RESTING MICROVASCULAR AND AUTONOMIC FUNCTION IN RELATION TO AGE AND SEX HORMONE CONCENTRATIONS

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Abstract

This study assessed the influence of estradiol, progesterone, testosterone and age on resting microvascular and autonomic function in younger and older males and females. Microvascular function was assessed with reactive hyperemia using the microvascular function index (MFI) technique (5mins baseline, circulatory occlusion and reperfusion) and was calculated using the area under the curve of pulse waveforms before and after occlusion/reperfusion. Autonomic function was assessed using 5 mins resting heart rate variability (HRV) and paced deep breathing. Multiple linear regressions were conducted for the influence of estradiol, progesterone, testosterone and age on resting HRV variables and MFI. Aging negatively correlated with respiratory sinus arrhythmia (RSA) indicating lower parasympathetic activity and positively correlated with diastolic blood pressure (DBP) and mean arterial pressure (MAP). Estradiol positively correlated with the low frequency/high frequency ratio of HRV, indicating higher sympathetic activity, and DBP and MAP. Progesterone and testosterone were not significantly correlated with any measures.

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List of Abbreviations

BMI: Body mass index

BP: Blood pressure

CVRR: coefficient of variance of RR

EI Ratio: Expiratory to inspiratory ratio

ELISA: Enzyme-linked immunosorbent assay

EndoPAT: Endothelial peripheral artery tonometry

eNOS: Endothelial nitric oxide synthase

FMD: Flow mediated dilation

HRV: Heart rate variability

HF: High frequency

LF: Low frequency

LF/HF: Low frequency to high frequency ratio

MAP: Mean arterial pressure

MFI: Microvascular function index

NO: Nitric oxide

PNS: Parasympathetic nervous system

pRR50: Percentage of difference higher than 50msec in RR intervals

DBP: Diastolic blood pressure

RHI: Reactive hyperemia index

RSA: Respiratory sinus arrythmia

SBP: Systolic blood pressure

SD1: Standard deviation perpendicular to the line of identity of the Poincaré scattergram plot

SD2: Standard deviation along the line of identity of the Poincaré scattergram plot

SDARR: Standard deviation of average RR Intervals

SDNN: Standard deviation of NN interval

SDRR: Standard deviation of RR Intervals

SDSD: Standard deviation of successive RR interval differences

SNS: Sympathetic nervous system

RMSSD: Root mean square of successive differences in RR intervals

VLF: Very low frequency

Chapter 1: Literature Review

1.1. Understanding The Vascular System

The vascular system is comprised of an extensive set of blood vessels ranging in size and function from arteries, arterioles, capillaries, venules, and veins, where they work together to provide a homeostatic environment for cells through the delivery of essential nutrients along with metabolic waste removal (reviewed in Pugsley & Tabrizchi, 2000). Arteries carry oxygenated blood away from the heart to the rest of the body branching into arterioles and then capillaries, which has the smallest diameter, to facilitate the gas exchange process across the capillary walls to tissues. Then the blood moves through the venules and veins carrying the deoxygenated blood back towards the heart (reviewed in Pugsley & Tabrizchi, 2000). With the exception of capillaries, the vascular wall of most blood vessels consists of three histologically distinct layers, the tunica adventitia, tunica media, and tunica intima (reviewed in Borysenko & Beringer, 1984). The outermost layer, tunica adventitia, primarily comprises fibro-elastic connective tissue for protection. The middle layer, tunica media, is predominately composed of smooth muscle cells and elastic tissue which assist in moving large volumes of blood. The inner layer, tunica intima, is the thinnest layer composed of a single layer of endothelial cells (reviewed in Borysenko & Beringer, 1984). Arterial and arteriolar blood vessels can increase (vasodilation) or decrease (vasoconstriction) their diameters to adjust blood delivery in response to tissue metabolic demand (Galley & Webster, 2004). The endothelium integrates multifaceted signals from the local microenvironment including metabolites, circulating hormones, and autonomic nervous activity which all play a central role in the regulation of vasodilatory capability. Measurements of endothelial dysfunction can be used as an early indicator of vascular diseases such as atherosclerosis (Davignon & Ganz, 2004).

1.2. Reactive hyperemia

Reactive hyperemia, the phenomenon of increased blood flow after a period of ischemia, can be used to assess a person's vasodilatory capability by measuring the degree of blood flow restoration in the limbs after a short period of arterial occlusion (Loscalzo & Vita, 1994). Experimentally, it has been established as a gold-standard technique to assess peripheral

