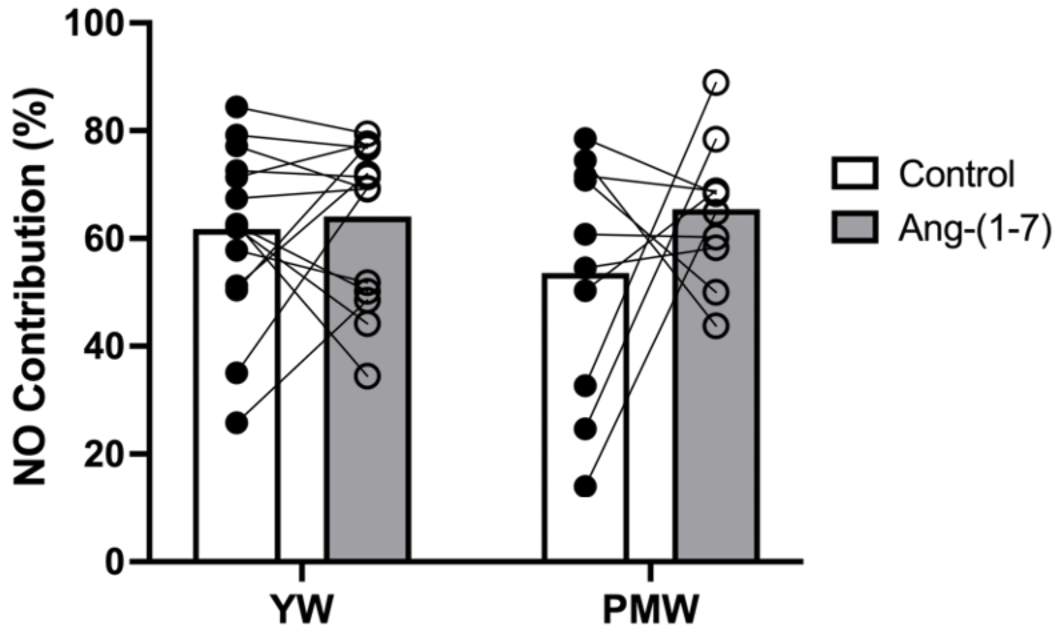

Figure 4.3 NO contribution to local heating response to Ang-(1-7). Mean is represented by bar with individual values in a subset of 14 young women (YW; closed circles) and 10 postmenopausal women (PMW; open circles). There were no significant differences between groups or drug treatment ($P>0.05$).

There was no significant correlation between $CVC_{\%max}$ during the local heating-induced plateau in the control site with total cholesterol (r= -0.26; P= 0.24), HDL cholesterol (r= -0.21; P= 0.36), LDL cholesterol (r= -0.25; P= 0.26), glucose (r= -0.26; P= 0.31), BMI (r= 0.21; P= 0.36), SBP (r= 0.17; P = 0.44), DBP (r= -0.12; P= 0.61), or MVPA (r= -0.24; P= 0.27). There was also no significant correlation between $CVC_{\%max}$ during the local heating-induced plateau in the Ang-(1-7) site with total cholesterol (r= -0.15; P= 0.52), HDL cholesterol (r= -0.09; P= 0.71), LDL cholesterol (r= -0.12; P= 0.61), glucose (r= -0.05; P= 0.83), BMI (r= -0.10; P= 0.67), SBP (r= -0.12; P= 0.63), DBP (r= 0.004; P= 0.99), or MVPA (r= 0.35; P = 0.12). There were no significant correlations between % NO contribution in the control site with total cholesterol (r= -0.12; P= 0.63), HDL cholesterol (r= 0.18; P= 0.41), LDL cholesterol (r= -0.20; P= 0.38), glucose (r= -0.49; P= 0.07), BMI (r= -0.52; P= 0.06) SBP (r= -0.21; P= 0.35), DBP (r= -0.10; P= 0.68), or MVPA (r= 0.23; P= 0.23) or the % NO contribution in the Ang-(1-7) site with total cholesterol (r= 0.18; P= 0.42), HDL cholesterol (r= 0.12; P= 0.60), LDL cholesterol (r= 0.10; P= 0.65), glucose (r=0.09; P= 0.68), BMI (r= 0.12; P= 0.64), SBP (r= 0.16; P= 0.48), DBP (r= 0.42; P= 0.06), or MVPA (r= -0.39; P= 0.07).

4.4 Discussion

This study is the first to examine whether increasing the bioavailability of Ang-(1-7) would improve endothelial function in PMW. Although the PMW in this study displayed a blunted response to local heating, indicative of impaired endothelial function, this response was not improved by local administration of Ang-(1-7). Additionally, there was no significant difference in the NO contribution to the local heating response to Ang-(1-7) in either group. These results suggest Ang-(1-7) may not play a role in the NO-dependent dilation in response to local heating in the microvasculature in healthy PMW. Finally, we did not see any association of total cholesterol, HDL cholesterol, LDL cholesterol, glucose, BMI, blood pressure, or physical activity on the endothelium-dependent vasodilation in the control or Ang-(1-7) sites or on the NO contribution to vasodilation in either site.

4.4.1 Ang-(1-7) Impact on Endothelial Function

Ang-(1-7) is known to impact endothelium-dependent vasodilation (Heitsch et al., 2001; Walkyria O. Sampaio et al., 2007; Walkyria Oliveira Sampaio et al., 2007; R

A Santos et al., 1992). Ang-(1-7) contributes to endothelial function through NO-dependent mechanisms (Brosnihan et al., 1996; Heitsch et al., 2001; Walkyria Oliveira Sampaio et al., 2007; R. A. Santos et al., 1992; Sh et al., 2018) and through other mechanisms not related to NO (Benter et al., 1995; Brosnihan et al., 1996). Given the evidence that Ang-(1-7) can improve endothelial function, we hypothesized that Ang-(1-7) could improve endothelium-mediated dilation in PMW. We found a blunted response to local heating in PMW compared to YW which agrees with the literature examining endothelial function within the microvasculature with menopause and aging in women. It was expected that local Ang-(1-7) administration would improve this response. However, Ang-(1-7) did not have an impact on the response to local heating in PMW in this study.

It is also notable that there was no significant impact of cholesterol, blood pressure, or physical activity on endothelium-dependent dilation in this study. This is surprising as these factors all contribute to microvascular endothelial function where higher lipid profiles (Davignon & Ganz, 2004; Schächinger & Zeiher, 2000), increased blood pressure (Panza et al., 1990), and decreased physical activity (Franzoni et al., 2005) are all associated with decreased endothelium-dependent dilation. Importantly, however, all the women included in this study were generally healthy. Though the PMW had significantly higher SBP and DBP than the YW, they were still normotensive. Thus, it is unclear if women who are not healthy as defined by these variables would respond similarly.

Physical activity is especially important to note in this study. Regular exercise is associated with a lower risk of CVD as it can maintain vascular function (Seals et al., 2009). However, there is a sex difference in the impact of exercise on vascular function with aging, where older women do not see the same benefits of exercise as older men (Moreau & Ozemek, 2017; Seals et al., 2019). Thus, there may be some mechanisms by which exercise training impacts older women differently than it does men. Further, it has been found in animal studies that exercise training could help to stimulate beneficial actions of Ang-(1-7) (Filho et al., 2008; Nunes-Silva et al., 2017; Rush & Aultman, 2008; Shah et al., 2012). Given these findings and the research suggesting Ang-(1-7) acts differently in the vasculature in women than men, the Ang-(1-7) pathway may be a potential mechanism impacting the benefits of exercise on endothelial function. There is high variability in the MVPA of the PMW in this study. This is due to one woman having reported MVPA of 17.6 MVPA hours/week and another with 11.9 MVPA hours/week. Without these women, the average MVPA of the PMW is 2.62$\pm$2.9 hours/week. However, excluding these women does not change the results of the correlation. Further, only 5 of the 13 PMW included had higher physical activity levels (MVPA>2.5 hours/wk) whereas 12 of the 15 YW included had higher physical activity. So, we did not see any significant contribution of physical activity to local heating response or NO contribution, but due to the high variability of MVPA in our sample size of PMW, future studies should still consider physical activity as a factor that might impact endothelium-dependent dilation in response to Ang-(1-7).

4.4.2 Ang-(1-7) and Estrogen

It has been well established that Ang-(1-7) interacts with estrogen to improve endothelial function. It has also been proposed that the presence of E2 enhances production of Ang-(1-7), further increasing the activity of Ang-(1-7) that might promote endothelial function (Mompeón et al., 2016). Other *in vitro* studies have also suggested pathways by which Ang-(1-7) and E2 together might specifically increase NO-dependent vasodilation (Saberi et al., 2016; Sh et al., 2018). In mouse models of menopause and aging, Ang-(1-7) only caused vasodilation when also in the presence of estrogen (Costa-Fraga et al., 2018; Neves et al., 2004).

In the only other studies examining Ang-(1-7) in the microvasculature in women, Ang-(1-7) improved the endothelial response to Ach perfusions and local heating (Stanhewicz & Alexander, 2020; Stewart et al., 2009). However, the populations from these studies were premenopausal women with circulating estrogen. This is important to consider when discussing Ang-(1-7) and endothelial function as much of the previous literature suggests estrogen interacts with the Ang-(1-7)/Mas_R pathway to improve endothelial function. Given that the women in our study are postmenopausal and therefore lacking in circulating estrogen, it is possible that they did not see improvements in endothelial function because estrogen must be present for these pathways. Also, Based on the literature and the lack of response in the current study, future studies should examine the response to Ang-(1-7) in PMW in the presence of estrogen to confirm whether estrogen is necessary for Ang-(1-7) to improve measurements of endothelial function in this population.

4.4.3 NO Contribution to Local Heating

We did not observe a significant difference in the NO contribution to the local heating response between groups or related to Ang-(1-7) administration in the current study. Previous literature has found that there is a significant contribution of NO to the local heating response and that this is lost with aging. NO contributions are generally between 70-80% in younger adults and about 60% in older men and women (Minson et al., 2002). In the current study, the mean NO contribution in the control site is

63.76% in YW and 57.7% in PMW. Thus, the NO contribution in YW is lower in this study than would be expected. The reason for this result is unclear since all other markers of cardiovascular health are normal in our sample. One woman who reported OCP use had an NO contribution in the control site at 50.8%, but the other 2 were 73.03% and 62.92% which is more representative of the mean. There is evidence that OCP use reduces endothelium-dependent dilation in healthy YW (Turner, Stanhewicz, Nielsen, Otis, et al., 2023; Turner, Stanhewicz, Nielsen, & Wong, 2023). However, in these studies, OCP use appears to increase NO contribution to endothelium-dependent dilation, despite endothelium-dependent dilation being blunted. Therefore, the driving factor of low NO contribution in the YW in this study is likely not OCP use. We did not see any association between NO contribution in this study with cholesterol, BMI, blood glucose, blood pressure, or physical activity. We ran these correlations to account for any variability or age-related baseline differences. However, based on the results of this study, it appears that Ang-(1-7) also does not impact the NO contribution to the response to local heating in YW and PMW as local administration of Ang-(1-7) did not improve the NO contribution in either group.

Regarding Ang-(1-7) and the NO contribution to local heating, it was expected that Ang-(1-7) would improve the NO contribution to local heating as Ang-(1-7) increases eNOS activation to increase production of NO (Walkyria Oliveira Sampaio et al., 2007). It has also been shown in animal studies that Ang-(1-7) is only able to induce vasodilation when the endothelium of the vessel is intact and eNOS is present (Brosnihan et al., 1996). Stanhewicz & Alexander found that the NO contribution to endothelium-dependent dilation is significantly blunted in women who have had preeclampsia compared with healthy controls, and that local administration of Ang-(1-

7) increased the NO contribution to dilation. Thus, Ang-(1-7) has been shown to improve the NO-contribution to endothelium-dependent dilation in humans with preclinical CVD. We did not find this in the current study. Though, there do appear to be a few PMW with lower NO contribution to the control site that show an improvement in the Ang-(1-7) site. However, these were only 3 women and there were no commonalities in any baseline characteristic variables among all 3. It is possible that in women with a very low NO contribution to the local heating response, Ang-(1-7) can help improve this, however more research is needed to confirm this.

Lastly, it is important to note that Ang-(1-7) may improve endothelial function independent of NO. Therefore, testing the NO contribution to local heating may not provide a full picture of the mechanisms Ang-(1-7) acts on the endothelium. There is evidence that Ang-(1-7) potentiates the bradykinin pathway and may also act through the bradykinin receptor (B2) to cause vasodilation (Brosnihan et al., 1996; Gorelik et al., 1998; Li et al., 1997) and can induce endothelium-dependent vasodilation via activation of prostaglandins (Andreatta-van Leyen et al., 1993; Santos, 2014). Ang-(1-7) is also known to counteract the actions of Angiotensin II (Ang II) which promote endothelial dysfunction. Given that Ang-(1-7) can cause endothelium-dependent vasodilation through mechanisms not including NO, those pathways should also be taken into consideration in future studies.

4.4.4 The Balance of Ang-(1-7) and Ang II

Ang-(1-7) is the main actor in the vasodilatory arm of the RAS that is known to counteract many of the negative impacts of Ang II on endothelial function. It is also known that Ang II is overactive in PMW and is a main contributor to the decline in endothelial function in these women (Javeshghani et al., 2006; Mirza et al., 2008).

Ang-(1-7) blocks the production of mitogen-activated protein kinases (MAPKs) by Ang II which contribute to cell proliferation, fibrosis, and cell remodeling which lead to endothelial dysfunction (Tallant & Clark, 2003). Ang-(1-7) also blocks Ang II-led production of reactive oxygen species (ROS) which leads to oxidative stress and also leads to endothelial dysfunction (Walkyria O. Sampaio et al., 2007). It has also been found that blockade of Angiotensin Converting Enzyme (ACE), the enzyme that drives production of Ang II, leads to increased levels of Ang-(1-7) (e Silva & Teixeira, 2016; Tom et al., 2003), further confirming that there is a balance of Ang II and Ang-(1-7). There is also evidence that Ang-(1-7) may interact with other aspects of the RAS such as ACE2 to downregulate Ang II (Ji et al., 2008). Therefore, it is possible that the mechanism through which Ang-(1-7) impacts endothelial function in women may be through a counteraction of Ang II rather than acting on the endothelium directly.

The relationship between Ang-(1-7) and Ang II is important because overactivity of the vasoconstrictive side of the RAS driven by Ang II leads to the pathology of CVD and causes endothelial dysfunction. Stanhewicz and Alexander (2018) found that Ang-(1-7) blunts Ang II mediated vasoconstriction in women who have had preeclampsia, a population known to have increased Ang II activity. It is possible that this may also be the case for PMW. A potential explanation for the negative findings of the current study is that we did not test the balance of Ang-(1-7) and Ang II. Therefore, it is important that future studies should consider the balance between Ang-(1-7) and Ang II when examining the role of Ang-(1-7) in endothelial function in PMW.

4.4.5 Limitations

This study is not without limitations. It is possible that a dose greater than the one used ($1x10^{-4}$ mmol) is needed to improve endothelial function in PMW. In the analysis from the previous aim (chapter 3), both PMW and YW on average did not start to see significant vasodilation until exposed to $1x10^{-2.5}$ mmol/min of Ang-(1-7). In this study we used $1x10^{-4}$ mmol/min of Ang-(1-7) based on previous studies that had found that this dose was successful in improving endothelium-dependent vasodilation in women with endothelial dysfunction (Stanhewicz & Alexander, 2020; Stewart et al., 2009), therefore it is possible that a more concentrated dose, such as $1x10^{-3}$ or $1x10^{-2.75}$ mmol/min, might impact endothelial function more effectively. Finally, we did not collect serum hormones in these women. PMW in this study are likely to have low levels of circulating estrogen and the YW in this study have relatively higher levels of estrogen. It is important that this is confirmed given the evidence in cell and animal studies that Ang-(1-7) interacts with estrogen to improve endothelial function as this could account for the results of the study. Further, we did not collect plasma Ang-(1-7) and thus cannot confirm on whether there is decreased bioavailability of Ang-(1-7) in PMW.

4.4.6 Conclusion

In conclusion, the findings in the current study suggest that although PMW show impaired endothelial function in the microvasculature compared to YW, this response is not improved by the local administration of Ang-(1-7). Future studies should further investigate the role of Ang-(1-7) in endothelial dysfunction in postmenopausal women, isolating different pathways, especially those related to estrogen. Future studies should also take the full RAS into perspective and consider the balance between Ang-(1-7) and Ang II in this population to determine whether the main mechanism that Ang-(1-7) can improve endothelial function is by counteracting Ang II.

The results from Chapter 3 suggest that PMW have preserved vascular sensitivity to Ang-(1-7), indicating they have the ability to respond to Ang-(1-7). In this investigation, we aimed to determine whether improving bioavailability of Ang-(1-7) would improve blunted endothelial function in PMW. However, we did not find that administering Ang-(1-7) improved endothelial function. Thus, it is possible that there are other issues with the pathway, such as in the balance of Ang-(1-7) and Ang II. There may also be a downstream issue with Ang-(1-7) signaling in which it is unable to enhance endothelial function. In chapter 5 we discuss an investigation into whether endothelial expression of the receptor for Ang-(1-7) is impacted by menopause as this could potentially explain the negative findings of the current analysis.

Chapter 5

ENDOTHELIAL CELL EXPRESSION OF THE MAS RECEPTOR FOR ANGIOTENSIN-(1-7) IN POSTMENOPAUSAL WOMEN

5.1 Introduction

Endothelial dysfunction is persistent in postmenopausal women (PMW) and is associated with future incidences of cardiovascular disease (CVD). This is largely due to declines in estrogen that occur with menopause, although the specific mechanisms related to this decline remain unknown. Ang-(1-7) is a vasodilator that mainly acts through Mas_R in the pathway commonly referred to as the Ang-(1-7)/Mas_R axis (Walkyria Oliveira Sampaio et al., 2007). It has been proposed that the Ang-(1-7)/Mas_R axis interacts with estrogen within the vasculature to cause vasodilation (Costa-Fraga et al., 2018; Mompeón et al., 2016; Neves et al., 2004; Saberi et al., 2016; Sobrino et al., 2017). There is data from animal and cell studies that have established that the presence of estradiol (E2) increases nitric oxide (NO) production caused by Ang-(1-7) through Mas_R within endothelial cells and that E2 and Mas_R are both necessary for Ang-(1-7)-mediated vasorelaxation animal models (Saberi et al., 2016; Sobrino et al., 2017). Given this potential interaction between estrogen and the Ang-(1-7)/Mas_R axis and the decline in endothelial function that occurs with menopause due to lack of estrogen, there is a need to study the role of Mas_R in endothelial cells in postmenopausal women (PMW).

As with most receptors and ligands, the density of Mas_R determines the ability of Ang-(1-7) to act on the vasculature. In older female mice, there is increased expression of Mas_R compared to younger female mice (Costa-Fraga et al., 2018). Costa Fraga and colleagues (2018) hypothesized Mas_R is upregulated to compensate for the absence of Ang-(1-7), although this has not yet been determined in humans. There are also discrepancies on the expression of Mas_R in disease states, where some studies confirm this upregulation hypothesis (Chang et al., 2018; Lu et al., 2013), but others show decreased receptor expression in diseased tissues (Dias-Peixoto et al., 2012). For instance, in animal models of acute ischemic stroke, Mas_R expression increases in diseased tissue (Lu et al., 2013) while in animal models of hypertension and myocardial infarction, Mas_R expression decreases in the diseased tissues (Dias-Peixoto et al., 2012). However, the presence of Mas_R in human endothelium has not yet been quantified. Thus, there is a need to develop a protocol to study endothelial Mas_R expression in humans.

Given the evidence in the literature that Mas_R plays a key role in mediating the actions by Ang-(1-7) and there is a possibility that the Ang-(1-7)/Mas_R axis plays a key role in the decline in endothelial function in postmenopausal women. Further, there is evidence in animal literature that Mas_R is directly impacted by aging and menopause in animal models. Thus, the aim of this study was exploratory and to determine the abundance of Mas_R in the endothelium of PMW and YW to determine how endothelial Mas_R is impacted by menopause.

5.2 Methods

5.2.1 Participants

20 YW (22$\pm$3 yrs) and 20 PMW (57$\pm$5 yrs) were included in this study. YW were all premenopausal and tested during the early follicular phase of the menstrual

cycle or during the placebo week if using oral contraceptives (OCPs). PMW were all between ages 50-65 and were classified as postmenopausal if it had been at least 1 year since their last menstrual period, and they were excluded if they reported use of any form of hormone therapy.

Institutional Review Board approval was obtained from the University of Delaware and all protocols adhered to the standards of the Declaration of Helsinki. All participants gave verbal and written informed consent prior to any participation in the study. Following consent, a screening visit including measurement of resting BP, height and weight to determine BMI, and a fasted blood draw for clinical labs was completed. All study visits occurred in the same laboratory setting to provide a controlled environment for data collection.

5.2.2 Endothelial Cell Collection and Isolation

Women were fasted for at least 3 hours, were asked not to consume any caffeine or alcohol 12 hours prior, or exercise 24 hours prior. To harvest endothelial cells from YW and PMW, we employed a J wire cell biopsy technique through the antecubital vein. The development of the human endothelial cell biopsy by Colombo and colleagues has provided new methodology to isolate proteins of interest in human cells (Colombo et al., 2002). This method has been used to study various proteins and has proven successful in providing viable cells for immunofluorescence staining of proteins (Donato et al., 2009; Feng et al., 1999; Kuczmarski et al., 2020; Silver et al., 2009; Tummala et al., 2024). This methodology may be useful in determining endothelial Mas_R expression in humans as it can provide a snapshot of a specific protein expression in human circulation. This is also a way to examine cells from human subjects using a feasible, reliable, and minimally invasive technique. However, a protocol has not yet been developed to use this methodology in studying Mas_R.

A research nurse inserted a catheter into the antecubital vein under sterile conditions. Venous endothelial cells were collected using a J-shaped guide wire inserted through the IV catheter. Two guide wires were used per participant, each advanced 2-4 cm past the tip of the IV catheter. After removing the J-wire from the catheter, it was cut and placed into a dissociation buffer (phosphate buffered saline (PBS), 100ug/ml heparin, 2mM EDTA, and 0.5% BSA) to bring the cells into suspension. Cells were pelleted by centrifugation (250 x g for 10min at 22ºC), the supernatant removed, and a red blood cell lysis buffer added for 10 min. Following centrifugation cells were washed using PBS, centrifuged again and then fixed using 4% paraformaldehyde for 10 minutes. Following fixation, cells were plated onto poly-L-Lysine coated #1.5 cover glass and dried at 50°C. Once dehydrated, cells were stored at -80ºC until staining and analysis.

5.2.3 Immunocytochemistry Staining

Coverslips from 20 YW and 20 PMW containing endothelial cells previously harvested from the antecubital vein of PMW and YW were stained for Mas_R using immunocytochemistry techniques with fluorescence. Coverslips were also stained with VE-Cadherin to confirm endothelial cells and DAPI to confirm intact nucleus (Donato et al., 2009; Kuczmarski et al., 2020; Silver et al., 2009). Cells were rehydrated using PBS and then a blocking solution (PBS, 5% normal donkey serum, and 2% cold water fish skin gelatin) was applied for 1 hour at room temperature. Primary antibodies for Mas_R ((Mas_R Polyclonal IgG Antibody, abcam, 1:200, Cat# ab235914), and VE-Cadherin (VE-Cadherin polyclonal antibody, Thermo Fisher Scientific, Cat# 36-1900) were diluted in blocking solution and applied overnight at 4°C. Coverslips were washed three times in PBS after which Alexa Fluor secondary antibodies for Mas_R and VE-Cad were applied for 1 hour at room temperature. Coverslips were then washed three times in PBS before being mounted onto microscope slides using prolong diamond mounting medium that contains DAPI. Slides were stored in the dark for 24 hours at room temperature to allow the mounting medium to harden prior to imaging.

5.2.4 Endothelial Cell Imaging and Analysis

All samples remained blinded during analysis. Imaging was done on a Zeiss Axio imager and HXP 120 V light source with an AxioCam MRm camera. Three channels were used, corresponding to the wavelength of the secondary antibody and DAPI stain. Exposure time and laser intensity were kept constant. Only cells that positively expressed DAPI and VE-Cad were included in analysis. Between 30-100 cells were imaged per participant based on the number of cells present on each slide. Images were analyzed using Zen blue software. The region of interest tool was used to select around the perimeter of the cell. Analysis was done in accordance with previous literature (Kuczmarski, 2021; Kuczmarski et al., 2020; Tummala et al., 2024). Intensity values from the cell were subtracted from the image background. All cells imaged per participant were averaged and normalized to a positive control (human umbilical vein endothelial cells; HUVECs; Figure 5.1) as endothelial cell intensity/HUVEC intensity. The normalization to a HUVEC as a positive control has been done previously to normalize variation in intensities between different cells and different staining sessions (Donato et al., 2009; Silver et al., 2009).

5.2.5 Statistical Analysis

A power analysis was calculated using data examining differences in receptor expression in endothelial cells of YW vs PMW (Kuczmarski et al., 2020) and determined that 15 participants per group would provide 80% power to detect differences using a large effect size (1.5) with an alpha set to $P < 0.05$. One coverslip per subject (20 per group) was stained and at minimum, 30 cells per participant were analyzed to account for errors in cell isolation, staining, and imaging. Unpaired t tests were used to compare baseline characteristics between groups (age, BMI, SBP, DBP, MAP, total cholesterol, LDL cholesterol, HDL cholesterol, and glucose). Unpaired t tests were also used to determine differences in endothelial cell expression of Mas_R between YW and PMW. Pearson's correlations were run to determine relations between Mas_R expression and blood cholesterol, blood glucose, BMI, and BP.

5.3 Results

Of the coverslips stained (20 YW; 20 PMW), 2 of the coverslips containing cells from YW and 1 coverslip containing cells from a PMW there were less than 30 cells found. Therefore, data from 18 YW and 19 PMW were used for analysis. On average, 42+13 cells were analyzed from YW, and 49+19 cells were analyzed from PMW (P= 0.14). As expected, PMW were significantly older than YW. PMW also had greater SBP and MAP. Blood cholesterol and glucose was measured in a subset of participants (17 YW; 18 PMW). In that subset, total cholesterol, HDL cholesterol, LDL cholesterol, and glucose were elevated in PMW compared to YW (table 5.1).

	YW (n = 18)	**PMW** (n = 19)
Age, yrs	22 ± 3	57 ± 5 *
BMI, kg/m^2	22 ± 7	24 ± 3
SBP, mmHg	108 ± 11	118 ± 13 *
DBP, mmHg	71 ± 8	75 ± 9
MAP. mmHg	82 ± 8	90 ± 10 *
Total Cholesterol, mg/dL	170 ± 25	208 ± 36 *
HDL, mg/dL	65 ± 13	77 ± 18 *
LDL, mg/dL	90 ± 24	114 ± 30 *
Glucose, mg/dL	79 ± 6	90 ± 11 *

Table 5.1 Participant characteristics. Values are mean±SD. YW, young women; PMW, postmenopausal women; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; HDL, high-density lipoprotein; LDL, low-density lipoprotein. Total cholesterol, HDL, LDL, and Glucose were measured in a subset (17 YW; 18 PMW). Groups were compared using unpaired t tests. *$P<0.05$ vs. YW.

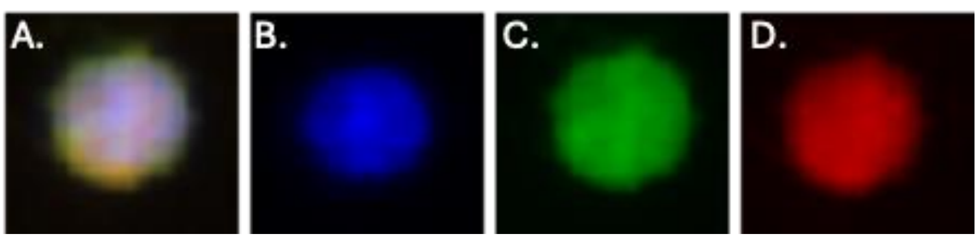

Figure 5.1 Representative images of HUVEC (positive control). Images are A. composite, B. DAPI, C. VECadherin, and D. MasR. All cells were included if they stained positive for DAPI and VECad. Data from positive control was used to normalize data from primary cells taken from participants (MasR intensity of primary cell/ MasR intensity of HUVEC = MasR intensity).

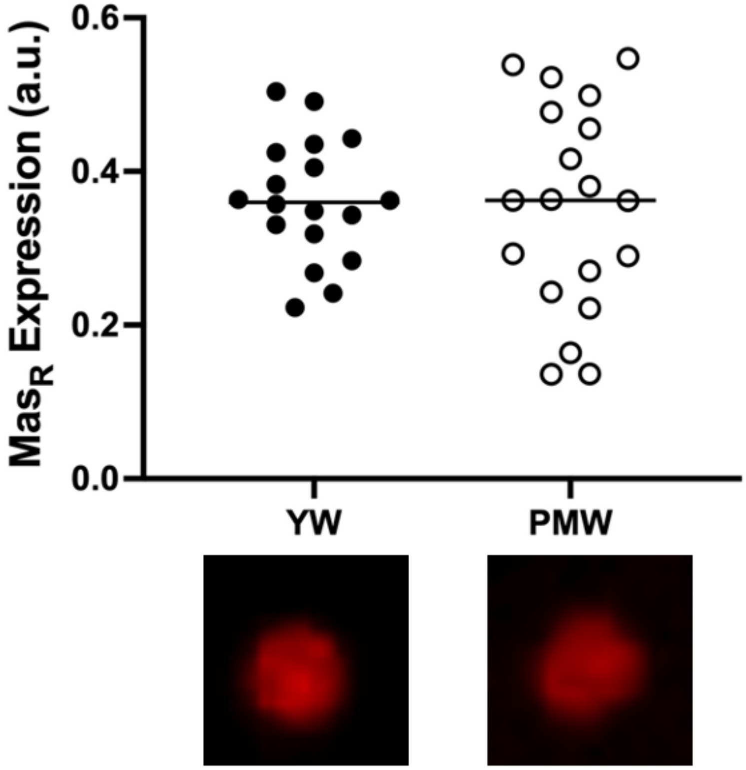

Figure 5.2 Individual values of MasR expression in young women (YW, n=18; closed circles) and postmenopausal women (PMW; n=19; open circles) in arbitrary units (A.U.). An unpaired t test was used to determine differences in cell expression between groups. There were no differences in MasR expression between groups (P= 0.77).

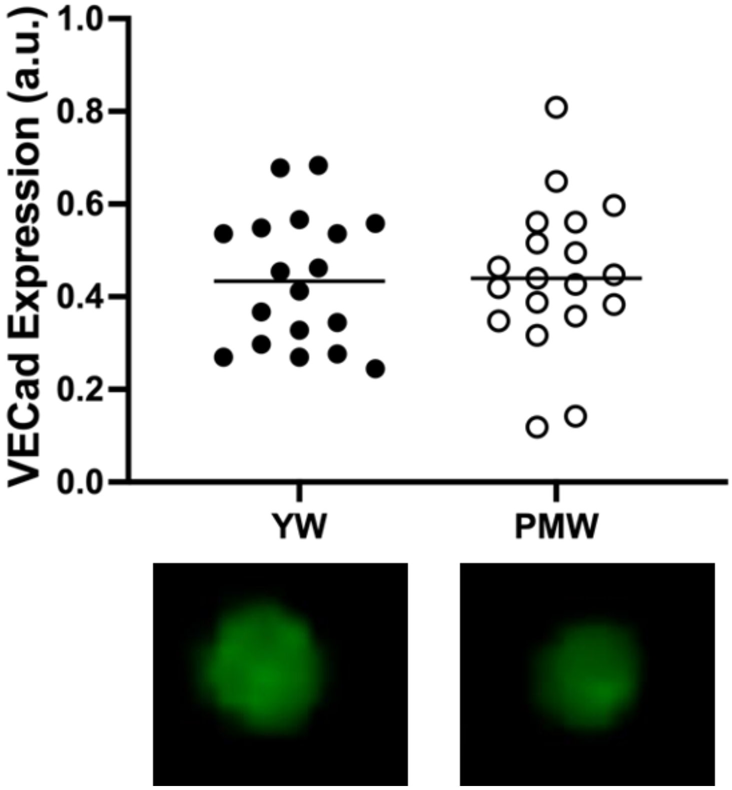

Figure 5.3 Individual values of vascular endothelial cadherin (VECad) expression in young women (YW, n=18; closed circles) and postmenopausal women (PMW; n=19; open circles) in arbitrary units (A.U.). An unpaired t test was used to determine differences in cell expression between groups. There were no differences in VECad expression between groups (P= 0.86).

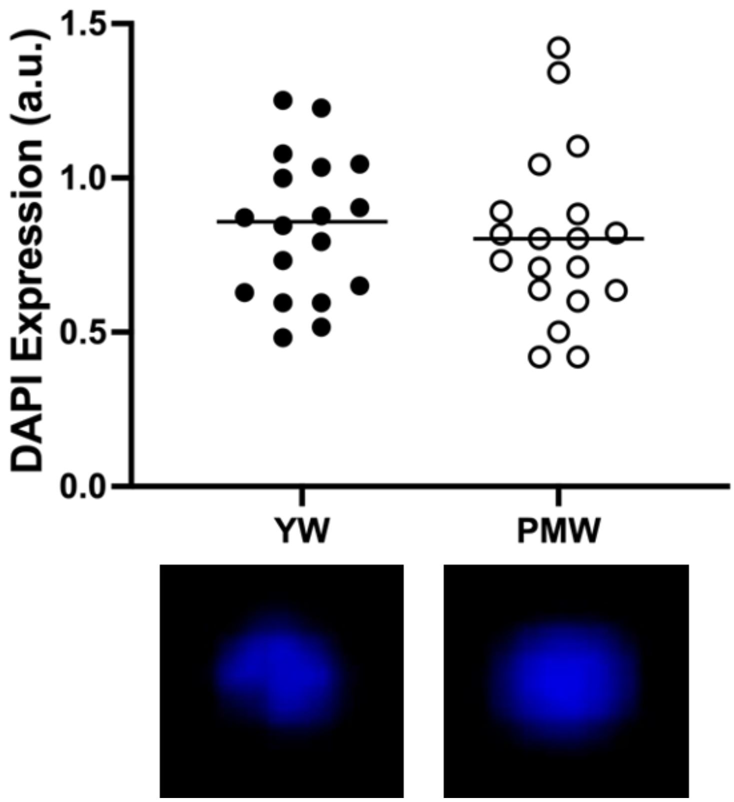

Figure 5.4 Individual values of DAPI nuclear stain expression in young women (YW, n=18; closed circles) and postmenopausal women (PMW; n=19; open circles) in arbitrary units (A.U.). An unpaired t test was used to determine differences in cell expression between groups. There were no differences in VECad expression between groups (P= 0.68).

There were no differences between groups in endothelial cell receptor expression of Mas_R (YW: 0.36±0.08 a.u. vs PMW: 0.35±0.13 a.u.; P= 0.77; Figure 5.2), VECad (YW: 0.44+0.14 a.u. vs. PMW: 0.44+0.16 a.u.; P= 0.86), or DAPI (YW: 0.84+0.23 a.u. vs PMW: 0.81+0.28 a.u.; P= 0.68). Mas_R cell expression in YW and PMW was not correlated to total cholesterol (r= 0.25; P= 0.147), LDL cholesterol (r= 0.21; P= 0.25), HDL cholesterol (r= 0.20; P= 0.26), BMI (r= -0.08; P= 0.65), SBP (r= 0.10; P= 0.58), DBP (r= 0.21; P= 0.24), MAP (r= 0.18; P= 0.32), triglycerides (r=

0.02; P= 0.93), or blood glucose (r= 0.13; P= 0.48). There were also no differences between groups in VECad expression (YW: 0.44$\pm$0.14 a.u. vs PMW: 0.43$\pm$0.16 a.u.; P= 0.86; Figure 5.3) or in DAPI expression (YW: 0.84$\pm$0.23 a.u. vs PMW: 0.81$\pm$0.28 a.u.; P= 0.68; Figure 5.4).

5.4 Discussion

This was the first study to assess Mas_R expression on venous endothelial cells in YW and PMW. The main finding of the current study is that there is no difference in endothelial Mas_R expression between YW and PMW.. This suggests Mas_R expression may not be impacted by the physiological changes that occur with menopause. These results coupled with results from the previous chapters, suggest the Ang-(1-7)/Mas_R axis may be preserved in PMW. There were also no significant correlations found between Mas_R expression and cholesterol, BP, blood glucose, triglycerides, or BMI.

There are discrepancies in the literature regarding the directionality of Mas_R expression in diseased tissues. Costa Fraga and colleagues (2018) found an upregulation of Mas_R in mouse models of aging and menopause. Other studies examining Mas_R in instances of CVD have found the opposite results of Costa Fraga et al. (2018) and have concluded lower Mas_R expression in models of myocardial infarction (Dias-Peixoto et al., 2012). The results of the current study do not provide insight into the direction of change in Mas_R expression due to endothelial dysfunction. However, it should be noted the women included in this study were all generally healthy based on traditional CVD risk factors. Despite the lack of differences in receptor expression between the YW and PMW in this study, we successfully stained for Mas_R in the human endothelium, providing valuable information for future investigations that aim to examine endothelial Mas_R expression in different populations. Further, these results may provide evidence that endothelial Mas_R expression is not impacted by menopause, warranting investigation into other proteins such as MrgD, another known receptor for Ang-(1-7), within the Ang-(1-7) pathway that might play a role in endothelial function in PMW.