microvascular function non-invasively and predict cardiovascular morbidity and mortality (Huang et al., 2007; Ishibashi et al., 2006). Reactive hyperemia can be assessed by wrapping a blood pressure (BP) cuff around a portion of a limb and inflating it to supra-systolic pressures, at least 50mmHg above resting systolic blood pressure (SBP), to sustain a 5-min arterial occlusion (Corretti et al., 2002). Arterial occlusion causes blood flow restriction, leading to tissues becoming ischemic, hence stimulating vasodilation upon the release of the cuff pressure (Patterson & Whelan, 1955). It has been demonstrated by both experimental and clinical studies that vasodilation during reperfusion is an endothelium-dependent mechanism (Nabel et al., 1990; Rubanyi et al., 1986). A primary vasodilatory molecule released from endothelial cells is nitric oxide (NO) (Laroia et al., 2003). First described by Furchgott and Zawadzki (1980), NO was originally called endothelium derived relaxing factor. Endothelial nitric oxide synthase (eNOS), an isoform of nitric oxide synthase, is expressed in endothelial cells (Förstermann et al., 1994) along with other places in the body, such as cardiac muscle, osteoclasts, and osteoblasts (Förstermann & Münzel, 2006). An increase in blood flow after ischemia causes shear stress on the vessels walls, resulting in calcium-activated potassium channels opening on the endothelial cell membrane (Cooke et al., 1991; Miura et al., 2001; Olesen et al., 1988). The efflux of potassium hyperpolarizes the endothelial cell, resulting in increased calcium entry into the cell, activating eNOS (Dimmeler et al., 1999). Activating eNOS creates NO by oxidizing the amino acid L-Arginine to L-citrulline (Palmer et al., 1988). NO then diffuses to the vascular smooth muscle cells leading to vasodilation (Schultz et al., 1977). A series of intracellular processes, such as calcium uptake by the sarco/endoplasmic reticulum calcium ATPase and inhibiting the calcium influx through the voltage-gated calcium channels. These processes reduce the intracellular concentration of Ca^{2+} and cause vascular smooth muscle relaxation, as a result of the dephosphorylation of myosin light chain (reviewed in Jackson, 2000; Sandoo et al., 2010).

1.3. Measures of Microvascular Function

1.3.1. Flow-Mediated Dilation

There are numerous different techniques that measure the response to reactive hyperemia. Original studies employed the venous occlusion plethysmography technique (Hewlett & Zwaluwenburg, 1910), where a strain gauge is positioned around the largest part of the forearm, to monitor the variation in limb volume, along with occluding BP cuffs placed on the upper arm

and wrist. In today's age, flow mediated dilation (FMD) has been established to be a gold-standard method (Uehata et al., 1997). FMD is a non-invasive method using duplex ultrasound developed by Celermajer and colleagues in 1992 to measure the change in artery diameter in response to shear stress in a conduit artery (typically the brachial artery) (Celermajer et al., 1992). The procedure for FMD typically consists of the measurement of arterial diameter for at least 1 min of resting baseline, 5 min arterial occlusion, and a recovery period of ≥3 min post-occlusion. FMD is calculated as the percent change between baseline diameter and the maximum reactive hyperemia diameter.

1.3.2. EndoPAT

Endothelial peripheral artery tonometry (EndoPAT) is a non-invasive device that captures changes in peripheral arterial tone using a pair of plethysmographic probes placed on the index fingers of both hands to measure changes in volume (Axtell et al., 2010) and is a useful device to test endothelial function (Hamburg & Benjamin, 2009). EndoPAT results correlate with FMD, as a linear relationship was observed between the measurements (Kuvin et al., 2003). However, while FMD and EndoPAT are good surrogates to examine vascular function, Nardone and colleagues (2020) found that these two methods assess different vasodilatory mechanisms; FMD is correlated with coronary vasodilation in response to adenosine and acetylcholine, whereas EndoPAT is correlated with coronary vasodilation in response to dobutamine and thus dilation through adrenergic stimulation. The protocol for EndoPAT is similar to that of FMD described above consisting of 5 mins of baseline, 5 mins of forearm arterial occlusion using a BP cuff inflated to suprasystolic pressure, and 5 mins of reactive hyperemia recovery. This technique provides a reactive hyperemia index (RHI), reflecting NO bioavailability (Nohria et al., 2006). RHI measures the average pulse wave amplitude for the second minute of reactive hyperemia compared to the average 210 secs prior to occlusion; this ratio is then normalized to the same ratio in the non-occluded arm (Kuvin et al., 2003).

1.3.3. Microvascular Function Index

The microvascular function index (MFI) method was introduced by Ramraj and colleagues (2024) where they found that MFI has a significant positive correlation with RHI, thus MFI can be used as an alternative method to the EndoPAT device, serving as an adequate measure of

microvascular function. The protocol for MFI is identical to the protocol conducted for EndoPAT, consisting of 5 mins of baseline, 5 mins of suprasystolic forearm occlusion (50mmHg above resting SBP), and 5 mins of recovery after occlusion. An important difference to note is the type of sensors. Rather than volumetric sensors encompassing the entire distal phalange of the index fingers (EndoPAT), piezoelectric pulse transducers (ADInstruments, USA) are placed on the pads of both middle fingers (MFI) and 10 secs around peak during reactive hyperemia are used for analysis (details in Methods).