Factors Impacting Endothelial Mas_R Expression

5.4.1 Estrogen

When examining the results of the current analysis in the context of menopause, ovarian hormones should be noted. Throughout menopause, endogenous estrogen levels decrease over time, leading to progressive physiologic adaptations. Therefore, a woman sooner after menopause may show different adaptations than a woman who has been postmenopausal for a greater amount of time. The PMW who participated in this study were all at least 1 year postmenopausal and are all relatively younger postmenopausal women. It is possible that the adaptations that occur due to loss of estrogen require more time without estrogen present to see compensatory mechanisms such as an upregulation of a receptor. This may explain the lack of differences in receptor expression between groups in the current study.

There is also evidence within the literature that estrogen interacts with the Ang-(1-7)/Mas_R axis to cause vasodilation, mainly through NO-mediated mechanisms (Saberi et al., 2016; Sobrino et al., 2017). However, these studies are exclusive to animal and cell research and have not yet been confirmed in humans. While there is evidence that Ang-(1-7) can improve endothelium-dependent dilation in women (Stanhewicz et al., 2018; Stewart et al., 2009), it has not specifically been confirmed that an interaction exists between estrogen and Ang-(1-7) in humans. Therefore, future research should confirm this relationship in women.

5.4.2 Cholesterol

There was no association between Mas_R and any measure of cholesterol. This analysis was performed to determine any other potential driving factors of endothelial Mas_R expression. There is a relationship between Mas_R and cholesterol in the literature in which Ang-(1-7) activation through Mas_R might be atheroprotective through increased NO release and endothelium-dependent dilation (Fraga-Silva et al., 2008; Silva et al., 2013). Greater Mas_R expression is also associated with lower lipid profiles (Yang et al., 2018). Endothelial Mas_R expression was compared with LDL cholesterol, HDL cholesterol, and total cholesterol. LDL, HDL, and total cholesterol were all not associated with Mas_R expression in the total sample.

5.4.3 Methodology

The difference in obtaining samples from mouse models and humans is significant and can, alone, account for the differences in results between these studies. Further, the samples used in Costa Fraga et al. (2018) were obtained from intact aorta while isolated venous endothelial cells were used in the current analysis. Therefore, the tissue or cell sample were different in each study, were taken from different host species, and different methodologies were used to test them. The methodology used in the current study was chosen based on its feasibility, and proven ability to provide a snapshot of protein expression within the human vascular endothelium (Colombo et al., 2002; Donato et al., 2009; Silver et al., 2009). However, given that these results do not align with the literature in this area, future research might consider different methodology to confirm that endothelial Mas_R expression is not affected by menopause in women.

While Mas_R is generally considered the primary receptor for Ang-(1-7), the full Ang-(1-7) pathway has still not yet been fully elucidated. This includes the possibility of other receptors within the pathway. For instance, a second GPCR has recently been identified as compatible with Ang-(1-7), referred to as MrgD (Tetzner et al., 2016). There is also evidence that other known receptors within the vasculature may interact with Ang-(1-7). Angiotensin type 2 receptor (AT2R) is among the most notable (Albrecht, 2007; Almeida-Santos et al., 2016; Bürgelová et al., 2009; Mansoori et al., 2016). It has also been suggested that the bradykinin receptor (B2) might play a role in the vasodilatory actions of Ang-(1-7) (Albrecht, 2007; Brosnihan et al., 1996; Li et al., 1997). Given this evidence, and the uncertainty of receptors within the Ang-(1-7) system, it is possible that we did not see any effect of menopause or aging on Mas_R in the current study because we only isolated one receptor. Future investigations might consider the roles of other possible receptors within this pathway in the context of menopause and aging.

5.4.4 Limitations

There are some limitations with the current analysis. First, Mas_R is a G-protein coupled receptor (GPCR) and it has previously been established that antibodies for GPCRs may lack in their ability to accurately stain for the protein of interest. Burghi and colleagues detailed some issues with current Mas_R antibodies that are commercially available (Burghi et al., 2017). They eventually determined only select antibodies produced successful physiological results in methods such as immunocytochemistry staining from Mas_R. The results from this analysis bring the validity of commercially available Mas_R antibodies into question. The antibody used in the current study was chosen based on pilot testing while considering the guidance in choosing an antibody from Burghi and colleagues (Burghi et al., 2017).

Second, the cells used in this study were all venous endothelial cells, not arterial endothelial cells. Therefore, they are not the main actors of vascular tone in measurements of endothelial function. However, the harvesting of venous endothelial cells is more feasible and less invasive than the harvest of arterial cells. It has been determined that protein expression on venous endothelial cells is correlated to protein expression on arterial endothelial cells (Silver et al., 2009), however, Mas_R was not specifically examined in this study. Thus, without evidence that Mas_R expression is the same on both the arterial and venous side of the circulation, it is possible that Mas_R might be an exception to the findings of Silver and colleagues (2009). Also, with this in mind, Costa Fraga and colleagues (2018) used aortic tissue which might be more representative of adaptations that occur on the arterial side of the vasculature. Another potential explanation for the disagreement in this analysis with the findings of Costa Fraga et al. is in methodology. Costa Fraga et al. used Western Blotting to determine Mas_R expression in their samples, while this study employed immunofluorescence staining techniques. While both methodologies have been proven to give representative expressions of protein (Burghi et al., 2017; Gaina et al., 2008; Prehn et al., 2023; Robert et al., 1996), it is possible that this difference in methodology may account for the difference in findings between this study and the study done by Costa Fraga and colleagues. Western blotting is a proven method of total cellular protein expression while the immunofluorescence staining technique used in the current study is targeting membrane proteins.

It should be noted that the PMW studied were all recently postmenopausal (5$\pm$3 years) and it is unknown if a more prolonged decreased exposure to estrogen is necessary to see differences in Ang-(1-7)-mediated vasodilation. It has been shown in the literature that women who are more recently menopausal have preserved vascular function. Moreau and Hildreth (2014) have shown that there is a stepwise decline from premenopause to late post menopause in measurements of endothelial function (Moreau et al., 2012) and arterial compliance (Hildreth et al., 2014). This is likely due to a combination of regular aging and increased time since chronic estrogen exposure.

Finally, in the current investigation, plasma Ang-(1-7) concentration was not assessed. It has previously been reported that women have a greater plasma concentration of Ang-(1-7) than young men and Ang-(1-7) plasma concentration (young women: 49.5$\pm$4.4 pg/mL; young men: 35.9$\pm$3.1 pg/mL; statistically significant at P= 0.04) is associated with endothelial function (Sullivan et al., 2015). These data along with the findings from Costa Fraga and colleagues suggest that endothelial Mas_R upregulation might be a compensatory mechanism due to a decrease in circulating Ang-(1-7) following menopause. Data on the plasma concentrations of Ang-(1-7) would provide greater insight into the presence of Ang-(1-7) as a ligand for Mas_R. This information could also potentially provide an explanation for the lack of differences between Mas_R expression between groups by showing if differences exist in plasma Ang-(1-7) concentration between groups. Further, it would provide insight into whether a compensatory upregulation is warranted in PMW, as they would need to show a decline in plasma Ang-(1-7) before an upregulation of Mas_R.

5.4.5 Conclusion

In conclusion, this is the first study to analyze endothelial Mas_R expression in humans and can inform future studies that may further examine endothelial Mas_R expression in humans. We did not observe differences in Mas_R between YW and PMW in venous endothelial cells. These data provide an important step in the isolation of the Ang-(1-7)/Mas_R axis as a main actor in regulating vascular function in women. The compensatory upregulation of Mas_R proposed does not appear to be present. However, this does not rule out the role of Mas_R in endothelial dysfunction that occurs with menopause. Given these findings and the limitations of this study, future investigations should study Mas_R expression along with expression of other potential receptors for Ang-(1-7) and with plasma concentration of Ang-(1-7).

Chapter 6

6.1 Summary

Ang-(1-7) has been categorized as a peptide opposite of Angiotensin II that counteracts many of the vasoconstrictive and generally pro-disease states caused by the classical arm of the Renin Angiotensin System (RAS) (Medina & Arnold, 2019; Roks et al., 1999; Walkyria O. Sampaio et al., 2007; Santos, 2014; Santos et al., 2018; Ueda et al., 2000). Thus, the arm of the RAS led by the actions of Ang-(1-7) through its receptor, Mas_R, has commonly been referred to as the vasoprotective arm of the RAS. More recently it was discovered that Ang-(1-7) interacts with estrogen and therefore may impact vascular function in women differently than in men. These discoveries began in cell and animal studies, where the presence of estrogen increased the activity of this pathway, specifically leading to overall greater vasodilatory states and improved vascular function (Mompeón et al., 2016; Neves et al., 2004; Saberi et al., 2016). Later research in humans showed that women had a greater plasma concentration of Ang-(1-7) than men, and that increased Ang-(1-7) plasma concentrations are associated with greater endothelial function (Sullivan et al., 2015). Further studies confirmed Ang-(1-7) may rescue endothelial dysfunction in younger women with high risk of developing CVD (Stanhewicz & Alexander, 2020; Stewart et al., 2009), further suggesting Ang-(1-7) might be a useful target in women with endothelial dysfunction. The potential for Ang-(1-7) to play a role in improving vascular endothelial function in women following menopause was solidified with a study done by Costa-Fraga et al. (2018), showing a blunted vasodilatory response to Ang-(1-7) in animal models of menopause and aging. This is notable as the mechanisms contributing to the decline in vascular function following menopause remain unknown (Costa-Fraga et al., 2018).

Given the existing literature, our research aimed to further elucidate the role of Ang-(1-7) in vascular function in postmenopausal women. We tested the vascular sensitivity to Ang-(1-7) in YW and PMW with the hypothesis that PMW would show a blunted sensitivity to Ang-(1-7). We also hypothesized that vasodilation caused by Ang-(1-7) would be NO-dependent. We did not find blunted sensitivity to Ang-(1-7) in PMW, nor was it apparent that dilation in response to Ang-(1-7) was mediated through an NO-dependent mechanism. However, we did establish a dose-response curve for Ang-(1-7) in the microvasculature in women, providing a depiction of the vascular sensitivity to local administration of escalating doses of Ang-(1-7). Further, the results of this aim provide evidence that vascular sensitivity to Ang-(1-7) is preserved in PMW.

We further aimed to show that Ang-(1-7) might improve blunted endothelial function in PMW. We did not conclude that Ang-(1-7) improved endothelial function on PMW, nor was the endothelial response to Ang-(1-7) determined to be NO-dependent. Though this finding is in contrast with our hypothesis, it provides rationale for future study of this pathway in women across the menopausal transition. The final aim of this dissertation was exploratory. We aimed to examine the endothelial expression of Mas_R by quantifying the presence of Mas_R in endothelial cells harvested from YW and PMW. Previous research suggested an upregulation of Mas_R might be present due to a decrease in production of Ang-(1-7) that might occur with age and potentially menopause (Costa-Fraga et al., 2018). However, there did not appear to be any differences in endothelial Mas_R expression between YW and PMW, indicating that there may not be any present upregulation.

The overall conclusion of this research is that Ang-(1-7) may not impact vascular endothelial function in PMW. This is not in agreement with our overall hypothesis. However, these data provide an interesting perspective and important first step in elucidating the role of the Ang-(1-7)/Mas_R axis in regulating vascular function in PMW. More importantly, these results in the context of the existing literature have the potential to generate further hypotheses in future study of the role of this pathway in regulating vascular endothelial function following the menopausal transition.

6.2 Perspectives

Women, specifically postmenopausal women, are a classically understudied group in physiology research. Further, CVD is a leading cause of death in these women and common methods of preventing or treating CVD in men do not appear to be as effective in women as they are in men of similar age (El Khoudary et al., 2020; Virani et al., 2021). CVD is generally preceded by declines in vascular function that occurs with normal aging or with coexisting disease states. Indeed, men and women see declines in vascular function with age, however, women show a sharp decline in vascular function following the time of menopause (Celermajer et al., 1994). Additionally. the rate of vascular decline occurs much faster in women than it does in men, and it has been determined that the physiological pathways responsible for this decline are different in women than they are in men. This decline in vascular function has been attributed to the loss of estrogen that occurs with menopause (Knowlton & Lee, 2012; Somani et al., 2019), however, the specific mechanisms leading to this decline remain unknown. Thus, it is important for research to examine different mechanisms that might impact vascular function in PMW. Given the current literature suggesting Ang-(1-7) might play a role in regulating vascular endothelial function in women it is important to study this pathway in this population.

The studies presented in this dissertation are the first to examine the role of the Ang-(1-7)/Mas_R axis in PMW. This pathway has been studied in cell and animal models, and in women with existing endothelial dysfunction, but had not yet been studied in PMW. The results from these studies provide an important next step in the literature examining the role of the Ang-(1-7)/Mas_R axis in vascular endothelial function in PMW, as these results combined with the current literature provide a strong rationale for further study of this pathway alongside estrogen in PMW. We showed that PMW have a preserved vascular sensitivity to Ang-(1-7), however local administration of Ang-(1-7) does not appear to improve microvascular endothelial dysfunction present in PMW. Given the dearth of literature suggesting estrogen might be necessary to see positive impacts of Ang-(1-7) on endothelial function, this study might provide further rationale to study this pathway alongside estrogen in this population.

Importantly, we are the first to establish a dose-response curve to test vascular sensitivity to Ang-(1-7) in both young and postmenopausal women. These curves might provide valuable information for future studies that aim to locally administer Ang-(1-7). We are also the first to successfully stain human endothelial cells for Mas_R using methods that had previously been established in other pathways such as oxidative stress and the endothelin pathway. Mas_R had previously been quantified in other human and animal cells, but not yet in human endothelial cells. Given the increasing interest in Mas_R as a receptor for Ang-(1-7), and its ability to improve vascular function and promote an overall vasoprotective state, Mas_R will be a popular target for future study. Thus, development of protocols to successfully stain for Mas_R will be important to further the knowledge on this receptor.

6.3 Future Directions

Though the findings of this dissertation disagree with the proposed hypotheses, the conclusions provide valuable information for future research that aims to elucidate the role of the Ang-(1-7)/Mas_R axis in vascular endothelial function in PMW. It is apparent that Ang-(1-7) may not improve endothelial function in PMW presently, but the cell and animal research in this area suggests estrogen may be necessary to elicit some of the effects of Ang-(1-7) on the vasculature. This is likely given previous research that Ang-(1-7) causes greater dilation in mouse models of menopause and aging when in the presence of estrogen (Costa-Fraga et al., 2018). Further, women with preeclampsia and POTS who show improved endothelial function from local Ang-(1-7) administration were all premenopausal women with circulating estrogen (Stanhewicz & Alexander, 2020; Stewart et al., 2009). The results of this dissertation along with this existing literature on Ang-(1-7) with estrogen provide rationale to study Ang-(1-7) alongside estrogen in PMW and in women earlier in the menopausal transition. Our lab has employed an estrogen knockdown and addback model to isolate the role of estrogen in past studies, specifically targeting the endothelin system. As our lab and others continue to study the Ang-(1-7)/Mas_R axis in PMW, methods such as these should be employed to determine whether the findings.

In context of the full menopausal transition, it is important to note that the decline in vascular function that occurs with menopause is progressive and begins earlier in the menopausal transition. Research on vascular function in women suggests earlier intervention, around the time of perimenopause or even earlier is best to prevent vascular dysfunction that occurs because of the loss of estrogen following menopause (El Khoudary et al., 2020; Hodis et al., 2016; Hodis et al., 2014). Studying only PMW might not provide a full picture of the physiological changes that occur with menopause. Future research should also consider the full menopausal transition.

Much of the research on Ang-(1-7) is currently focused on the direction of development of therapeutics, as Ang-(1-7) provides an ideal target for metrics of cardiovascular health such as blood pressure control and endothelial function (Miller & Arnold, 2023). The results of this dissertation also point to another direction in the future of research on this pathway. Research on the Ang-(1-7)/Mas_R axis in women, specifically postmenopausal women is new, yet promising, in providing information on another pathway that might be impacted by the decline in estrogen production following menopause. Thus, studying this pathway in the context of menopause, estrogen, and women's vascular health will likely improve our current understanding of the physiological pathways impacted by menopause and potentially provide a new pathway to target when considering treatment and prevention of CVD in postmenopausal women.

REFERENCES

Abularrage, C. J., Sidawy, A. N., Aidinian, G., Singh, N., Weiswasser, J. M., & Arora, S. (2005). Evaluation of the microcirculation in vascular disease. *Journal of Vascular Surgery*, *42*(3), 574-581.

Adler, T. E., Usselman, C. W., Takamata, A., & Stachenfeld, N. S. (2018). Blood pressure predicts endothelial function and the effects of ethinyl estradiol exposure in young women. *American Journal of Physiology-Heart and Circulatory Physiology*, *315*(4), H925-H933. https://doi.org/10.1152/ajpheart.00188.2018

Agapitov, A. V., & Haynes, W. G. (2002). Role of endothelin in cardiovascular disease. *Journal of the Renin-Angiotensin-Aldosterone System*, *3*(1), 1-15.

Agarwal, S. C., Allen, J., Murray, A., & Purcell, I. F. (2009). Comparative reproducibility of dermal microvascular blood flow changes in response to acetylcholine iontophoresis, hyperthermia and reactive hyperaemia. *Physiological measurement*, *31*(1), 1.

Albrecht, D. (2007). Angiotensin-(1-7)-induced plasticity changes in the lateral amygdala are mediated by COX-2 and NO. *Learning & Memory*, *14*(3), 177-184. https://doi.org/10.1101/lm.425907

Alenina, N., Xu, P., Rentzsch, B., Patkin, E. L., & Bader, M. (2008). Genetically altered animal models for Mas and angiotensin-(1-7). *Experimental Physiology*, *93*(5), 528-537. https://doi.org/10.1113/expphysiol.2007.040345

Alexander, S. P., Mathie, A., & Peters, J. A. (2011). Guide to receptors and channels (GRAC). *British Journal of Pharmacology*, *164*, S1-S2.

Almeida-Santos, Ana F., Kangussu, Lucas M., Moreira, Fabrício A., Santos, Robson A. S., Aguiar, Daniele C., & Campagnole-Santos, Maria J. (2016). Anxiolytic- and antidepressant-like effects of angiotensin-(1–7) in hypertensive transgenic (mRen2)27 rats. *Clinical Science*, *130*(14), 1247-1255. https://doi.org/10.1042/cs20160116

Andreatta-van Leyen, S., Romero, M. F., Khosla, M. C., & Douglas, J. G. (1993). Modulation of phospholipase A2 activity and sodium transport by angiotensin-(1-7). *Kidney International*, *44*(5), 932-936.