1.4. Understanding the Autonomic Nervous System

The autonomic nervous system is a major part of the peripheral nervous system and controls involuntary bodily functions such as heart rate, blood pressure, breathing, digestion, and sexual arousal. It is divided into the parasympathetic nervous system (PNS) and the sympathetic nervous system (SNS), and communicates with the brain which translates sensory and motor inputs through afferent nerves and elicits appropriate responses through efferent nerves (reviewed by Waxenbaum et al., 2024). The PNS is colloquially known as "rest and digest," where the nerves signal the body to relax, whereas the SNS is colloquially known as "fight or flight," where the nerves signal the body to prepare for action. SNS directly affects the microvasculature of skeletal muscle through the innervation of the blood vessel wall and release of norepinephrine, which predominantly binds to the alpha-1 adrenergic receptor on smooth muscle to cause vasoconstriction (reviewed by Bevan et al., 2011). In addition, epinephrine is primarily released from the adrenal medulla into the bloodstream, which has a higher affinity towards the beta-2 adrenergic receptor to cause vasodilation (reviewed by Bevan et al., 2011). The PNS does not directly affect microvascular function in skeletal muscle due to a lack of parasympathetic nerve innervation of the skeletal muscle vasculature (reviewed by (Undem, 2009). However, it can cause vasodilation in specific vascular beds such as the cerebral and enteric vasculature (Talman et al., 2007).

1.5. Measurements of the Autonomic Nervous System

1.5.1. Heart Rate Variability

Heart rate variability (HRV) is the variation in time intervals between consecutive heartbeats (Saul, 1990). It has been established as a non-invasive assessment method for autonomic

function where higher HRV represents greater PNS and lower SNS activity and conversely lower HRV represents lower PNS and greater SNS activity (Rajendra Acharya et al., 2006). The current study aims to assess the balance of SNS and PNS by employing frequency, time, and non-linear domain analysis of HR. Frequency domain analysis uses fast Fourier transform functionality to breakdown the variability of RR intervals into its components. This includes a low frequency (LF) component (i.e. slow changes in RR interval), which represents a combined activity of SNS and PNS; a high frequency (HF) component (i.e. fast changes in RR interval), which predominately represents PNS activity; and the LF/HF ratio, which represents the activity of SNS (Malik et al., 1996). In addition, Goldstein and colleagues (2011) discussed the relationship between LF and baroreflex sensitivity, an autonomic mechanism that regulates blood pressure and heart rate. They addressed the previously long-held belief that LF is not solely an indicator of cardiac sympathetic tone; it can assess baroreceptor sensitivity. The time domain includes parameters of HRV that all represent PNS control, for example the standard deviation of RR intervals (SDRR; this is also referred to as SDNN which is defined as the standard deviation of NN interval), the standard deviation of average RR intervals for every 5 min cycle of a measurement (SDARR), the root mean square of successive differences in RR intervals (RMSSD), and the percentage of difference higher than 50msec in RR intervals (pRR50) (Malik et al., 1996). The non-linear domain includes variables shown in Figure 1, such as the standard deviations perpendicular (SD1) and parallel (SD1) to the line of identity of the Poincaré scattergram plot developed when comparing an RR interval against the previous RR interval. SD1 represents PNS dominance and SD2 represents SNS dominance (Malik et al., 1996).

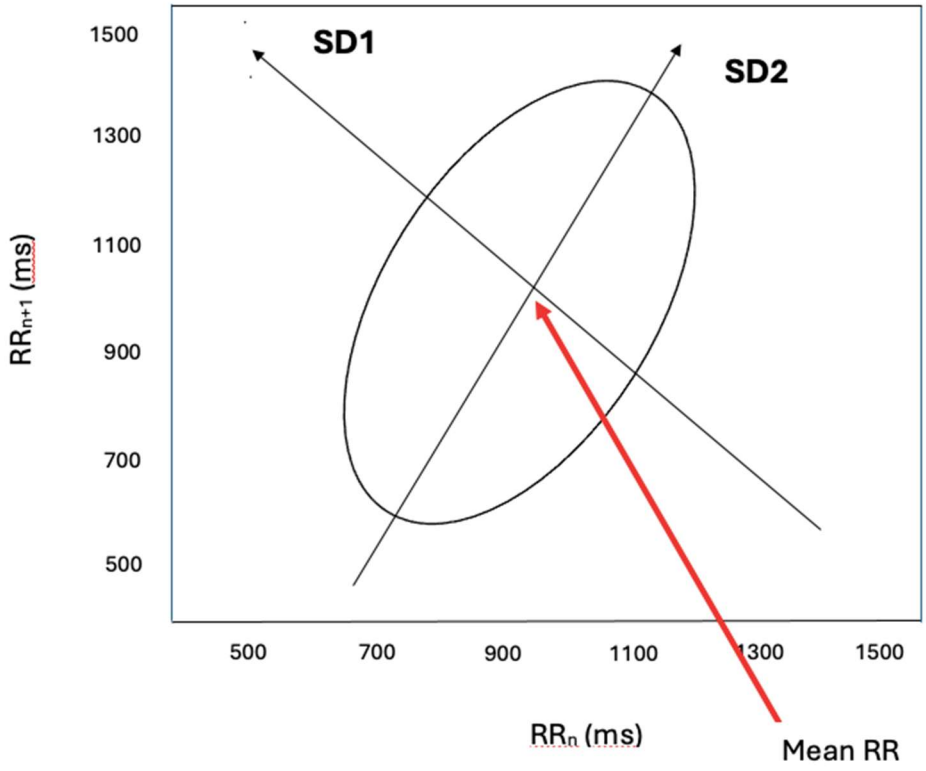

Figure 1: Poincaré scatterplot for non-linear analysis of heart rate variability. Standard deviation perpendicular (SD1) and parallel (SD2) to the line of identity of the Poincaré scattergram plot developed when comparing RR interval against the previous RR interval. RR represents the R-R intervals between each heartbeat.