Arora, S., Veves, A., Caballaro, A. E., Smakowski, P., & LoGerfo, F. W. (1998). Estrogen improves endothelial function. *Journal of Vascular Surgery*, *27*(6), 1141-1147. https://doi.org/https://doi.org/10.1016/S0741-5214(98)70016-3

Babcock, M., DuBose, L., Stauffer, B., & Moreau, K. (2023). Endothelin-1 Contributes to Reduced Endothelial Function in Middle-aged/Older Men with Low Testosterone. *Physiology*, *38*(S1), 5729567.

Baker, L., Meldrum, K. K., Wang, M., Sankula, R., Vanam, R., Raiesdana, A., Tsai, B., Hile, K., Brown, J. W., & Meldrum, D. R. (2003). The role of estrogen in cardiovascular disease. *Journal of surgical research*, *115*(2), 325-344.

Ballermann, B. J., Dardik, A., Eng, E., & Liu, A. (1998). Shear stress and the endothelium. *Kidney International*, *54*, S100-S108.

Baracho, N. C. V., Simões-e-Silva, A. C., Khosla, M. C., & Santos, R. A. S. (1998). Effect of selective angiotensin antagonists on the antidiuresis produced by angiotensin-(1-7) in water-loaded rats. *Brazilian Journal of Medical and Biological Research*, *31*.

Barton, M., Cremer, J., & Mügge, A. (1998). 17β-Estradiol acutely improves endothelium-dependent relaxation to bradykinin in isolated human coronary arteries. *European journal of pharmacology*, *362*(1), 73-76.

Bauersachs, J., Popp, R. d., Hecker, M., Sauer, E., Fleming, I., & Busse, R. (1996). Nitric oxide attenuates the release of endothelium-derived hyperpolarizing factor. *Circulation*, *94*(12), 3341-3347.

Becker, L. K., Etelvino, G. M., Walther, T., Santos, R. A., & Campagnole-Santos, M. J. (2007). Immunofluorescence localization of the receptor Mas in cardiovascular-related areas of the rat brain. *American Journal of Physiology-Heart and Circulatory Physiology*, *293*(3), H1416-H1424.

Bellanti, F., Matteo, M., Rollo, T., De Rosario, F., Greco, P., Vendemiale, G., & Serviddio, G. (2013). Sex hormones modulate circulating antioxidant enzymes: impact of estrogen therapy. *Redox biology*, *1*(1), 340-346.

Benter, I. F., Ferrario, C. M., Morris, M., & Diz, D. I. (1995). Antihypertensive actions of angiotensin-(1-7) in spontaneously hypertensive rats. *American Journal of Physiology-Heart and Circulatory Physiology*, *269*(1), H313-H319. https://doi.org/10.1152/ajpheart.1995.269.1.H313

Benter, I. F., Yousif, M. H., Anim, J., Cojocel, C., & Diz, D. (2006). Angiotensin-(1–7) prevents development of severe hypertension and end-organ damage in spontaneously hypertensive rats treated with L-NAME. *American Journal of Physiology-Heart and Circulatory Physiology*, *290*(2), H684-H691.

Best, P. J., Berger, P. B., Miller, V. M., & Lerman, A. (1998). The effect of estrogen replacement therapy on plasma nitric oxide and endothelin-1 levels in postmenopausal women. *Annals of Internal Medicine*, *128*(4), 285-288.

Bircher, A., de Boer, E. M., Agner, T., Wahlberg, J. E., & Serup, J. (1994). Guidelines for measurement of cutaneous blood flow by laser Doppler flowmetry. *Contact Dermatitis*, *30*(2), 65-72. https://doi.org/https://doi.org/10.1111/j.1600-0536.1994.tb00565.x

Bomtempo, C. A., Santos, G. F., Santos, R. A., & Campagnole-Santos, M. J. (1998). Interaction of bradykinin and angiotensin-(1–7) in the central modulation of the baroreflex control of the heart rate. *Journal of hypertension*, *16*(12), 1797-1804.

Botelho-Santos, G. A., Bader, M., Alenina, N., & Santos, R. A. S. (2012). Altered regional blood flow distribution in Mas-deficient mice. *Therapeutic Advances*

in Cardiovascular Disease, *6*(5), 201-211. https://doi.org/10.1177/1753944712461164
Brandes, R. P. (2014). Endothelial dysfunction and hypertension. *Hypertension*, *64*(5), 924-928.
Brevetti, G., Silvestro, A., Schiano, V., & Chiariello, M. (2003). Endothelial dysfunction and cardiovascular risk prediction in peripheral arterial disease: additive value of flow-mediated dilation to ankle-brachial pressure index. *Circulation*, *108*(17), 2093-2098.
Brosnihan, K., Neves, L., Anton, L., Joyner, J., Valdes, G., & Merrill, D. (2004). Enhanced expression of Ang-(1-7) during pregnancy. *Brazilian Journal of Medical and Biological Research*, *37*, 1255-1262.
Brosnihan, K. B., Li, P., & Ferrario, C. M. (1996). Angiotensin-(1-7) dilates canine coronary arteries through kinins and nitric oxide. *Hypertension*, *27*(3), 523-528.
Brosnihan, K. B., Li, P., Ganten, D., & Ferrario, C. M. (1997). Estrogen protects transgenic hypertensive rats by shifting the vasoconstrictor-vasodilator balance of RAS. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, *273*(6), R1908-R1915. https://doi.org/10.1152/ajpregu.1997.273.6.R1908
Buchanan, C. H., Brown, E. A., Bishu, K. G., Weeda, E., Axon, R. N., Taber, D. J., & Gebregziabher, M. (2022). The Magnitude and Potential Causes of Sex Disparities in Statin Therapy in Veterans with Type 2 Diabetes: A 10-year Nationwide Longitudinal Cohort Study. *Women's Health Issues*, *32*(3), 274-283. https://doi.org/https://doi.org/10.1016/j.whi.2021.10.003
Bürgelová, M., Kramer, H. J., Teplan, V., Veličková, G., Vítko, Š., Heller, J., Malý, J., & Červenka, L. (2002). Intrarenal Infusion of Angiotensin-(1–7) Modulates Renal Functional Responses to Exogenous Angiotensin II in the Rat. *Kidney and Blood Pressure Research*, *25*(4), 202-210. https://doi.org/10.1159/000066340
Bürgelová, M., Vanourková, Z., Thumová, M., Dvorák, P., Opocenský, M., Kramer, H. J., Želízko, M., Malý, J., Bader, M., & Cervenka, L. (2009). Impairment of the angiotensin-converting enzyme 2–angiotensin-(1-7)-Mas axis contributes to the acceleration of two-kidney, one-clip Goldblatt hypertension. *Journal of hypertension*, *27*(10), 1988-2000. https://doi.org/10.1097/HJH.0b013e32832f0d06
Burger, H. G., Dudley, E. C., Robertson, D. M., & Dennerstein, L. (2002). Hormonal changes in the menopause transition. *Recent progress in hormone research*, *57*, 257-276.
Burghi, V., Fernández, N. C., Gándola, Y. B., Piazza, V. G., Quiroga, D. T., Guilhen Mario, É., Felix Braga, J., Bader, M., Santos, R. A. S., & Dominici, F. P. (2017). Validation of commercial Mas receptor antibodies for utilization in Western Blotting, immunofluorescence and immunohistochemistry studies. *PLOS ONE*, *12*(8), e0183278.

Busse, R., Edwards, G., Félétou, M., Fleming, I., Vanhoutte, P. M., & Weston, A. H. (2002). EDHF: bringing the concepts together. *Trends in pharmacological sciences*, *23*(8), 374-380.

Callera, G. E., Tostes, R. C., Yogi, A., Montezano, A. C., & Touyz, R. M. (2006). Endothelin-1-induced oxidative stress in DOCA-salt hypertension involves NADPH-oxidase-independent mechanisms. *Clinical Science*, *110*(2), 243-253.

Campagnole-Santos, M. J., Heringer, S. B., Batista, E. N., Khosla, M. C., & Santos, R. (1992). Differential baroreceptor reflex modulation by centrally infused angiotensin peptides. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, *263*(1), R89-R94.

Cao, X., Yang, F.-Y., Xin, Z., Xie, R.-R., & Yang, J.-K. (2014). The ACE2/Ang-(1–7)/Mas axis can inhibit hepatic insulin resistance. *Molecular and cellular endocrinology*, *393*(1-2), 30-38.

Celermajer, D. S., Sorensen, K. E., Spiegelhalter, D. J., Georgakopoulos, D., Robinson, J., & Deanfield, J. E. (1994). Aging is associated with endothelial dysfunction in healthy men years before the age-related decline in women. *Journal of the American College of Cardiology*, *24*(2), 471-476.

Chakrabarti, S., Lekontseva, O., & Davidge, S. T. (2008). Estrogen is a modulator of vascular inflammation. *IUBMB Life*, *60*(6), 376-382. https://doi.org/https://doi.org/10.1002/iub.48

Chambliss, K. L., & Shaul, P. W. (2002). Estrogen Modulation of Endothelial Nitric Oxide Synthase. *Endocrine Reviews*, *23*(5), 665-686. https://doi.org/10.1210/er.2001-0045

Chan, S. Y., Mancini, G. J., Kuramoto, L., Schulzer, M., Frohlich, J., & Ignaszewski, A. (2003). The prognostic importance of endothelial dysfunction and carotid atheromaburden in patients with coronary artery disease. *Journal of the American College of Cardiology*, *42*(6), 1037-1043.

Chang, R. L., Chang, C. F., Ju, D. T., Ho, T. J., Chang, T. T., Lin, J. W., Li, J. C., Cheng, S. M., Day, C. H., & Viswanadha, V. P. (2018). Short-term hypoxia upregulated Mas receptor expression to repress the AT1R signaling pathway and attenuate Ang II-induced cardiomyocyte apoptosis. *Journal of cellular biochemistry*, *119*(3), 2742-2749.

Chen, Q., Yang, Y., Huang, Y., Pan, C., Liu, L., & Qiu, H. (2013). Angiotensin-(1-7) attenuates lung fibrosis by way of Mas receptor in acute lung injury. *Journal of surgical research*, *185*(2), 740-747.

Chen, Y.-F., Naftilan, A. J., & Oparil, S. (1992). Androgen-dependent angiotensinogen and renin messenger RNA expression in hypertensive rats. *Hypertension*, *19*(5), 456-463.

Chen, Y.-P., Lu, Y.-P., Li, J., Liu, Z.-W., Chen, W.-J., Liang, X.-J., Chen, X., Wen, W.-R., Xiao, X.-M., & Reichetzeder, C. (2014). Fetal and maternal angiotensin (1-7) are associated with preterm birth. *Journal of hypertension*, *32*(9), 1833-1841.

Chen, Z., Yuhanna, I. S., Galcheva-Gargova, Z., Karas, R. H., Mendelsohn, M. E., & Shaul, P. W. (1999). Estrogen receptor α mediates the nongenomic activation of endothelial nitric oxide synthase by estrogen. *The Journal of clinical investigation*, *103*(3), 401-406.

Clark, J. F., & Pyne-Geithman, G. (2005). Vascular smooth muscle function: the physiology and pathology of vasoconstriction. *Pathophysiology*, *12*(1), 35-45.

Collier, M. L., Ji, G., Wang, Y., & Kotlikoff, M. I. (2000). Calcium-induced calcium release in smooth muscle: loose coupling between the action potential and calcium release. *J Gen Physiol*, *115*(5), 653-662. https://doi.org/10.1085/jgp.115.5.653

Colombo, P. C., Ashton, A. W., Celaj, S., Talreja, A., Banchs, J. E., Dubois, N. B., Marinaccio, M., Malla, S., Lachmann, J., & Ware, J. A. (2002). Biopsy coupled to quantitative immunofluorescence: a new method to study the human vascular endothelium. *Journal of applied physiology*, *92*(3), 1331-1338.

Costa, A. P., Fagundes-Moura, C. R., Pereira, V. M., Silva, L. F., Vieira, M. A. R., Santos, R. A., & Dos Reis, A. M. (2003). Angiotensin-(1–7): a novel peptide in the ovary. *Endocrinology*, *144*(5), 1942-1948.

Costa-Fraga, F. P., Goncalves, G. K., Souza-Neto, F. P., Reis, A. M., Capettini, L. A., Santos, R. A., Fraga-Silva, R. A., Stergiopulos, N., & da Silva, R. F. (2018). Age-related changes in vascular responses to angiotensin-(1-7) in female mice. *Journal of the Renin-Angiotensin-Aldosterone System*, *19*(3), 1470320318789332.

Cowley, S. M., Hoare, S., Mosselman, S., & Parker, M. G. (1997). Estrogen receptors α and β form heterodimers on DNA. *Journal of Biological Chemistry*, *272*(32), 19858-19862.

Cunningham, K. S., & Gotlieb, A. I. (2005). The role of shear stress in the pathogenesis of atherosclerosis. *Laboratory Investigation*, *85*(1), 9-23. https://doi.org/10.1038/labinvest.3700215

Davenport, A. P., Hyndman, K. A., Dhaun, N., Southan, C., Kohan, D. E., Pollock, J. S., Pollock, D. M., Webb, D. J., & Maguire, J. J. (2016). Endothelin. *Pharmacological reviews*, *68*(2), 357-418.

Davie, A. P., & McMurray, J. J. (1999). ACE inhibitors and heart failure in hospital: any difference between cardiologists and general physicians? *Postgraduate Medical Journal*, *75*(882), 219-222. https://doi.org/10.1136/pgmj.75.882.219

Davies, M. J., Richardson, P. D., Woolf, N., Katz, D. R., & Mann, J. (1993). Risk of thrombosis in human atherosclerotic plaques: role of extracellular lipid, macrophage, and smooth muscle cell content. *Heart*, *69*(5), 377-381.

Davignon, J., & Ganz, P. (2004). Role of endothelial dysfunction in atherosclerosis. *Circulation*, *109*(23_suppl_1), III-27-III-32.

Davis, S. R., Lambrinoudaki, I., Lumsden, M., Mishra, G. D., Pal, L., Rees, M., Santoro, N., & Simoncini, T. (2015). Menopause. *Nature Reviews Disease Primers*, *1*(1), 15004. https://doi.org/10.1038/nrdp.2015.4

Dawson, E. A., Green, D. J., Cable, N. T., & Thijssen, D. H. J. (2013). Effects of acute exercise on flow-mediated dilatation in healthy humans. *Journal of applied physiology*, *115*(11), 1589-1598. https://doi.org/10.1152/japplphysiol.00450.2013

De Wet, H., Allen, M., Holmes, C., Stobbart, M., Lippiat, J. D., De Wet, H., Allen, M., Holmes, C., Stobbart, M., & Lippiat, J. D. (2006). Modulation of the BK channel by estrogens: examination at single channel level. *Molecular membrane biology*, *23*(5), 420-429.

Debbabi, H., Bonnin, P., Ducluzeau, P. H., Lefthériotis, G., & Levy, B. I. (2010). Noninvasive assessment of endothelial function in the skin microcirculation. *American journal of hypertension*, *23*(5), 541-546.

Dehghani, A., Saberi, S., & Nematbakhsh, M. (2015). Nitric oxide synthases blockade; L-NAME and estradiol alter renal blood flow response to angiotensin 1-7 in ovariectomized rats. *Indian J Physiol Pharmacol*, *59*(3), 266-274.

DelliPizzi, A., Hilchey, S. D., & Bell-Quilley, C. P. (1994). Natriuretic action of angiotensin(1–7). *British Journal of Pharmacology*, *111*(1), 1-3. https://doi.org/https://doi.org/10.1111/j.1476-5381.1994.tb14014.x

Dhaun, N., Goddard, J., & Webb, D. (2006). The endothelin system and its antagonism in chronic kidney disease. *Journal of the American Society of Nephrology*, *17*(4), 943-955.

Dhawan, S. S., Avati Nanjundappa, R. P., Branch, J. R., Taylor, W. R., Quyyumi, A. A., Jo, H., McDaniel, M. C., Suo, J., Giddens, D., & Samady, H. (2010). Shear stress and plaque development. *Expert Rev Cardiovasc Ther*, *8*(4), 545-556. https://doi.org/10.1586/erc.10.28

Dias-Peixoto, M. F., Ferreira, A. J., Almeida, P. W., Braga, V. B., Coutinho, D. C., Melo, D. S., Gomes Filho, A., Melo, M. B., Greco, L., & Campagnole-Santos, M. J. (2012). The cardiac expression of Mas receptor is responsive to different physiological and pathological stimuli. *Peptides*, *35*(2), 196-201.

Donato, A. J., Gano, L. B., Eskurza, I., Silver, A. E., Gates, P. E., Jablonski, K., & Seals, D. R. (2009). Vascular endothelial dysfunction with aging: endothelin-1 and endothelial nitric oxide synthase. *American Journal of Physiology-Heart and Circulatory Physiology*, *297*(1), H425-H432.

Downward, J. (1998). Mechanisms and consequences of activation of protein kinase B/Akt. *Current opinion in cell biology*, *10*(2), 262-267.

Dwyer, A. A., & Quinton, R. (2019). Anatomy and physiology of the hypothalamic-pituitary-gonadal (HPG) axis. *Advanced Practice in Endocrinology Nursing*, 839-852.

e Silva, A. C. S., & Teixeira, M. M. (2016). ACE inhibition, ACE2 and angiotensin-(1 7) axis in kidney and cardiac inflammation and fibrosis. *Pharmacological research*, *107*, 154-162.

El Khoudary, S. R., Aggarwal, B., Beckie, T. M., Hodis, H. N., Johnson, A. E., Langer, R. D., Limacher, M. C., Manson, J. E., Stefanick, M. L., & Allison, M.

A. (2020). Menopause Transition and Cardiovascular Disease Risk: Implications for Timing of Early Prevention: A Scientific Statement From the American Heart Association. *Circulation*, *142*(25). https://doi.org/10.1161/cir.0000000000000912

Elahi, M. M., Kong, Y. X., & Matata, B. M. (2009). Oxidative stress as a mediator of cardiovascular disease. *Oxidative Medicine and Cellular Longevity*, *2*(5), 259-269.

Ellison, K. E., Ingelfinger, J. R., Pivor, M., & Dzau, V. J. (1989). Androgen regulation of rat renal angiotensinogen messenger RNA expression. *The Journal of clinical investigation*, *83*(6), 1941-1945.

Ergul, A., Jupin, D., Johnson, M. H., & Prisant, L. M. (2006). Elevated endothelin-1 levels are associated with decreased arterial elasticity in hypertensive patients. *The Journal of Clinical Hypertension*, *8*(8), 549-554.