1.5.2. Paced Deep Breathing

Another way to assess HRV is via paced deep breathing while measuring changes in HR. Participants lie supine and perform cycles of deep inhalation (5 secs) and deep exhalation (5 secs) for eight breathing cycles with one minute rest between trials (Novak, 2011). The rhythmic fluctuations in heart rate that occur with breathing are known as respiratory sinus arrhythmia (RSA), as shown in Figure 2, and is a phenomenon of cardiorespiratory coupling (Ja & B, 1981). RSA is calculated as the difference between the average maximum heart rate (average HR max) and the average minimum heart rate (average HR min). The expiratory-to-inspiratory ratio (E/I ratio) is the quotient of average HR max and average HR min.

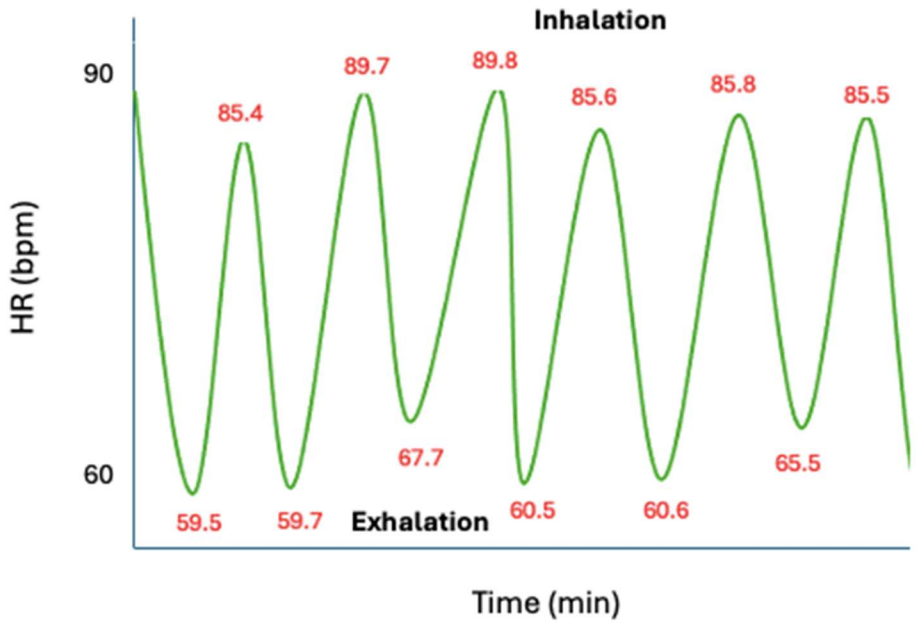

Figure 2: Changes in heart rate (HR) with multiple inhalations and exhalations.

1.6. Sex hormones

Varying amounts of estrogen, progesterone and testosterone are found in men and women depending on time of day, menstrual cycle, age, andropause, and menopause (depending on the specific hormone). Estrogen is a steroid hormone that is typically known for influencing female secondary sex characteristics and reproduction (Liang & Shang, 2013). There are three types of estrogen that carry a different physiological function, for example, estrone is produced in women after menopause; estradiol is the most potent type of estrogen produced in premenopausal women; estriol is mainly produced in women during pregnancy (Cui et al., 2013). For the purpose of this study, we will be focusing on estradiol, rather than estrone, since it is the major biologically active estrogen, and is closely linked to physiological processes such as maintaining bone health (Vermeulen et al., 2002). Progestogens are endogenous steroid hormones and similar to estrogen, there are different forms, whereby progesterone is the major progestogen in the body, and typically known for regulating female reproduction function. Progestins are a group of synthetic progesterone which subdivides into pregnanes, estranes, and gonanes and can be used as contraceptives (García-Sáenz et al., 2023). Testosterone is a type of androgen, which is a group of sex steroid hormones that are typically known for regulating male reproductive function with

precursors or metabolites such as dehydroepiandrosterone and dihydrotestosterone, androstenedione (Alemany, 2022). Each of these hormones has a much more complicated role in human physiology than their roles in sexual development and pregnancy, and each of these hormones are important in the physiological functioning of both biological sexes. For example, estrogen modulates vasodilatory capacity (Hamilton et al., 2017), progesterone plays an important role in neuroprotection during traumatic brain injury (Espinoza & Wright, 2011), testosterone is responsible for skeletal muscle hypertrophy by stimulating protein synthesis, and inhibiting protein degradation (Vingren et al., 2010). Despite the important roles that sex hormones play in regulating human physiology, there remains a significant gap in the literature, as most studies have been conducted primarily in males. This has led to a dearth of information in females health, particularly the hormonal fluctuations that occur across the menstrual cycle and menopause. Therefore, the inclusion of premenopausal women and postmenopausal women is essential to provide a comprehensive understanding of how endogenous sex hormones relate to microvascular and autonomic function.

In the past, estrogen and progesterone were known as "female" sex hormones and testosterone was regarded as a "male" sex hormone; however, there is a large cross over between groups in terms of the concentration of sex hormones. For example, young men and women have similar concentrations of estrogen and progesterone when examining women in the early follicular phase of the menstrual cycle (Sherman & Korenman, 1975). As women age and reach menopause, they experience a decline in estrogen and progesterone (Burger et al., 2002) leading to higher levels of estrogen and progesterone in older men compared to postmenopausal women (Vermeulen et al., 2002). In addition, the amount of testosterone produced by males in their testes is 7 to 8 greater than the amount produced by females in their ovaries (Franchimont, 1983) and it begins to decline at age 30 in men by approximately 1% each year (Harman et al., 2001), and in women after menopause (Rannevik et al., 1986). Women with polycystic ovarian syndrome (PCOS) have higher levels of testosterone than regularly cycling premenopausal women, but not greater than men, along with higher estrogen and lower progesterone (Abdelazim et al., 2020), (Luan et al., 2022). Interestingly, women have higher circulating testosterone than estrogen throughout the menstrual cycle (Muneyvirci-Delale et al., 1998).

Premenopausal women experience cyclical changes of hormones throughout the menstrual cycle highlighted in Figure 3 The first phase is the early follicular phase, when menstruation

occurs, and very low estrogen and progesterone concentrations are present. The second phase is the late follicular phase, when there is increasing/high estrogen with low progesterone. The third phase is ovulation, where the egg releases from the ovaries and travels to the fallopian tubes, where there is high estrogen with low progesterone. The fourth phase takes place immediately after ovulation, the early luteal phase, where there are increasing levels of estrogen and progesterone. Lastly, in the luteal phase, there are higher levels of both estrogen and progesterone. The cycle then starts over again leading to the early follicular phase/menstruation with falling levels of estrogen and progesterone (Mihm et al., 2011). Testosterone stays constant throughout the menstrual phases, except for brief elevation around the time of ovulation (Figure 3).