Eun, H. C. (1995). Evaluation of skin blood flow by laser Doppler flowmetry. *Clinics in Dermatology*, *13*(4), 337-347. https://doi.org/https://doi.org/10.1016/0738-081X(95)00080-Y

Evans, E., Rendell, M., Bartek, J., Connor, S., Bamisedun, O., Dovgan, D., & Giitter, M. (1993). Thermally-Induced Cutaneous Vasodilatation in Aging. *Journal of Gerontology*, *48*(2), M53-M57. https://doi.org/10.1093/geronj/48.2.M53

Feng, L., Stern, D. M., & Pile-Spellman, J. (1999). Human endothelium: endovascular biopsy and molecular analysis. *Radiology*, *212*(3), 655-664.

Fernandes, L., Fortes, Z. B., Nigro, D., Tostes, R. C. A., Santos, R. A. S., & Carvalho, M. H. C. d. (2001). Potentiation of Bradykinin by Angiotensin-(1-7) on Arterioles of Spontaneously Hypertensive Rats Studied In Vivo. *Hypertension*, *37*(2), 703-709. https://doi.org/doi:10.1161/01.HYP.37.2.703

Ferrario, C. M., Jessup, J., Gallagher, P. E., Averill, D. B., Brosnihan, K. B., Ann Tallant, E., Smith, R. D., & Chappell, M. C. (2005). Effects of renin-angiotensin system blockade on renal angiotensin-(1-7) forming enzymes and receptors. *Kidney International*, *68*(5), 2189-2196. https://doi.org/10.1111/j.1523-1755.2005.00675.x

Ferreira, A. J., Pinheiro, S. V., Castro, C. H., Silva, G. A., e Silva, A. C. S., Almeida, A. P., Bader, M., Rentzsch, B., Reudelhuber, T. L., & Santos, R. A. (2006). Renal function in transgenic rats expressing an angiotensin-(1–7)-producing fusion protein. *Regulatory peptides*, *137*(3), 128-133.

Ferreira, A. J., & Santos, R. A. S. (2005). Cardiovascular actions of angiotensin-(1-7). *Brazilian Journal of Medical and Biological Research*, *38*(4), 499-507. https://doi.org/10.1590/s0100-879x2005000400003

Filardo, E. J., Quinn, J. A., Bland, K. I., & Frackelton Jr, A. R. (2000). Estrogen-induced activation of Erk-1 and Erk-2 requires the G protein-coupled receptor homolog, GPR30, and occurs via trans-activation of the epidermal growth factor receptor through release of HB-EGF. *Molecular endocrinology*, *14*(10), 1649-1660.

Filho, A. G., Ferreira, A. J., Santos, S. H. S., Neves, S. R., Silva Camargos, E. R., Becker, L. K., Belchior, H. A., Dias-Peixoto, M. F., Pinheiro, S. V., & Santos, R. A. (2008). Selective increase of angiotensin (1–7) and its receptor in hearts of spontaneously hypertensive rats subjected to physical training. *Experimental Physiology*, *93*(5), 589-598.

Fischer, M. (2002). Renin angiotensin system and gender differences in the cardiovascular system. *Cardiovascular Research*, *53*(3), 672-677. https://doi.org/10.1016/s0008-6363(01)00479-5

Fraga-Silva, R. A., Pinheiro, S. V. B., Gonçalves, A. C. C., Alenina, N., Bader, M., & Souza Santos, R. A. (2008). The antithrombotic effect of angiotensin-(1-7) involves mas-mediated NO release from platelets. *Molecular Medicine*, *14*, 28-35.

Franzoni, F., Ghiadoni, L., Galetta, F., Plantinga, Y., Lubrano, V., Huang, Y., Salvetti, G., Regoli, F., Taddei, S., & Santoro, G. (2005). Physical activity, plasma antioxidant capacity, and endothelium-dependent vasodilation in young and older men. *American journal of hypertension*, *18*(4), 510-516.

Fukaya, Y., & Ohhashi, T. (1996). Acetylcholine-and flow-induced production and release of nitric oxide in arterial and venous endothelial cells. *American Journal of Physiology-Heart and Circulatory Physiology*, *270*(1), H99-H106.

Fulton, D., Gratton, J.-P., McCabe, T. J., Fontana, J., Fujio, Y., Walsh, K., Franke, T. F., Papapetropoulos, A., & Sessa, W. C. (1999). Regulation of endothelium-derived nitric oxide production by the protein kinase Akt. *Nature*, *399*(6736), 597-601. https://doi.org/10.1038/21218

Gaina, G., Manole, E., Matanie, C., Mihalcea, A., & Ionica, E. (2008). Muscular dystrophies proteins evaluation by western blot and immunofluorescence. *Romanian Biotechnological Letters*, *13*(3), 3729-3736.

Gavin, K. M., Seals, D. R., Silver, A. E., & Moreau, K. L. (2009). Vascular Endothelial Estrogen Receptor α Is Modulated by Estrogen Status and Related to Endothelial Function and Endothelial Nitric Oxide Synthase in Healthy Women. *The Journal of Clinical Endocrinology & Metabolism*, *94*(9), 3513-3520. https://doi.org/10.1210/jc.2009-0278

Giani, J. F., Gironacci, M. M., Muñoz, M. C., Peña, C., Turyn, D., & Dominici, F. P. (2007). Angiotensin-(1–7) stimulates the phosphorylation of JAK2, IRS-1 and Akt in rat heart in vivo: role of the AT1 and Mas receptors. *American Journal of Physiology-Heart and Circulatory Physiology*, *293*(2), H1154-H1163. https://doi.org/10.1152/ajpheart.01395.2006

Giani, J. F., Mayer, M. A., Munoz, M. C., Silberman, E. A., Hocht, C., Taira, C. A., Gironacci, M. M., Turyn, D., & Dominici, F. P. (2009). Chronic infusion of angiotensin-(1–7) improves insulin resistance and hypertension induced by a high-fructose diet in rats. *American Journal of Physiology-Endocrinology and Metabolism*, *296*(2), E262-E271.

Giani, J. F., Muñoz, M. C., Pons, R. A., Cao, G., Toblli, J. E., Turyn, D., & Dominici, F. P. (2011). Angiotensin-(1–7) reduces proteinuria and diminishes structural damage in renal tissue of stroke-prone spontaneously hypertensive rats. *American Journal of Physiology-Renal Physiology*, *300*(1), F272-F282.

Glass, C. K., & Witztum, J. L. (2001). Atherosclerosis: the road ahead. *Cell*, *104*(4), 503-516.

Gorelik, G., Carbini, L., & Scicli, A. (1998). Angiotensin 1–7 induces bradykinin-mediated relaxation in porcine coronary artery. *Journal of Pharmacology and Experimental Therapeutics*, *286*(1), 403-410.

Haas, E., Bhattacharya, I., Brailoiu, E., Damjanović, M., Brailoiu, G. C., Gao, X., Mueller-Guerre, L., Marjon, N. A., Gut, A., Minotti, R., Meyer, M. R., Amann, K., Ammann, E., Perez-Dominguez, A., Genoni, M., Clegg, D. J., Dun, N. J., Resta, T. C., Prossnitz, E. R., & Barton, M. (2009). Regulatory Role of G Protein–Coupled Estrogen Receptor for Vascular Function and Obesity. *Circulation Research*, *104*(3), 288-291. https://doi.org/doi:10.1161/CIRCRESAHA.108.190892

Hafezi-Moghadam, A., Simoncini, T., Yang, Z., Limbourg, F. P., Plumier, J.-C., Rebsamen, M. C., Hsieh, C.-M., Chui, D.-S., Thomas, K. L., & Prorock, A. J. (2002). Acute cardiovascular protective effects of corticosteroids are mediated by non-transcriptional activation of endothelial nitric oxide synthase. *Nature medicine*, *8*(5), 473-479.

Handa, R. K., Ferrario, C. M., & Strandhoy, J. W. (1996). Renal actions of angiotensin-(1-7): in vivo and in vitro studies. *American Journal of Physiology-Renal Physiology*, *270*(1), F141-F147. https://doi.org/10.1152/ajprenal.1996.270.1.F141

Hansell, J., Henareh, L., Agewall, S., & Norman, M. (2004). Non-invasive assessment of endothelial function–relation between vasodilatory responses in skin microcirculation and brachial artery. *Clinical physiology and functional imaging*, *24*(6), 317-322.

Harlow, S. D., Gass, M., Hall, J. E., Lobo, R., Maki, P., Rebar, R. W., Sherman, S., Sluss, P. M., & De Villiers, T. J. (2012). Executive Summary of the Stages of Reproductive Aging Workshop + 10: Addressing the Unfinished Agenda of Staging Reproductive Aging. *The Journal of Clinical Endocrinology & Metabolism*, *97*(4), 1159-1168. https://doi.org/10.1210/jc.2011-3362

Hashimoto, M., Akishita, M., Eto, M., Ishikawa, M., Kozaki, K., Toba, K., Sagara, Y., Taketani, Y., Orimo, H., & Ouchi, Y. (1995). Modulation of Endothelium-Dependent Flow-Mediated Dilatation of the Brachial Artery by Sex and Menstrual Cycle. *Circulation*, *92*(12), 3431-3435. https://doi.org/doi:10.1161/01.CIR.92.12.3431

Haynes, M. P., Sinha, D., Russell, K. S., Collinge, M., Fulton, D., Morales-Ruiz, M., Sessa, W. C., & Bender, J. R. (2000). Membrane estrogen receptor engagement activates endothelial nitric oxide synthase via the PI3-kinase–Akt pathway in human endothelial cells. *Circulation Research*, *87*(8), 677-682.

Heitsch, H., Brovkovych, S., Malinski, T., & Wiemer, G. (2001). Angiotensin-(1-7)–Stimulated Nitric Oxide and Superoxide Release From Endothelial Cells. *Hypertension*, *37*(1), 72-76. https://doi.org/10.1161/01.hyp.37.1.72

Heller, J., Kramer, H. J., Malý, J., Červenka, L., & Horáček, V. (2000). Effect of Intrarenal Infusion of Angiotensin–(1–7) in the Dog. *Kidney and Blood Pressure Research*, *23*(2), 89-94. https://doi.org/10.1159/000025959

Hilchey, S. D., & Bell-Quilley, C. P. (1995). Association Between the Natriuretic Action of Angiotensin-(1-7) and Selective Stimulation of Renal Prostaglandin I₂ Release. *Hypertension*, *25*(6), 1238-1244. https://doi.org/doi:10.1161/01.HYP.25.6.1238

Hildreth, K. L., Kohrt, W. M., & Moreau, K. L. (2014). Oxidative stress contributes to large elastic arterial stiffening across the stages of the menopausal transition. *Menopause*, *21*(6), 624-632.

Hishikawa, K., Nakaki, T., Marumo, T., Suzuki, H., Kato, R., & Saruta, T. (1995). Up-regulation of nitric oxide synthase by estradiol in human aorticendothelial cells. *FEBS letters*, *360*(3), 291-293.

Hodis, H. N., Mack, W. J., Henderson, V. W., Shoupe, D., Budoff, M. J., Hwang-Levine, J., Li, Y., Feng, M., Dustin, L., & Kono, N. (2016). Vascular effects of early versus late postmenopausal treatment with estradiol. *New England Journal of Medicine*, *374*(13), 1221-1231.

Hodis, H. N., Mack, W. J., Shoupe, D., Azen, S. P., Stanczyk, F. Z., Hwang-Levine, J., Budoff, M. J., & Henderson, V. W. (2014). Testing the menopausal hormone therapy timing hypothesis: the Early versus Late Intervention Trial with Estradiol. *Circulation*, *130*(suppl_2), A13283-A13283.

Holowatz, L. A. (2010). Aging and the control of human skin blood flow. *Frontiers in Bioscience*, *15*(1), 718. https://doi.org/10.2741/3642

Holowatz, L. A., Houghton, B. L., Wong, B. J., Wilkins, B. W., Harding, A. W., Kenney, W. L., & Minson, C. T. (2003). Nitric oxide and attenuated reflex cutaneous vasodilation in aged skin. *American Journal of Physiology-Heart and Circulatory Physiology*, *284*(5), H1662-H1667.

Holowatz, L. A., Thompson, C. S., Minson, C. T., & Kenney, W. L. (2005). Mechanisms of acetylcholine-mediated vasodilatation in young and aged human skin. *The Journal of physiology*, *563*(3), 965-973.

Holowatz, L. A., Thompson-Torgerson, C. S., & Kenney, W. L. (2008). The human cutaneous circulation as a model of generalized microvascular function. *Journal of applied physiology*, *105*(1), 370-372.

Horinouchi, T., Terada, K., Higashi, T., & Miwa, S. (2013). Endothelin receptor signaling: new insight into its regulatory mechanisms. *Journal of pharmacological sciences*, *123*(2), 85-101.

Hornig, B., & Drexler, H. (1997). Endothelial function and bradykinin in humans. *Drugs*, *54 Suppl 5*, 42-47. https://doi.org/10.2165/00003495-199700545-00007

Humphries, K. H., Izadnegahdar, M., Sedlak, T., Saw, J., Johnston, N., Schenck-Gustafsson, K., Shah, R., Regitz-Zagrosek, V., Grewal, J., & Vaccarino, V.

(2017). Sex differences in cardiovascular disease–impact on care and outcomes. *Frontiers in Neuroendocrinology*, *46*, 46-70.
Iwai, M., & Horiuchi, M. (2009). Devil and angel in the renin–angiotensin system: ACE–angiotensin II–AT1 receptor axis vs. ACE2–angiotensin-(1–7)–Mas receptor axis. *Hypertension Research*, *32*(7), 533-536. https://doi.org/10.1038/hr.2009.74
Janes, L. (2020). Hypothalamic-Pituitary-Gonadal (HPG) Axis. *Encyclopedia of Personality and Individual Differences*, 2112-2114.
Javeshghani, D., Sairam, M. R., Neves, M. F., Schiffrin, E. L., & Touyz, R. M. (2006). Angiotensin II induces vascular dysfunction without exacerbating blood pressure elevation in a mouse model of menopause-associated hypertension. *Journal of hypertension*, *24*(7), 1365-1373.
Jensen-Urstad, K., Johansson, J., & Jensen-Urstad, M. (1997). Vascular function correlates with risk factors for cardiovascular disease in a healthy population of 35-year-old subjects. *Journal of internal medicine*, *241*(6), 507-513.
Ji, H., Menini, S., Zheng, W., Pesce, C., Wu, X., & Sandberg, K. (2008). Role of angiotensin-converting enzyme 2 and angiotensin (1–7) in 17β-oestradiol regulation of renal pathology in renal wrap hypertension in rats. *Experimental Physiology*, *93*(5), 648-657.
Kang, L. S., Chen, B., Reyes, R. A., LeBlanc, A. J., Teng, B., Mustafa, S. J., & Muller-Delp, J. M. (2011). Aging and estrogen alter endothelial reactivity to reactive oxygen species in coronary arterioles. *American Journal of Physiology-Heart and Circulatory Physiology*, *300*(6), H2105-H2115.
Kannel, W. B., McGee, D., & Gordon, T. (1976). A general cardiovascular risk profile: the Framingham Study. *The American journal of cardiology*, *38*(1), 46-51.
Kauser, K., & Rubanyi, G. M. (2008). Potential Cellular Signaling Mechanisms Mediating Upregulation of Endothelial Nitric Oxide Production by Estrogen. *Journal of Vascular Research*, *34*(3), 229-236. https://doi.org/10.1159/000159227
Keaney Jr, J. F., Shwaery, G. T., Xu, A., Nicolosi, R. J., Loscalzo, J., Foxall, T. L., & Vita, J. A. (1994). 17 beta-estradiol preserves endothelial vasodilator function and limits low-density lipoprotein oxidation in hypercholesterolemic swine. *Circulation*, *89*(5), 2251-2259.
Kellogg Jr, D., Zhao, J., Coey, U., & Green, J. (2005). Acetylcholine-induced vasodilation is mediated by nitric oxide and prostaglandins in human skin. *Journal of applied physiology*, *98*(2), 629-632.
Khalil, R. A. (2005). Sex Hormones as Potential Modulators of Vascular Function in Hypertension. *Hypertension*, *46*(2), 249-254. https://doi.org/10.1161/01.hyp.0000172945.06681.a4
Kim, K. H., & Bender, J. R. (2005). Rapid, Estrogen Receptor–Mediated Signaling: Why Is the Endothelium So Special? *Science's STKE*, *2005*(288), pe28-pe28.

Kim, S. C., Boese, A. C., Moore, M. H., Cleland, R. M., Chang, L., Delafontaine, P., Yin, K.-J., Lee, J.-P., & Hamblin, M. H. (2018). Rapid estrogen receptor-α signaling mediated by ERK activation regulates vascular tone in male and ovary-intact female mice. *American Journal of Physiology-Heart and Circulatory Physiology*, *314*(2), H330-H342.

Knowlton, A., & Lee, A. (2012). Estrogen and the cardiovascular system. *Pharmacology & Therapeutics*, *135*(1), 54-70.

Konukoglu, D., & Uzun, H. (2017). Endothelial dysfunction and hypertension. *Hypertension: from basic research to clinical practice*, 511-540.

Kreilgaard, M. (2002). Assessment of cutaneous drug delivery using microdialysis. *Advanced Drug Delivery Reviews*, *54*, S99-S121. https://doi.org/https://doi.org/10.1016/S0169-409X(02)00117-5

Kuczmarski, A. V. (2021). *Role of the Endothelin System in Vascular Dysfunction in Women* (Publication Number 28548683) [Ph.D., University of Delaware]. Dissertations & Theses @ University of Delaware; ProQuest Dissertations & Theses Global. United States -- Delaware. https://www.proquest.com/dissertations-theses/role-endothelin-system-vascular-dysfunction-women/docview/2586538709/se-2?accountid=10457

https://delcat.primo.exlibrisgroup.com/openurl/01UDEL_INST/01UDEL_INST:01UDEL_INST??url_ver=Z39.88-2004&rft_val_fmt=info:ofi/fmt:kev:mtx:dissertation&genre=dissertations&sid=ProQ:ProQuest+Dissertations+%26+Theses+Global&atitle=&title=Role+of+the+Endothelin+System+in+Vascular+Dysfunction+in+Women&issn=&date=2021-01-01&volume=&issue=&spage=&au=Kuczmarski%2C+Andrew+Vincent&isbn=9798471107083&jtitle=&btitle=&rft_id=info:eric/&rft_id=info:doi/

Kuczmarski, A. V., Shoemaker, L. N., Hobson, J. C., Edwards, D. G., & Wenner, M. M. (2020). Altered endothelial ETB receptor expression in postmenopausal women. *American Journal of Physiology-Heart and Circulatory Physiology*, *319*(1), H242-H247.