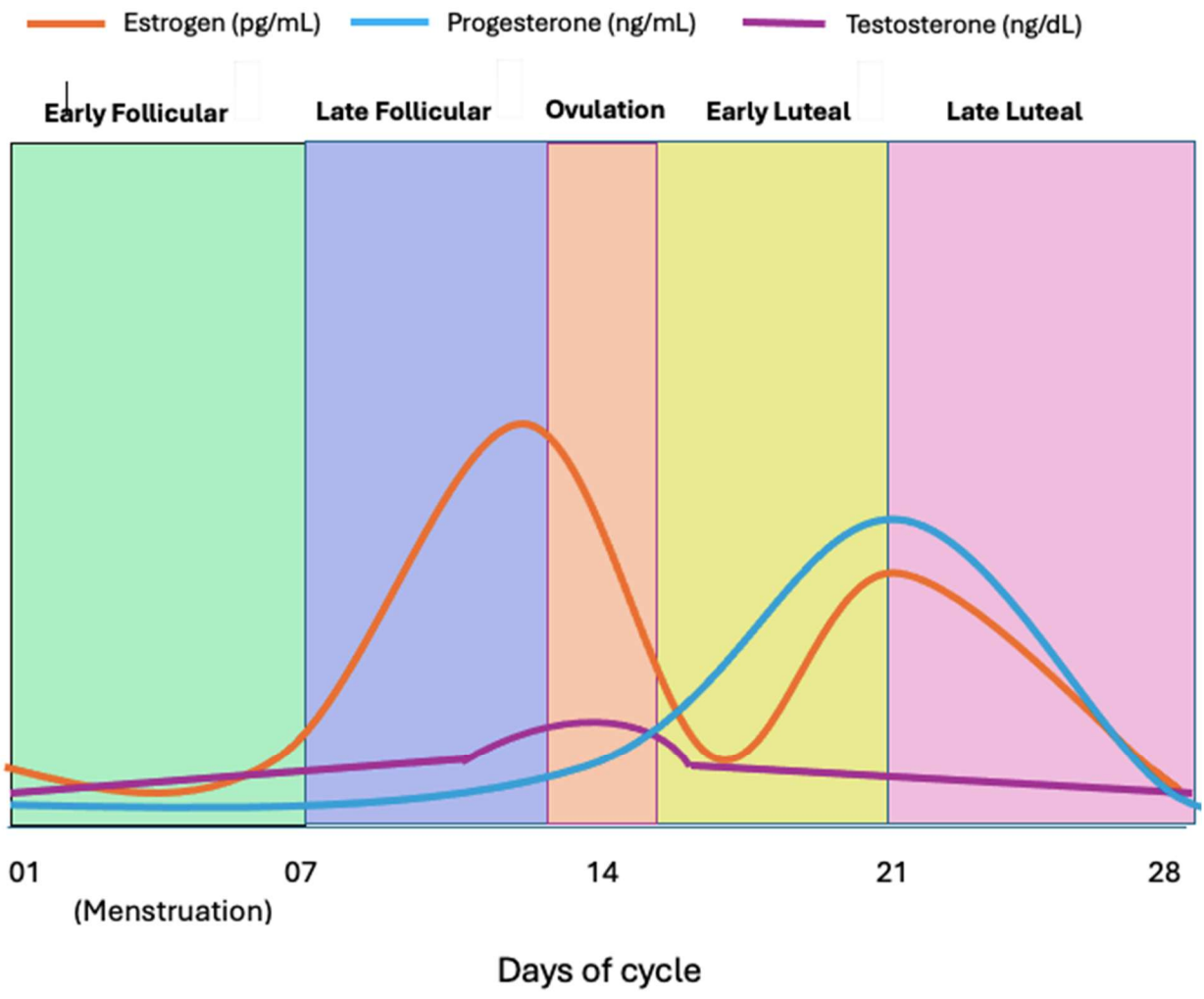

Figure 3: Schematic representing changes in sex hormones over different phases of the menstrual cycle including estrogen (orange), progesterone (blue), and testosterone (purple).

1.7. Impact of Age and Sex on Autonomic Function, Microvascular Function and Blood Pressure

Kuo and colleagues (1999) observed a sex difference between the frequency domains of resting HRV, where females have higher HF than males who had higher LF/HF ratio and LF, illustrating that females have greater resting PNS function and males have greater SNS function. In addition, Abhishekh and colleagues (2013) found that LF was lower, yet HF was higher, in females, suggesting greater resting PNS function in females. Interestingly, Dantas and colleagues (2018) found some conflicting results where males had lower HF than females, yet higher SDRR LF, and LF/HF. These results may suggest that males have greater SNS compared to females, yet unclear changes in PNS function, making the impact of sex unclear on HRV. With regard to changes due to aging, Zhang and colleagues (2007) observed an inverse relationship between age and LF and HF, indicating a decrease in PNS control with an increase in age. Further, Abhishekh and colleagues (2013) found that SDRR, RMSSD, and HF all decreased with increasing age, whereas LF/HF ratio increased with age, suggesting an increase in SNS function and a decrease in PNS function with increasing age. An elevation in SBP and MAP was observed with increasing age, whereas diastolic blood pressure (DBP) increases until 50 and declines thereafter, suggesting that SBP and MAP are positively correlated with age whereas DBP is negatively correlated with older age (Burt et al., 1995; Franklin et al., 1997).

Celermajer and colleagues (1994) found that aging is associated with endothelial dysfunction, as reduced FMD was observed in an older aged cohort. Furthermore, in the same study, sex differences were also noted, where FMD levels started to decline at the age of 40 in men whereas the decline was seen in women during their early 50s, indicating that this impairment in vascular function could be due to the loss of sex hormones in either sex (i.e. andropause and menopause) rather than age *per se*. Contrarily, Jensen-Urstad and Johansson (2001) observed that FMD in men was the same at age 35 and at age 55, perhaps indicating that andropause does not play a role; however, in women, FMD was reduced at age 55 compared to age 35, which could be a result of menopause.

1.8. Estrogen and Progesterone in relation to Autonomic Function

As previously discussed, estrogen and progesterone vary through a normal menstrual cycle and with age in women, thus it is important to note that each hormone has the potential to influence

autonomic function. Grrishma and colleagues (2015) found that PNS components of HRV, such as, RMSSD, pRR50 and HF decreased in the late luteal phase (indicating lower PNS function), whereas SNS components of HRV such as the LF/HF ratio, were significantly higher in the late luteal phase (indicating higher SNS function). These findings suggest that the presence of progesterone in the luteal phase decreases PNS and increases SNS function. Indeed, Sato and colleagues (1995) observed similar findings where LF and LF/HF ratio increased along with a reduction in HF in the late luteal phase compared to the follicular phase, suggesting greater SNS and lower PNS function in the luteal phase potentially due to increased progesterone levels.

Neves and colleagues (2007) found that postmenopausal women with estrogen therapy decreased LF and LF/HF ratio while increasing HF, showing that estrogen therapy decreases SNS and increases PNS activity in older women. In support of this, Yang and colleagues (2013) also showed that postmenopausal women treated with estrogen therapy alone had lower LF and higher HF, whereas HRV in women who took combined estrogen and progesterone therapy had similar responses to untreated postmenopausal women. Together, these results suggest that estrogen increases HRV yet progesterone may attenuate the effect of estrogen.

1.9. Influence of Estrogen and Progesterone on Microvascular Function and Blood Pressure

Miner and colleagues (2011) discovered that after supressing endogenous estrogen and progesterone in premenopausal women using a gonadotropin releasing hormone antagonist, those who subsequently received a combined therapy of estrogen and progesterone had lower FMD; however, upon the administration of estrogen alone, FMD increased. These findings suggest that estrogen can increase endothelial function, yet progesterone may oppose the vasodilatory effect of estrogen in young women. Indeed, Lizarelli and colleagues (2009) investigated the effect of either combined oral contraceptives (containing both synthetic estrogen and progesterone) or oral contraceptives containing only synthetic progesterone on blood vessel function in premenopausal women and found that FMD was significantly lower in the groups taking either combined oral contraceptives or progesterone only compared to controls not taking any contraceptives, suggesting that progesterone worsens microvascular function in younger women (Lizarelli et al., 2009). Further, Peterson and colleagues (2000) found that postmenopausal women taking estrogen therapy had significantly higher peak hyperemic blood flow after arterial occlusion compared to postmenopausal women not taking estrogen therapy. Interestingly, the postmenopausal women

taking estrogen had a similar peak hyperemic blood velocity response to premenopausal women, illustrating that estrogen treatment enhances microvascular function to that of younger women. Indeed, Sanada and colleagues (2001) showed that postmenopausal women taking estrogen therapy had higher blood flow in their forearms during reactive hyperemia, further suggesting that estrogen increases microvascular function. Similarly, Lieberman and colleagues (1994) found that postmenopausal women who received estrogen therapy experienced greater FMD compared to an age-matched control group, supporting the idea that microvascular function positively correlates with estrogen in postmenopausal women.

Mercuro and colleagues (1998) found that postmenopausal women taking estrogen therapy had significantly lower SBP and diastolic blood pressure (DBP), suggesting that estrogen decreases BP. Indeed, Seely and colleagues (1999) found a decrease in SBP and DBP in postmenopausal women taking estrogen therapy. Similarly, Shi and colleagues (2023) found that progesterone was also negatively associated with DBP in postmenopausal women and older men. suggesting that progesterone decreases DBP. Further, Barbagallo and colleagues (2001) found that progesterone administered to rats also reduced SBP.

1.10. Testosterone and Autonomic Function

Ermis and colleagues (2010) observed a positive correlation between testosterone and various time and frequency parameters of HRV in young men with hypogonadism, a condition where the body is unable to produce enough testosterone, such as SDNN, SDANN, LF, HF, and LF/HF, showing that higher testosterone levels increased both PNS and SNS function. Indeed, Poliwczak and colleagues (2013) found that testosterone supplementation in men with hypoandrogen-metabolic syndrome increased SDNN, SDANN, and LF in men with testosterone deficiency and metabolic syndrome, indicating that testosterone increases PNS activity (Poliwczak et al., 2013). However, Doğru and colleagues (2010) showed that testosterone level in men with cardiac disease (i.e. not hypoandrogenic) was positively correlated with HF and negatively correlated with LF and LF/HF, indicating that testosterone increases PNS yet decreases SNS function in men with functioning reproductive systems. The contrasting results could be attributed to the different baseline concentration of testosterone, which could be influenced by age and health condition of participants. For example, Ermis and colleagues (2010) recruited younger men with hypogonadism, while Poliwczak and colleagues (2013) recruited older men with hypogonadism,

and Doğru and colleagues (2010) recruited older men with cardiac disease. Interestingly, Yildirir and colleagues (2006) found that women with polycystic ovarian syndrome (PCOS; a condition where there is a higher testosterone in females) had higher levels of LF and LF/HF along with lower HF compared to controls without PCOS, showing that higher testosterone levels in women decreases HRV via lower PNS and higher SNS. There is a limited understanding of the relationship between testosterone concentrations and autonomic function in young and older women.

Table 1: Summary of the effect of sex hormones on autonomic and microvascular function

	Estradiol	**Progesterone**	**Testosterone**
HF	↑	↓	Conflicting results
LF/HF	↓	↑	Conflicting results
LF	↓	↑	Conflicting results
SDRR	Insufficient information	Insufficient information	↑
SDARR	Insufficient information	Insufficient information	↑
pRR50	Insufficient information	↓	Conflicting results
RMSSD	Insufficient information	↓	Insufficient information
PNS	Likely increased	Likely decreased	Likely increased
SNS	Likely decreased	Likely increased	Conflicting results
FMD	↑	↓	Conflicting results
EndoPAT	Insufficient information	Insufficient information	↑

EndoPAT, Endothelial peripheral artery tonometry; FMD, Flow mediated dilation; HF, High frequency; LF, Low frequency; LF/HF, Low frequency to high frequency; PNS, Parasympathetic nervous system; pRR50: Percentage of difference higher than 50msec in RR intervals; RMSSD, Root mean square of successive differences in RR intervals; SDARR, Standard deviation of

average RR Intervals SDRR: Standard deviation of normal RR Intervals; SNS, Sympathetic nervous system