Kuczmarski, A. V., Welti, L. M., Moreau, K. L., & Wenner, M. M. (2021). ET-1 as a sex-specific mechanism impacting age-related changes in vascular function. *Frontiers in Aging*, *2*, 727416.

Lakshmi, S. V., Padmaja, G., Kuppusamy, P., & Kutala, V. K. (2009). Oxidative stress in cardiovascular disease.

LeBlanc, A. J., Reyes, R., Kang, L. S., Dailey, R. A., Stallone, J. N., Moningka, N. C., & Muller-Delp, J. M. (2009). Estrogen replacement restores flow-induced vasodilation in coronary arterioles of aged and ovariectomized rats. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, *297*(6), R1713-R1723.

Lee, R. T., & Libby, P. (1997). The unstable atheroma. *Arteriosclerosis, Thrombosis, and Vascular Biology*, *17*(10), 1859-1867.

Lerman, A., Holmes Jr, D. R., Bell, M. R., Garratt, K. N., Nishimura, R. A., & Burnett Jr, J. C. (1995). Endothelin in coronary endothelial dysfunction and early atherosclerosis in humans. *Circulation*, *92*(9), 2426-2431.

Li, L., Hisamoto, K., Kim, K. H., Haynes, M. P., Bauer, P. M., Sanjay, A., Collinge, M., Baron, R., Sessa, W. C., & Bender, J. R. (2007). Variant estrogen receptor–c-Src molecular interdependence and c-Src structural requirements for endothelial NO synthase activation. *Proceedings of the National Academy of Sciences*, *104*(42), 16468-16473.

Li, P., Chappell, M. C., Ferrario, C. M., & Brosnihan, K. B. (1997). Angiotensin-(1–7) Augments Bradykinin-Induced Vasodilation by Competing With ACE and Releasing Nitric Oxide. *Hypertension*, *29*(1), 394-398. https://doi.org/10.1161/01.hyp.29.1.394

Li, Y., Cao, Y., Zeng, Z., Liang, M., Xue, Y., Xi, C., Zhou, M., & Jiang, W. (2015). Angiotensin-converting enzyme 2/angiotensin-(1–7)/Mas axis prevents lipopolysaccharide–induced apoptosis of pulmonary microvascular endothelial cells by inhibiting JNK/NF–κB pathways. *Scientific reports*, *5*(1), 8209.

Llorens, S., Jordán, J., & Nava, E. (2002). The nitric oxide pathway in the cardiovascular system. *Journal of physiology and biochemistry*, *58*(3), 179-188.

Lloyd-Jones, D. M., Allen, N. B., Anderson, C. A. M., Black, T., Brewer, L. C., Foraker, R. E., Grandner, M. A., Lavretsky, H., Perak, A. M., Sharma, G., & Rosamond, W. (2022). Life's Essential 8: Updating and Enhancing the American Heart Association's Construct of Cardiovascular Health: A Presidential Advisory From the American Heart Association. *Circulation*, *146*(5), e18-e43. https://doi.org/doi:10.1161/CIR.0000000000001078

Lu, D., & Kassab, G. S. (2011). Role of shear stress and stretch in vascular mechanobiology. *Journal of the royal society interface*, *8*(63), 1379-1385.

Lu, J., Jiang, T., Wu, L., Gao, L., Wang, Y., Zhou, F., Zhang, S., & Zhang, Y. (2013). The expression of angiotensin-converting enzyme 2–angiotensin-(1–7)–Mas receptor axis are upregulated after acute cerebral ischemic stroke in rats. *Neuropeptides*, *47*(5), 289-295.

Luksha, L., Agewall, S., & Kublickiene, K. (2009). Endothelium-derived hyperpolarizing factor in vascular physiology and cardiovascular disease. *Atherosclerosis*, *202*(2), 330-344. https://doi.org/https://doi.org/10.1016/j.atherosclerosis.2008.06.008

Ma, X., Xu, D., Ai, Y., Zhao, S., Zhang, L., Ming, G., & Liu, Z. (2016). Angiotensin-(1-7)/Mas signaling inhibits lipopolysaccharide-induced ADAM17 shedding activity and apoptosis in alveolar epithelial cells. *Pharmacology*, *97*(1-2), 63-71.

MacRitchie, A. N., Jun, S. S., Chen, Z., German, Z., Yuhanna, I. S., Sherman, T. S., & Shaul, P. W. (1997). Estrogen upregulates endothelial nitric oxide synthase gene expression in fetal pulmonary artery endothelium. *Circulation Research*, *81*(3), 355-362.

Magalhães, G., Rodrigues-Machado, M., Motta-Santos, D., Silva, A., Caliari, M., Prata, L., Abreu, S., Rocco, P., Barcelos, L., & Santos, R. (2015). A ngiotensin-(1-7) attenuates airway remodelling and hyperresponsiveness in a model of chronic allergic lung inflammation. *British Journal of Pharmacology*, *172*(9), 2330-2342.

Malek, A. M., Alper, S. L., & Izumo, S. (1999). Hemodynamic shear stress and its role in atherosclerosis. *JAMA*, *282*(21), 2035-2042.

Maleki, M., & Nematbakhsh, M. (2016). Renal Blood Flow Response to Angiotensin 1-7 versus Hypertonic Sodium Chloride 7.5% Administration after Acute Hemorrhagic Shock in Rats. *Int J Vasc Med*, *2016*, 6562017. https://doi.org/10.1155/2016/6562017

Man, A. W., Li, H., & Xia, N. (2020). Impact of lifestyles (diet and exercise) on vascular health: oxidative stress and endothelial function. *Oxidative Medicine and Cellular Longevity*, *2020*(1), 1496462.

Mansoori, A., Oryan, S., & Nematbakhsh, M. (2016). Role of Mas receptor antagonist (A779) in renal hemodynamics in condition of blocked angiotensin II receptors in rats. *Acta Physiologica Hungarica*, *103*(1), 13-20. https://doi.org/https://doi.org/10.1556/036.103.2016.1.2

McArdle, A., & Jackson, M. J. (2000). Exercise, oxidative stress and ageing. *The Journal of Anatomy*, *197*(4), 539-541.

McEniery, C. M., Qasem, A., Schmitt, M., Avolio, A. P., Cockcroft, J. R., & Wilkinson, I. B. (2003). Endothelin-1 regulates arterial pulse wave velocity in vivo. *Journal of the American College of Cardiology*, *42*(11), 1975-1981.

McGuire, J. J., Ding, H., & Triggle, C. R. (2001). Endothelium-derived relaxing factors: a focus on endothelium-derived hyperpolarizing factor (s). *Canadian journal of physiology and pharmacology*, *79*(6), 443-470.

McNeill, A. M., Kim, N., Duckles, S. P., & Krause, D. N. (1999). Chronic Estrogen Treatment Increases Levels of Endothelial Nitric Oxide Synthase Protein in Rat Cerebral Microvessels. *Stroke*, *30*(10), 2186-2190. https://doi.org/doi:10.1161/01.STR.30.10.2186

Medina, D., & Arnold, A. C. (2019). Angiotensin-(1-7): translational avenues in cardiovascular control. *American journal of hypertension*, *32*(12), 1133-1142.

Medina, D., Mehay, D., & Arnold, A. C. (2020). Sex differences in cardiovascular actions of the renin–angiotensin system. *Clinical Autonomic Research*, *30*, 393-408.

Meendering, J. R., Torgrimson, B. N., Miller, N. P., Kaplan, P. F., & Minson, C. T. (2008). Estrogen, medroxyprogesterone acetate, endothelial function, and biomarkers of cardiovascular risk in young women. *American Journal of Physiology-Heart and Circulatory Physiology*, *294*(4), H1630-H1637. https://doi.org/10.1152/ajpheart.01314.2007

Mendelsohn, M. E. (2002). Genomic and nongenomic effects of estrogen in the vasculature. *The American journal of cardiology*, *90*(1), F3-F6.

Mendelsohn, M. E., & Karas, R. H. (1999). The protective effects of estrogen on the cardiovascular system. *New England Journal of Medicine*, *340*(23), 1801-1811.

Meng, Y., Yu, C.-H., Li, W., Li, T., Luo, W., Huang, S., Wu, P.-S., Cai, S.-X., & Li, X. (2014). Angiotensin-converting enzyme 2/angiotensin-(1-7)/Mas axis protects against lung fibrosis by inhibiting the MAPK/NF-κB pathway. *American journal of respiratory cell and molecular biology*, *50*(4), 723-736.

Merrill, D. C., Karoly, M., Chen, K., Ferrario, C. M., & Brosnihan, K. B. (2002). Angiotensin-(1–7) in normal and preeclamptic pregnancy. *Endocrine*, *18*, 239-245.

Miller, A., & Arnold, A. (2023). Angiotensin-(1-7) improves blood pressure and cardiovascular autonomic function in human aging. *Physiology*, *38*(S1), 5732602.

Miller, V. M., & Duckles, S. P. (2008). Vascular actions of estrogens: functional implications. *Pharmacological reviews*, *60*(2), 210-241.

Milner, P., Kirkpatrick, K. A., Ralevic, V., Toothill, V., Pearson, J., & Burnstock, G. (1990). Endothelial cells cultured from human umbilical vein release ATP, substance P and acetylcholine in response to increased flow. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, *241*(1302), 245-248.

Miner, J. A., Martini, E. R., Smith, M. M., Brunt, V. E., Kaplan, P. F., Halliwill, J. R., & Minson, C. T. (2011). Short-term oral progesterone administration antagonizes the effect of transdermal estradiol on endothelium-dependent vasodilation in young healthy women. *American Journal of Physiology-Heart and Circulatory Physiology*, *301*(4), H1716-H1722. https://doi.org/10.1152/ajpheart.00405.2011

Minson, C. T., Berry, L. T., & Joyner, M. J. (2001). Nitric oxide and neurally mediated regulation of skin blood flow during local heating. *Journal of applied physiology*, *91*(4), 1619-1626.

Minson, C. T., Holowatz, L. A., Wong, B. J., Kenney, W. L., & Wilkins, B. W. (2002). Decreased nitric oxide-and axon reflex-mediated cutaneous vasodilation with age during local heating. *Journal of applied physiology*, *93*(5), 1644-1649.

Mirza, F. S., Ong, P., Collins, P., Okamura, K., Gerhard-Herman, M., Williams, G. H., & Seely, E. W. (2008). Effects of estradiol and the angiotensin II receptor blocker irbesartan on vascular function in postmenopausal women. *Menopause*, *15*(1), 44-50.

Mitchell, G. F., Parise, H., Vita, J. A., Larson, M. G., Warner, E., Keaney Jr, J. F., Keyes, M. J., Levy, D., Vasan, R. S., & Benjamin, E. J. (2004). Local shear stress and brachial artery flow-mediated dilation: the Framingham Heart Study. *Hypertension*, *44*(2), 134-139.

Miyauchi, T., & Masaki, T. (1999). Pathophysiology of endothelin in the cardiovascular system. *Annual review of physiology*, *61*(1), 391-415.

Mompeón, A., Lázaro-Franco, M., Bueno-Betí, C., Pérez-Cremades, D., Vidal-Gómez, X., Monsalve, E., Gironacci, M. M., Hermenegildo, C., & Novella, S. (2016). Estradiol, acting through ERα, induces endothelial non-classic renin-angiotensin system increasing angiotensin 1–7 production. *Molecular and cellular endocrinology*, *422*, 1-8.
Moreau, K., Kahn, Z., & Seals, D. R. (2007). Endothelium-Dependent Dilation is Greater in Habitually Exercising Postmenopausal Women and is Related to Reduced Vascular Oxidative Stress. In: Wiley Online Library.
Moreau, K. L., Gavin, K. M., Plum, A. E., & Seals, D. R. (2006). Oxidative stress explains differences in large elastic artery compliance between sedentary and habitually exercising postmenopausal women. *Menopause*, *13*(6), 951-958.
Moreau, K. L., & Hildreth, K. L. (2014). Vascular aging across the menopause transition in healthy women. *Advances in vascular medicine*, *2014*(1), 204390.
Moreau, K. L., Hildreth, K. L., Klawitter, J., Blatchford, P., & Kohrt, W. M. (2020). Decline in endothelial function across the menopause transition in healthy women is related to decreased estradiol and increased oxidative stress. *Geroscience*, *42*(6), 1699-1714.
Moreau, K. L., Hildreth, K. L., Meditz, A. L., Deane, K. D., & Kohrt, W. M. (2012). Endothelial Function Is Impaired across the Stages of the Menopause Transition in Healthy Women. *The Journal of Clinical Endocrinology & Metabolism*, *97*(12), 4692-4700. https://doi.org/10.1210/jc.2012-2244
Moreau, K. L., & Ozemek, C. (2017). Vascular adaptations to habitual exercise in older adults: time for the sex talk. *Exercise and sport sciences reviews*, *45*(2), 116-123.
Moreau, K. L., Stauffer, B. L., Kohrt, W. M., & Seals, D. R. (2013). Essential Role of Estrogen for Improvements in Vascular Endothelial Function With Endurance Exercise in Postmenopausal Women. *The Journal of Clinical Endocrinology & Metabolism*, *98*(11), 4507-4515. https://doi.org/10.1210/jc.2013-2183
Mori, J., Patel, V. B., Ramprasath, T., Alrob, O. A., DesAulniers, J., Scholey, J. W., Lopaschuk, G. D., & Oudit, G. Y. (2014). Angiotensin 1–7 mediates renoprotection against diabetic nephropathy by reducing oxidative stress, inflammation, and lipotoxicity. *American Journal of Physiology-Renal Physiology*, *306*(8), F812-F821. https://doi.org/10.1152/ajprenal.00655.2013
Mosca, L., Barrett-Connor, E., & Kass Wenger, N. (2011). Sex/gender differences in cardiovascular disease prevention: what a difference a decade makes. *Circulation*, *124*(19), 2145-2154.
Mudau, M., Genis, A., Lochner, A., & Strijdom, H. (2012). Endothelial dysfunction: the early predictor of atherosclerosis. *Cardiovascular journal of Africa*, *23*(4), 222-231.
Mullen, M. J., Kharbanda, R. K., Cross, J., Donald, A. E., Taylor, M., Vallance, P., Deanfield, J. E., & MacAllister, R. J. (2001). Heterogenous nature of flow-mediated dilatation in human conduit arteries in vivo: relevance to endothelial dysfunction in hypercholesterolemia. *Circulation Research*, *88*(2), 145-151.

Murga, C., Laguinge, L., Wetzker, R., Cuadrado, A., & Gutkind, J. S. (1998). Activation of Akt/Protein Kinase B by G protein-coupled receptors: a role for α and βγ subunits of heterotrimeric g proteins acting through phosphatidylinositol-3-OH kinaseγ. *Journal of Biological Chemistry*, *273*(30), 19080-19085.
Nadal, A., Ropero, A. B., Laribi, O., Maillet, M., Fuentes, E., & Soria, B. (2000). Nongenomic actions of estrogens and xenoestrogens by binding at a plasma membrane receptor unrelated to estrogen receptor α and estrogen receptor β. *Proceedings of the National Academy of Sciences*, *97*(21), 11603-11608.
Navab, M., Berliner, J. A., Watson, A. D., Hama, S. Y., Territo, M. C., Lusis, A. J., Shih, D. M., Van Lenten, B. J., Frank, J. S., & Demer, L. L. (1996). The yin and yang of oxidation in the development of the fatty streak: a review based on the 1994 George Lyman Duff Memorial Lecture. *Arteriosclerosis, Thrombosis, and Vascular Biology*, *16*(7), 831-842.
Neves, L. A., Averill, D. B., Ferrario, C. M., Aschner, J. L., & Brosnihan, K. B. (2004). Vascular responses to angiotensin-(1–7) during the estrous cycle. *Endocrine*, *24*, 161-165.
Nogueira, A. I., Santos, R. A. S., e Silva, A. C. S., Cabral, A. C. V., Vieira, R. L. P., Drumond, T. C., de Campos Machado, L. J., Freire, C. M. V., & Ribeiro-Oliveira Jr, A. (2007). The pregnancy-induced increase of plasma angiotensin-(1–7) is blunted in gestational diabetes. *Regulatory peptides*, *141*(1-3), 55-60.
Nunes-Silva, A., Rocha, G. C., Magalhaes, D. M., Vaz, L. N., Salviano de Faria, M. H., & Simoes e Silva, A. C. (2017). Physical exercise and ACE2-angiotensin-(1-7)-mas receptor axis of the renin angiotensin system. *Protein and peptide letters*, *24*(9), 809-816.
Panza, J. A., Quyyumi, A. A., Brush Jr, J. E., & Epstein, S. E. (1990). Abnormal endothelium-dependent vascular relaxation in patients with essential hypertension. *New England Journal of Medicine*, *323*(1), 22-27.
Papapetropoulos, A., García-Cardeña, G., Madri, J. A., & Sessa, W. C. (1997). Nitric oxide production contributes to the angiogenic properties of vascular endothelial growth factor in human endothelial cells. *The Journal of clinical investigation*, *100*(12), 3131-3139.
Papinska, A., Mordwinkin, N., Meeks, C., Jadhav, S., & Rodgers, K. (2015). Angiotensin-(1–7) administration benefits cardiac, renal and progenitor cell function in db/db mice. *British Journal of Pharmacology*, *172*(18), 4443-4453.
Paulsson, G., Zhou, X., Törnquist, E., & Hansson, G. r. K. (2000). Oligoclonal T cell expansions in atherosclerotic lesions of apolipoprotein E–deficient mice. *Arteriosclerosis, Thrombosis, and Vascular Biology*, *20*(1), 10-17.
Pereira, M., FitzerGerald, S., & Gregg, E. (1997). A collection of physical activity questionnaires for health-related research. Kriska and Caspersen, Eds. Centers for Disease Control and Prevention. *Med Sci Sports Exerc*, *29*(6 Suppl), S1-205.

Pereira, V. M., Reis, F. M., Santos, R. A., Cassali, G. D., Santos, S. H., Honorato-Sampaio, K., & dos Reis, A. M. (2009). Gonadotropin stimulation increases the expression of angiotensin-(1—7) and mas receptor in the rat ovary. *Reproductive sciences*, *16*(12), 1165-1174.

Perticone, F., Ceravolo, R., Pujia, A., Ventura, G., Iacopino, S., Scozzafava, A., Ferraro, A., Chello, M., Mastroroberto, P., & Verdecchia, P. (2001). Prognostic significance of endothelial dysfunction in hypertensive patients. *Circulation*, *104*(2), 191-196.

Pinheiro, S. V. B., Ferreira, A. J., Kitten, G. T., da Silveira, K. D., da Silva, D. A., Santos, S. H. S., Gava, E., Castro, C. H., Magalhães, J. A., da Mota, R. K., Botelho-Santos, G. A., Bader, M., Alenina, N., Santos, R. A. S., & Simoes e Silva, A. C. (2009). Genetic deletion of the angiotensin-(1–7) receptor Mas leads to glomerular hyperfiltration and microalbuminuria. *Kidney International*, *75*(11), 1184-1193. https://doi.org/10.1038/ki.2009.61

Polderman, K. H., Stehouwer, C. D., Van Kamp, G. J., Dekker, G. A., Verheugt, F. W., & Gooren, L. J. (1993). Influence of sex hormones on plasma endothelin levels. *Annals of Internal Medicine*, *118*(6), 429-432.

Pollock, D. M., & Pollock, J. S. (2005). Endothelin and oxidative stress in the vascular system. *Current vascular pharmacology*, *3*(4), 365-367.

Popat, V., Prodanov, T., Calis, K., & Nelson, L. (2008). The Menstrual Cycle A Biological Marker of General Health in Adolescents. *Annals of the New York Academy of Sciences*, *1135*, 43-51. https://doi.org/10.1196/annals.1429.040

Prehn, A., Hobusch, C., Härtig, W., Michalski, D., Krueger, M., & Flachmeyer, B. (2023). Increasing reproducibility in preclinical stroke research: The correlation of immunofluorescence intensity measurements and Western blot analyses strongly depends on antibody clonality and tissue pre-treatment in a mouse model of focal cerebral ischemia. *Frontiers in Cellular Neuroscience*, *17*, 1183232.

Randolph Jr, J. F., Sowers, M., Gold, E. B., Mohr, B. A., Luborsky, J., Santoro, N., McConnell, D. S., Finkelstein, J. S., Korenman, S. G., & Matthews, K. A. (2003). Reproductive hormones in the early menopausal transition: relationship to ethnicity, body size, and menopausal status. *The Journal of Clinical Endocrinology & Metabolism*, *88*(4), 1516-1522.

Randolph Jr, J. F., Zheng, H., Sowers, M. R., Crandall, C., Crawford, S., Gold, E. B., & Vuga, M. (2011). Change in follicle-stimulating hormone and estradiol across the menopausal transition: effect of age at the final menstrual period. *The Journal of Clinical Endocrinology & Metabolism*, *96*(3), 746-754.

Ren, Y., Garvin, J. L., & Carretero, O. A. (2002). Vasodilator action of angiotensin-(1-7) on isolated rabbit afferent arterioles. *Hypertension*, *39*(3), 799-802.

Revankar, C. M., Cimino, D. F., Sklar, L. A., Arterburn, J. B., & Prossnitz, E. R. (2005). A transmembrane intracellular estrogen receptor mediates rapid cell signaling. *Science*, *307*(5715), 1625-1630.

Robert, F., Weil, B., Kassis, N., & Dupouy-Camet, J. (1996). Investigation of immunofluorescence cross-reactions against Trichinella spiralis by Western blot (immunoblot) analysis. *Clinical Diagnostic Laboratory Immunology*, *3*(5), 575-577.

Robles, N. R., Cerezo, I., & Hernandez-Gallego, R. (2014). Renin–Angiotensin System Blocking Drugs. *Journal of Cardiovascular Pharmacology and Therapeutics*, *19*(1), 14-33. https://doi.org/10.1177/1074248413501018

Rodrigues-Machado, M., Magalhães, G., Cardoso, J., Kangussu, L., Murari, A., Caliari, M., Oliveira, M., Cara, D., Noviello, M., & Marques, F. (2013). AVE 0991, a non-peptide mimic of angiotensin-(1–7) effects, attenuates pulmonary remodelling in a model of chronic asthma. *British Journal of Pharmacology*, *170*(4), 835-846.

Roks, A. J., van Geel, P. P., Pinto, Y. M., Buikema, H., Henning, R. H., de Zeeuw, D., & van Gilst, W. H. (1999). Angiotensin-(1–7) is a modulator of the human renin-angiotensin system. *Hypertension*, *34*(2), 296-301.

Rosenthal, T., & Oparil, S. (2000). Hypertension in women. *Journal of human hypertension*, *14*(10), 691-704.

Ross, R. (1999). Atherosclerosis—an inflammatory disease. *New England Journal of Medicine*, *340*(2), 115-126.

Ruan, Y., Kan, H., & Malik, K. U. (1997). Beta adrenergic receptor stimulated prostacyclin synthesis in rabbit coronary endothelial cells is mediated by selective activation of phospholipase D: inhibition by adenosine 3′ 5′-cyclic monophosphate. *Journal of Pharmacology and Experimental Therapeutics*, *281*(3), 1038-1046.

Rush, J. W., & Aultman, C. D. (2008). Vascular biology of angiotensin and the impact of physical activity. *Applied physiology, nutrition, and metabolism*, *33*(1), 162-171.

Saberi, S., Dehghani, A., & Nematbakhsh, M. (2016). Role of Mas receptor in renal blood flow response to angiotensin-(1-7) in ovariectomized estradiol treated rats. *Res Pharm Sci*, *11*(1), 65-72.

Sampaio, W. O., Henrique De Castro, C., Santos, R. A. S., Schiffrin, E. L., & Touyz, R. M. (2007). Angiotensin-(1-7) Counterregulates Angiotensin II Signaling in Human Endothelial Cells. *Hypertension*, *50*(6), 1093-1098. https://doi.org/10.1161/hypertensionaha.106.084848

Sampaio, W. O., Nascimento, A. A. S., & Santos, R. A. S. (2003). Systemic and regional hemodynamic effects of angiotensin-(1–7) in rats. *American Journal of Physiology-Heart and Circulatory Physiology*, *284*(6), H1985-H1994. https://doi.org/10.1152/ajpheart.01145.2002

Sampaio, W. O., Santos, R. A. S. d., Faria-Silva, R., Machado, L. T. d. M., Schiffrin, E. L., & Touyz, R. M. (2007). Angiotensin-(1-7) Through Receptor Mas Mediates Endothelial Nitric Oxide Synthase Activation via Akt-Dependent Pathways. *Hypertension*, *49*(1), 185-192. https://doi.org/doi:10.1161/01.HYP.0000251865.35728.2f

Santoro, N., & Randolph, J. F. (2011). Reproductive hormones and the menopause transition. *Obstetrics and Gynecology Clinics*, *38*(3), 455-466.

Santos, R. A. (2014). Angiotensin-(1–7). *Hypertension*, *63*(6), 1138-1147. https://doi.org/10.1161/hypertensionaha.113.01274

Santos, R. A., & Baracho, N. C. (1992). Angiotensin-(1-7) is a potent antidiuretic peptide in rats. *Brazilian journal of medical and biological research = Revista brasileira de pesquisas medicas e biologicas*, *25*(6), 651-654. http://europepmc.org/abstract/MED/1342241

Santos, R. A., Brosnihan, K. B., Jacobsen, D. W., DiCorleto, P. E., & Ferrario, C. M. (1992). Production of angiotensin-(1-7) by human vascular endothelium. *Hypertension*, *19*(2_supplement), II56. https://doi.org/doi:10.1161/01.HYP.19.2_Suppl.II56

Santos, R. A., Brosnihan, K. B., Jacobsen, D. W., Dicorleto, P. E., & Ferrario, C. M. (1992). Production of angiotensin-(1-7) by human vascular endothelium. *Hypertension*, *19*(2_supplement), II56-II56. https://doi.org/10.1161/01.hyp.19.2_suppl.ii56

Santos, R. A. S., Ferreira, A. J., & Simões E Silva, A. C. (2008). Recent advances in the angiotensin-converting enzyme 2-angiotensin(1-7)-Mas axis. *Experimental Physiology*, *93*(5), 519-527. https://doi.org/10.1113/expphysiol.2008.042002

Santos, R. A. S., Sampaio, W. O., Alzamora, A. C., Motta-Santos, D., Alenina, N., Bader, M., & Campagnole-Santos, M. J. (2018). The ACE2/Angiotensin-(1–7)/MAS Axis of the Renin-Angiotensin System: Focus on Angiotensin-(1–7). *Physiological Reviews*, *98*(1), 505-553. https://doi.org/10.1152/physrev.00023.2016

Santos-Parker, J. R., Strahler, T. R., Vorwald, V. M., Pierce, G. L., & Seals, D. R. (2017). Habitual aerobic exercise does not protect against micro-or macrovascular endothelial dysfunction in healthy estrogen-deficient postmenopausal women. *Journal of applied physiology*, *122*(1), 11-19.

Sasaki, S., Higashi, Y., Nakagawa, K., Matsuura, H., Kajiyama, G., & Oshima, T. (2001). Effects of Angiotensin-(1-7) on Forearm Circulation in Normotensive Subjects and Patients With Essential Hypertension. *Hypertension*, *38*(1), 90-94. https://doi.org/doi:10.1161/01.HYP.38.1.90

Scaling, A. L., Prossnitz, E. R., & Hathaway, H. J. (2014). GPER mediates estrogen-induced signaling and proliferation in human breast epithelial cells and normal and malignant breast. *Hormones and Cancer*, *5*, 146-160.

Schächinger, V., Britten, M. B., & Zeiher, A. M. (2000). Prognostic impact of coronary vasodilator dysfunction on adverse long-term outcome of coronary heart disease. *Circulation*, *101*(16), 1899-1906.

Schächinger, V., & Zeiher, A. (2000). Atherosclerosis-associated endothelial dysfunction. *Zeitschrift für Kardiologie*, *89*, IX70-IX74.

Schiffrin, E. L. (2001). Role of endothelin-1 in hypertension and vascular disease. *American journal of hypertension*, *14*(S3), 83S-89S.

Schiffrin, E. L. (2005). Vascular endothelin in hypertension. *Vascular pharmacology*, *43*(1), 19-29.
Schinzari, F., Tesauro, M., Veneziani, A., Mores, N., Daniele, N. D., & Cardillo, C. (2018). Favorable Vascular Actions of Angiotensin-(1–7) in Human Obesity. *Hypertension*, *71*(1), 185-191. https://doi.org/doi:10.1161/HYPERTENSIONAHA.117.10280
Schulz, R., & Triggle, C. R. (1994). Role of NO in vascular smooth muscle and cardiac muscle function. *Trends in pharmacological sciences*, *15*(7), 255-259.
Scott, J. (2004). Pathophysiology and biochemistry of cardiovascular disease. *Current Opinion in Genetics & Development*, *14*(3), 271-279. https://doi.org/https://doi.org/10.1016/j.gde.2004.04.012
Seals, D. R., Nagy, E. E., & Moreau, K. L. (2019). Aerobic exercise training and vascular function with ageing in healthy men and women. *The Journal of physiology*, *597*(19), 4901-4914.
Seals, D. R., Walker, A. E., Pierce, G. L., & Lesniewski, L. A. (2009). Habitual exercise and vascular ageing. *The Journal of physiology*, *587*(23), 5541-5549.
Sebzda, K. N., Kuczmarski, A. V., Pohlig, R. T., Lennon, S. L., Edwards, D. G., & Wenner, M. M. (2018). Ovarian hormones modulate endothelin-1 receptor responses in young women. *Microcirculation*, *25*(7), e12490.
Sh, S., Nematbakhsh, M., & Dehghani, A. (2018). The Role of Mas Receptor and Bradykinin on Nitric Oxide Production Response to Angiotensin 1-7 in Ovariectomized Rats Treated with Estradiol. *HMJ*, *22*(1), 12-17.
Shah, A., Oh, Y.-B., Lee, S. H., Lim, J. M., & Kim, S. H. (2012). Angiotensin-(1–7) attenuates hypertension in exercise-trained renal hypertensive rats. *American Journal of Physiology-Heart and Circulatory Physiology*, *302*(11), H2372-H2380.
Sharma, G., & Prossnitz, E. R. (2011). Mechanisms of estradiol-induced insulin secretion by the G protein-coupled estrogen receptor GPR30/GPER in pancreatic β-cells. *Endocrinology*, *152*(8), 3030-3039.
Shaul, P. W. (1999). Rapid activation of endothelial nitric oxide synthase by estrogen. *Steroids*, *64*(1-2), 28-34.
Shi, Y., Lo, C.-S., Padda, R., Abdo, S., Chenier, I., Filep, J. G., Ingelfinger, J. R., Zhang, S.-L., & Chan, J. S. (2015). Angiotensin-(1–7) prevents systemic hypertension, attenuates oxidative stress and tubulointerstitial fibrosis, and normalizes renal angiotensin-converting enzyme 2 and Mas receptor expression in diabetic mice. *Clinical Science*, *128*(10), 649-663.
Shibata, H., Spencer, T. E., Onate, S. A., Jenster, G., Tsai, S. Y., Tsai, M.-J., & O'Malley, B. W. (1997). Role of co-activators and co-repressors in the mechanism of steroid/thyroid receptor action. *Recent progress in hormone research*, *52*, 141-164; discussion 164.
Shihoya, W., Nishizawa, T., Okuta, A., Tani, K., Dohmae, N., Fujiyoshi, Y., Nureki, O., & Doi, T. (2016). Activation mechanism of endothelin ETB receptor by endothelin-1. *Nature*, *537*(7620), 363-368.

Shoemaker, L. N., Haigh, K. M., Kuczmarski, A. V., McGinty, S. J., Welti, L. M., Hobson, J. C., Edwards, D. G., Feinberg, R. F., & Wenner, M. M. (2021). ETB receptor-mediated vasodilation is regulated by estradiol in young women. *American Journal of Physiology-Heart and Circulatory Physiology*, *321*(3), H592-H598.

Sies, H. (1986). Biochemistry of oxidative stress. *Angewandte Chemie International Edition in English*, *25*(12), 1058-1071.

Silva, A. R., Aguilar, E. C., Alvarez-Leite, J. I., da Silva, R. F., Arantes, R. M., Bader, M., Alenina, N., Pelli, G., Lenglet, S., & Galan, K. (2013). Mas receptor deficiency is associated with worsening of lipid profile and severe hepatic steatosis in ApoE-knockout mice. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, *305*(11), R1323-R1330.

Silveira, K. D., Barroso, L. C., Vieira, A. T., Cisalpino, D., Lima, C. X., Bader, M., Arantes, R. M. E., dos Santos, R. A. S., Simões-e-Silva, A. C., & Teixeira, M. M. (2013). Beneficial Effects of the Activation of the Angiotensin-(1–7) Mas Receptor in a Murine Model of Adriamycin-Induced Nephropathy. *PLOS ONE*, *8*(6), e66082. https://doi.org/10.1371/journal.pone.0066082

Silver, A. E., Christou, D. D., Donato, A. J., Beske, S. D., Moreau, K. L., Magerko, K. A., & Seals, D. R. (2009). Protein expression in vascular endothelial cells obtained from human peripheral arteries and veins. *Journal of Vascular Research*, *47*(1), 1-8.

Simoncini, T., Hafezi-Moghadam, A., Brazil, D. P., Ley, K., Chin, W. W., & Liao, J. K. (2000). Interaction of oestrogen receptor with the regulatory subunit of phosphatidylinositol-3-OH kinase. *Nature*, *407*(6803), 538-541.

Singh, T., Singh, K., & Sharma, P. (2010). Ameliorative potential of angiotensin1-7/Mas receptor axis in streptozotocin-induced diabetic nephropathy in rats. *Methods and findings in experimental and clinical pharmacology*, *32*(1), 19-25.

Sitia, S., Tomasoni, L., Atzeni, F., Ambrosio, G., Cordiano, C., Catapano, A., Tramontana, S., Perticone, F., Naccarato, P., & Camici, P. (2010). From endothelial dysfunction to atherosclerosis. *Autoimmunity reviews*, *9*(12), 830-834.

Sobrino, A., Vallejo, S., Novella, S., Lázaro-Franco, M., Mompeón, A., Bueno-Betí, C., Walther, T., Sánchez-Ferrer, C., Peiró, C., & Hermenegildo, C. (2017). Mas receptor is involved in the estrogen-receptor induced nitric oxide-dependent vasorelaxation. *Biochemical Pharmacology*, *129*, 67-72.

Somani, Y. B., Pawelczyk, J. A., De Souza, M. J., Kris-Etherton, P. M., & Proctor, D. N. (2019). Aging women and their endothelium: probing the relative role of estrogen on vasodilator function. *American Journal of Physiology-Heart and Circulatory Physiology*, *317*(2), H395-H404.

Sorensen, K. E., Dorup, I., Hermann, A. P., & Mosekilde, L. (1998). Combined hormone replacement therapy does not protect women against the age-related

decline in endothelium-dependent vasomotor function. *Circulation*, *97*(13), 1234-1238.
Stanhewicz, A. E., & Alexander, L. M. (2020). Local angiotensin-(1–7) administration improves microvascular endothelial function in women who have had preeclampsia. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, *318*(1), R148-R155.
Stanhewicz, A. E., Wenner, M. M., & Stachenfeld, N. S. (2018). Sex differences in endothelial function important to vascular health and overall cardiovascular disease risk across the lifespan. *American Journal of Physiology-Heart and Circulatory Physiology*, *315*(6), H1569-H1588.
Stegbauer, J., Oberhauser, V., Vonend, O., & Rump, L. C. (2004). Angiotensin-(1–7) modulates vascular resistance and sympathetic neurotransmission in kidneys of spontaneously hypertensive rats. *Cardiovascular Research*, *61*(2), 352-359. https://doi.org/10.1016/j.cardiores.2003.11.017
Stegbauer, J., Vonend, O., Oberhauser, V., & Rump, L. C. (2003). Effects of angiotensin-(1-7) and other bioactive components of the renin–angiotensin system on vascular resistance and noradrenaline release in rat kidney. *Journal of hypertension*, *21*(7), 1391-1399. https://journals.lww.com/jhypertension/fulltext/2003/07000/effects_of_angiotensin__1_7__and_other_bioactive.30.aspx
Steinberg, D., & Witztum, J. (1999). Lipoproteins, lipoprotein oxidation, and atherogenesis. *Molecular basis of cardiovascular disease*, *1499*, 458-475.
Stern, M. D. (1975). In vivo evaluation of microcirculation by coherent light scattering. *Nature*, *254*(5495), 56-58. https://doi.org/10.1038/254056a0
Stewart, J. M., Ocon, A. J., Clarke, D., Taneja, I., & Medow, M. S. (2009). Defects in cutaneous angiotensin-converting enzyme 2 and angiotensin-(1-7) production in postural tachycardia syndrome. *Hypertension*, *53*(5), 767-774.
Stewart, J. M., Taneja, I., Glover, J., & Medow, M. S. (2008). Angiotensin II type 1 receptor blockade corrects cutaneous nitric oxide deficit in postural tachycardia syndrome. *American Journal of Physiology-Heart and Circulatory Physiology*, *294*(1), H466-H473.
Stirone, C., Duckles, S. P., Krause, D. N., & Procaccio, V. (2005). Estrogen increases mitochondrial efficiency and reduces oxidative stress in cerebral blood vessels. *Molecular Pharmacology*, *68*(4), 959-965.
Su, C., Xue, J., Ye, C., & Chen, A. (2021). Role of the central renin-angiotensin system in hypertension. *International journal of molecular medicine*, *47*(6), 1-16.
Su, Z., Zimpelmann, J., & Burns, K. D. (2006). Angiotensin-(1–7) inhibits angiotensin II-stimulated phosphorylation of MAP kinases in proximal tubular cells. *Kidney International*, *69*(12), 2212-2218. https://doi.org/10.1038/sj.ki.5001509
Sullivan, J. C., Rodriguez-Miguelez, P., Zimmerman, M. A., & Harris, R. A. (2015). Differences in angiotensin (1–7) between men and women. *American Journal*

of Physiology-Heart and Circulatory Physiology, *308*(9), H1171-H1176. https://doi.org/10.1152/ajpheart.00897.2014

Taddei, S., Virdis, A., Ghiadoni, L., Mattei, P., Sudano, I., Bernini, G., Pinto, S., & Salvetti, A. (1996). Menopause is associated with endothelial dysfunction in women. *Hypertension*, *28*(4), 576-582.

Tagawa, T., Imaizumi, T., Endo, T., Shiramoto, M., Harasawa, Y., & Takeshita, A. (1994). Role of nitric oxide in reactive hyperemia in human forearm vessels. *Circulation*, *90*(5), 2285-2290.

Tallant, E. A., & Clark, M. A. (2003). Molecular mechanisms of inhibition of vascular growth by angiotensin-(1-7). *Hypertension*, *42*(4), 574-579.

Tetzner, A., Gebolys, K., Meinert, C., Klein, S., Uhlich, A., Trebicka, J., Villacañas, Ó., & Walther, T. (2016). G-Protein–Coupled Receptor MrgD Is a Receptor for Angiotensin-(1–7) Involving Adenylyl Cyclase, cAMP, and Phosphokinase A. *Hypertension*, *68*(1), 185-194. https://doi.org/doi:10.1161/HYPERTENSIONAHA.116.07572

Thijssen, D. H., Black, M. A., Pyke, K. E., Padilla, J., Atkinson, G., Harris, R. A., Parker, B., Widlansky, M. E., Tschakovsky, M. E., & Green, D. J. (2011). Assessment of flow-mediated dilation in humans: a methodological and physiological guideline. *American Journal of Physiology-Heart and Circulatory Physiology*, *300*(1), H2-H12.

Tian, J., Zhang, L., Zhou, Y., Xiao, J., Li, S., Chen, Y., Qiao, Z., Niu, J., & Gu, Y. (2014). Angiotensin-(1-7) attenuates damage to podocytes induced by preeclamptic serum through MAPK pathways. *Int J Mol Med*, *34*(4), 1057-1064. https://doi.org/10.3892/ijmm.2014.1870

Tom, B., Dendorfer, A., & Jan Danser, A. H. (2003). Bradykinin, angiotensin-(1–7), and ACE inhibitors: how do they interact? *The International Journal of Biochemistry & Cell Biology*, *35*(6), 792-801. https://doi.org/https://doi.org/10.1016/S1357-2725(02)00273-X

Tousoulis, D., Kampoli, A.-M., Tentolouris Nikolaos Papageorgiou, C., & Stefanadis, C. (2012). The role of nitric oxide on endothelial function. *Current vascular pharmacology*, *10*(1), 4-18.

Tummala, S., Kuczmarski, A. V., Del Vecchio, A. R., Schwab, A. I., Edwards, D. G., & Wenner, M. M. (2024). Antecubital venous endothelial ETB receptor protein expression is preserved with aging in men. *American Journal of Physiology-Heart and Circulatory Physiology*, *326*(1), H110-H115.

Turner, C. G., Stanhewicz, A. E., Nielsen, K. E., Otis, J. S., Feresin, R. G., & Wong, B. J. (2023). Effects of biological sex and oral contraceptive pill use on cutaneous microvascular endothelial function and nitric oxide-dependent vasodilation in humans. *Journal of applied physiology*, *134*(4), 858-867.

Turner, C. G., Stanhewicz, A. E., Nielsen, K. E., & Wong, B. J. (2023). Microvascular endothelial function following cessation of long-term oral contraceptive pill use: A case report. *Experimental Physiology*, *108*(1), 5-11.

Twist, D. J. L. v., Houben, A. J. H. M., Haan, M. W. d., Mostard, G. J. M., Kroon, A. A., & Leeuw, P. W. d. (2013). Angiotensin-(1–7)–Induced Renal Vasodilation in Hypertensive Humans Is Attenuated by Low Sodium Intake and Angiotensin II Co-Infusion. *Hypertension*, *62*(4), 789-793. https://doi.org/doi:10.1161/HYPERTENSIONAHA.113.01814

Ueda, S., Masumori-Maemoto, S., Ashino, K., Nagahara, T., Gotoh, E., Umemura, S., & Ishii, M. (2000). Angiotensin-(1-7) Attenuates Vasoconstriction Evoked by Angiotensin II but Not by Noradrenaline in Man. *Hypertension*, *35*(4), 998-1001. https://doi.org/10.1161/01.hyp.35.4.998

Unger, T. (2002). The role of the renin-angiotensin system in the development of cardiovascular disease. *The American journal of cardiology*, *89*(2), 3-9.

Vallon, V., Heyne, N., Richter, K., Khosla, M. C., & Fechter, K. (1998). [7-D-ALA]-Angiotensin 1-7 Blocks Renal Actions of Angiotensin 1-7 in the Anesthetized Rat. *Journal of cardiovascular pharmacology*, *32*(1), 164-167. https://journals.lww.com/cardiovascularpharm/fulltext/1998/07000/_7_d_ala__angiotensin_1_7_blocks_renal_actions_of_.25.aspx

van der Wouden, E. A., Ochodnický, P., van Dokkum, R. P., Roks, A. J., Deelman, L. E., de Zeeuw, D., & Henning, R. H. (2006). The role of angiotensin(1–7) in renal vasculature of the rat. *Journal of hypertension*, *24*(10), 1971-1978. https://doi.org/10.1097/01.hjh.0000244945.42169.c0

van Twist, D. J. L., Houben, A. J. H. M., de Haan, M. W., de Leeuw, P. W., & Kroon, A. A. (2016). Renal hemodynamics and renin–angiotensin system activity in humans with multifocal renal artery fibromuscular dysplasia. *Journal of hypertension*, *34*(6), 1160-1169. https://doi.org/10.1097/hjh.0000000000000917

Van Twist, D. J. L., Houben, A. J. H. M., De Haan, M. W., Mostard, G. J. M., De Leeuw, P. W., & Kroon, A. A. (2014). Angiotensin-(1-7)-induced renal vasodilation is reduced in human kidneys with renal artery stenosis. *Journal of hypertension*, *32*(12), 2428-2432. https://doi.org/10.1097/hjh.0000000000000351

Vane, J., & Corin, R. E. (2003). Prostacyclin: A Vascular Mediator. *European Journal of Vascular and Endovascular Surgery*, *26*(6), 571-578. https://doi.org/https://doi.org/10.1016/S1078-5884(03)00385-X

Vane, J. R., Änggård, E. E., & Botting, R. M. (1990). Regulatory functions of the vascular endothelium. *New England Journal of Medicine*, *323*(1), 27-36.

Velloso, E., Vieira, R., Cabral, A., Kalapothakis, E., & Santos, R. (2007). Reduced plasma levels of angiotensin-(1-7) and renin activity in preeclamptic patients are associated with the angiotensin I-converting enzyme deletion/deletion genotype. *Brazilian Journal of Medical and Biological Research*, *40*, 583-590.

Verma, S., & Anderson, T. J. (2002). Fundamentals of endothelial function for the clinical cardiologist. *Circulation*, *105*(5), 546-549.

Viana, G. E., Pereira, V. M., Honorato-Sampaio, K., Oliveira, C. A., Santos, R. A., & Reis, A. M. (2011). Angiotensin-(1–7) induces ovulation and steroidogenesis in perfused rabbit ovaries. *Experimental Physiology*, *96*(9), 957-965.

Vierhapper, H., Wagner, O., Nowotny, P., & Waldhäusl, W. (1990). Effect of endothelin-1 in man. *Circulation*, *81*(4), 1415-1418.

Virani, S. S., Alonso, A., Aparicio, H. J., Benjamin, E. J., Bittencourt, M. S., Callaway, C. W., Carson, A. P., Chamberlain, A. M., Cheng, S., Delling, F. N., Elkind, M. S. V., Evenson, K. R., Ferguson, J. F., Gupta, D. K., Khan, S. S., Kissela, B. M., Knutson, K. L., Lee, C. D., Lewis, T. T., . . . Tsao, C. W. (2021). Heart Disease and Stroke Statistics—2021 Update. *Circulation*, *143*(8). https://doi.org/10.1161/cir.0000000000000950

Wagenaar, G. T., Laghmani, E. H., Fidder, M., Sengers, R. M., De Visser, Y. P., De Vries, L., Rink, R., Roks, A. J., Folkerts, G., & Walther, F. J. (2013). Agonists of MAS oncogene and angiotensin II type 2 receptors attenuate cardiopulmonary disease in rats with neonatal hyperoxia-induced lung injury. *American Journal of Physiology-Lung Cellular and Molecular Physiology*, *305*(5), L341-L351.

Wagner, D. C., BonDurant, R. H., & Sischo, W. M. (2001). Reproductive effects of estradiol cypionate in postparturient dairy cows. *Journal of the American Veterinary Medical Association*, *219*(2), 220-223.

Waldron, G., Ding, H., Lovren, F., Kubes, P., & Triggle, C. (1999). Acetylcholine-induced relaxation of peripheral arteries isolated from mice lacking endothelial nitric oxide synthase. *British Journal of Pharmacology*, *128*(3), 653-658.

Wang, Y., Pringle, K. G., Chen, Y. X., Zakar, T., & Lumbers, E. R. (2012). Regulation of the renin-angiotensin system (RAS) in BeWo and HTR-8/SVneo trophoblast cell lines. *Placenta*, *33*(8), 634-639. https://doi.org/https://doi.org/10.1016/j.placenta.2012.05.001

Weiss, G., Skurnick, J. H., Goldsmith, L. T., Santoro, N. F., & Park, S. J. (2004). Menopause and hypothalamic-pituitary sensitivity to estrogen. *JAMA*, *292*(24), 2991-2996. https://doi.org/10.1001/jama.292.24.2991

Wenner, M. M., & Moreau, K. L. (2019). Endothelin-1 Contributes to Vascular Dysfunction in Peri-Menopausal Women. *Hypertension*, *74*(Suppl_1), A071-A071.

Wenner, M. M., Sebzda, K. N., Kuczmarski, A. V., Pohlig, R. T., & Edwards, D. G. (2017). ETB receptor contribution to vascular dysfunction in postmenopausal women. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, *313*(1), R51-R57.

Wenner, M. M., Shenouda, N., Shoemaker, L., Kuczmarski, A., Haigh, K., Del Vecchio, A., Schwab, A., McGinty, S. J., Edwards, D. G., & Pohlig, R. T. (2024). Characterizing vascular and hormonal changes in women across the life span: a cross-sectional analysis. *American Journal of Physiology-Heart and Circulatory Physiology*, *327*(5), H1286-H1295.

Wenner, M. M., Taylor, H. S., & Stachenfeld, N. S. (2011). Endothelin B receptor contribution to peripheral microvascular function in women with polycystic ovary syndrome. *The Journal of physiology*, *589*(19), 4671-4679.

Wenner, M. M., Wilson, T. E., Davis, S. L., & Stachenfeld, N. S. (2011). Pharmacological curve fitting to analyze cutaneous adrenergic responses. *Journal of applied physiology*, *111*(6), 1703-1709.

White, R. E. (2002). Estrogen and vascular function. *Vascular pharmacology*, *38*(2), 73-80.

Wilcox, J. G., Hatch, I. E., Gentzschein, E., Stanczyk, F. Z., & Lobo, R. A. (1997). Endothelin levels decrease after oral and nonoral estrogen in postmenopausal women with increased cardiovascular risk factors. *Fertility and sterility*, *67*(2), 273-277.

Williams, M. R. I., Westerman, R. A., Kingwell, B. A., Paige, J., Blombery, P. A., Sudhir, K., & Komesaroff, P. A. (2001). Variations in Endothelial Function and Arterial Compliance during the Menstrual Cycle. *The Journal of Clinical Endocrinology & Metabolism*, *86*(11), 5389-5395. https://doi.org/10.1210/jcem.86.11.8013

Wilsdorf, T., Gainer, J. V., Murphey, L. J., Vaughan, D. E., & Brown, N. J. (2001). Angiotensin-(1-7) Does Not Affect Vasodilator or TPA Responses to Bradykinin in Human Forearm. *Hypertension*, *37*(4), 1136-1140. https://doi.org/10.1161/01.hyp.37.4.1136

Wilson, C., Lee, M. D., & McCarron, J. G. (2016). Acetylcholine released by endothelial cells facilitates flow-mediated dilatation. *The Journal of physiology*, *594*(24), 7267-7307.

Wise, P. M., Suzuki, S., & Brown, C. M. (2009). Estradiol: a hormone with diverse and contradictory neuroprotective actions. *Dialogues Clin Neurosci*, *11*(3), 297-303. https://doi.org/10.31887/DCNS.2009.11.3/pmwise

Wong, B. J. (2013). Sensory nerves and nitric oxide contribute to reflex cutaneous vasodilation in humans. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, *304*(8), R651-R656. https://doi.org/10.1152/ajpregu.00464.2012

Wright, H. M., & Malik, K. U. (1996). Prostacyclin formation elicited by endothelin-1 in rat aorta is mediated via phospholipase D activation and not phospholipase C or A2. *Circulation Research*, *79*(2), 271-276.

Yamada, Y., Doi, T., Hamakubo, T., & Kodama, T. (1998). Scavenger receptor family proteins: roles for atherosclerosis, host defence and disorders of the central nervous system. *Cellular and Molecular Life Sciences CMLS*, *54*, 628-640.

Yanes, L. L., Romero, D. G., Cucchiarelli, V. E., Fortepiani, L. A., Gomez-Sanchez, C. E., Santacruz, F., & Reckelhoff, J. F. (2005). Role of endothelin in mediating postmenopausal hypertension in a rat model. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, *288*(1), R229-R233.

Yang, G., Istas, G., Höges, S., Yakoub, M., Hendgen-Cotta, U., Rassaf, T., Rodriguez-Mateos, A., Hering, L., Grandoch, M., & Mergia, E. (2018). Angiotensin-(1-7)-induced Mas receptor activation attenuates atherosclerosis through a nitric oxide-dependent mechanism in apolipoproteinE-KO mice. *Pflügers Archiv-European Journal of Physiology*, *470*, 661-667.

Yang, S., Bae, L., & Zhang, L. (2000). Estrogen increases eNOS and NOx release in human coronary artery endothelium. *Journal of cardiovascular pharmacology*, *36*(2), 242-247.

Ylikorkala, O., Orpana, A., Puolakka, J., Pyörälä, T., & Viinikka, L. (1995). Postmenopausal hormonal replacement decreases plasma levels of endothelin-1. *The Journal of Clinical Endocrinology & Metabolism*, *80*(11), 3384-3387.

Yung, L. M., Wong, W. T., Tian, X. Y., Leung, F. P., Yung, L. H., Chen, Z. Y., Yao, X., Lau, C. W., & Huang, Y. (2011). Inhibition of renin-angiotensin system reverses endothelial dysfunction and oxidative stress in estrogen deficient rats. *PLOS ONE*, *6*(3), e17437.

Zeng, G., & Quon, M. J. (1996). Insulin-stimulated production of nitric oxide is inhibited by wortmannin. Direct measurement in vascular endothelial cells. *The Journal of clinical investigation*, *98*(4), 894-898.

Zhang, F., Xu, Y., Pan, Y., Sun, S., Chen, A., Li, P., Bao, C., Wang, J., Tang, H., & Han, Y. (2019). Effects of Angiotensin-(1-7) and Angiotensin II on Acetylcholine-Induced Vascular Relaxation in Spontaneously Hypertensive Rats. *Oxidative Medicine and Cellular Longevity*, *2019*, 6512485. https://doi.org/10.1155/2019/6512485

Zheng, Y., Tang, L., Huang, W., Yan, R., Ren, F., Luo, L., & Zhang, L. (2015). Anti-inflammatory effects of Ang-(1-7) in ameliorating HFD-induced renal injury through LDLr-SREBP2-SCAP pathway. *PLOS ONE*, *10*(8), e0136187.

Appendix A

Human Subjects Approval

Institutional Review Board
210H Hullihen Hall
Newark, DE 19716
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Fax: 302-831-2828

DATE:	January 22, 2024
TO:	Angelica Del Vecchio
FROM:	University of Delaware IRB
STUDY TITLE:	[2146686-1] Vascular Effects of Angiotensin-(1-7) in Postmenopausal Women
SUBMISSION TYPE:	New Project
ACTION:	APPROVED
APPROVAL DATE:	January 22, 2024
EXPIRATION DATE:	January 16, 2025
REVIEW TYPE:	Full Committee Review

Thank you for your New Project submission to the University of Delaware Institutional Review Board (UD IRB). The UD IRB has reviewed and APPROVED the proposed research and submitted documents via Full Committee Review in compliance with the pertinent federal regulations.

As the Principal Investigator for this study, you are responsible for and agree that:

- All research must be conducted in accordance with the protocol and all other study forms as approved in this submission. Any revisions to the approved study procedures or documents must be reviewed and approved by the IRB prior to their implementation. Please use the UD amendment form to request the review of any changes to approved study procedures or documents.
- Informed consent is a process that must allow prospective participants sufficient opportunity to discuss and consider whether to participate. IRB-approved and stamped consent documents must be used when enrolling participants and a written copy shall be given to the person signing the informed consent form.
- Unanticipated problems, serious adverse events involving risk to participants, and all non-compliance issues must be reported to this office in a timely fashion according with the UD requirements for reportable events. All sponsor reporting requirements must also be followed.

Oversight of this study by the UD IRB REQUIRES the submission of a CONTINUING REVIEW seeking the renewal of this IRB approval, which will expire on January 16, 2025. A continuing review/progress report form and up-to-date copies of the protocol form and all other approved study materials must be submitted to the UD IRB at least 45 days prior to the expiration date to allow for the required IRB review of that report.

If you have any questions, please contact the UD IRB Office at (302) 831-2137 or via email at hsrb-research@udel.edu. Please include the study title and reference number in all correspondence with this office.

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