1.11. Testosterone and Microvascular Function and Blood Pressure

A study of younger and older men found that testosterone level positively correlates with RHI, suggesting that higher testosterone improves microvascular function (Corrigan et al., 2015; Kumagai et al., 2021). Similarly, an increase in RHI was found when testosterone therapy was given to men with hypogonadism further illustrating that testosterone improves microvascular function (Shoskes et al., 2016). On the other hand, studies which used FMD instead of RHI have conflicting results whereby Sader and colleagues (2003) examined the effect of testosterone therapy on FMD in hypogonadal men. The study concluded that FMD was lower after testosterone therapy, suggesting that a greater amount of testosterone is linked with endothelial dysfunction. Similarly, Herman and colleagues (1997) measured FMD among three groups of men with varying amounts of testosterone and found that men with the highest amount of testosterone resulted in lowest FMD, showing that higher testosterone levels decrease endothelial function. As mentioned previously, while FMD and EndoPAT RHI are correlated, Nardone and colleagues (2020) found that they measured different physiological mechanisms that result in vasodilation. FMD is correlated with coronary vasodilation to adenosine and acetylcholine, whereas EndoPAT is correlated with coronary vasodilation to dobutamine and dilation through adrenergic stimulation. Taken together, testosterone appears to improve microvascular function using EndoPAT but deteriorates the function measured by FMD in men. In contrast to what was observed in males, postmenopausal women with low testosterone had lower FMD compared to postmenopausal women with higher testosterone, suggesting greater endothelial function in the presence of high testosterone in older women (Monalcini et al., 2007). There is a limited understanding of the relationship between testosterone concentrations and microvascular function in young women. Interestingly, Gulamhusein and colleagues (2023) suggested that instead of testosterone alone, it was rather the testosterone-to-estradiol ratio that demonstrated a positive correlation with FMD in premenopausal women with chronic kidney disease. These findings highlight that the relationship between testosterone and FMD in women is not independent; instead, it could be the balance between testosterone and estrogen that influences FMD.

In younger and older men testosterone levels are negatively correlated with SBP and DBP, suggesting that testosterone decreases BP in men (Khaw & Barrett-Connor, 1988). On the other hand, Huisman and colleagues (2006) found that higher testosterone levels were linked with higher SBP in younger and older women with no association in younger and older men, suggesting that testosterone increases BP in women. Indeed, in an animal model, Loh and Salleh (2017) suggested that testosterone therapy increases MAP in female mice, and reduces MAP in testosterone deprived male mice. The contrasting results could be potentially attributed to the different cohorts recruited where Khaw & Barrett-Connor, 1988 recruited only men and Huisman and colleagues (2006) and Loh and Salleh (2017) recruited both men and women. Nonetheless, this evidence suggests that there may be a sexually dimorphic influence of testosterone on blood pressure.

Chapter 2: Proposed Study Design

2.1. Research Objectives

This correlational and cross-sectional study aims to determine:

1. If sex hormones, age, and/or biological sex have a relationship with resting microvascular function.
2. If sex hormones, age, and/or biological sex have a relationship with resting autonomic function.

2.2. Hypotheses

We hypothesized that:

1. Due to the negative relationship between age and FMD, and age and HRV, we hypothesized that age would decrease microvascular function, increase BP and decrease PNS/increase SNS function.

2. Due to the positive relationship between estradiol and FMD, and estradiol and HRV, we hypothesized that estradiol would enhance microvascular function, decrease BP and increase PNS/decrease SNS function.

3. Given the negative relationship between progesterone and FMD, and progesterone and HRV, we hypothesized that progesterone would reduce microvascular function and decrease PNS/increase SNS function, yet decrease BP.

4. As a result of the positive relationship between testosterone and FMD, and testosterone and HRV, we hypothesized that testosterone would improve microvascular function and increase PNS/decrease SNS function.

2.3. Methods

2.3.1. Participants

All protocols were approved by the Research Ethics Board at York University, and informed consent was obtained before participation. The following groups were recruited : 1) self-identified premenopausal women (ages 18-50) who had a regular menstrual cycle (24-35 days in duration) in their i) late follicular phase (n=6; days 7-12 of cycle when there is high estradiol and low progesterone, and ii) mid-luteal phase (n=6; days 18-24 of cycle when there is high estradiol and high progesterone), 2) age-matched younger men (n=7; higher testosterone compared to women), 3) women with polycystic ovarian syndrome (PCOS), (n=6; elevated testosterone), 4)

postmenopausal women, one year past their last period (n=6; lower estradiol, progesterone and testosterone compared to young women), 5) age-matched older men (n=7; higher estradiol, progesterone, and testosterone compared to postmenopausal women). Five participants were removed as outliers, one female with PCOS, another female from the late follicular and mid-luteal phase, along with one older and younger male. The removal of outliers is further described in section 2.3.3 below, and the anthropometrics of the remaining 33 participants was described in Table 2. The purpose of recruiting these groups was to include a wide range of concentrations of all 3 major sex hormones with overlap between the groups where possible. Participants were excluded if they had any previously diagnosed cardiovascular, cerebrovascular, metabolic, or respiratory conditions (other than PCOS), such as sleep apnea, congenital valve disease, asthma, tuberculosis, emphysema, or genetic blood disorders (not an exhaustive list). Participants who smoked regularly within the last six months, or took oral contraceptives/hormonal replacement therapy within the last three months were also excluded. All participants were cis-gender and thus the phrases male/female and men/women are used interchangeably.

2.3.2. Experimental Protocol

2.3.2.1. Patient Anthropometric

All testing occurred between 8 a.m. and 11 a.m. A stadiometer was used to measure height and body mass. Participants were asked to refrain from fatty food, alcohol, caffeine, heavy exercise, and smoking 12 hours prior to testing and were instructed to remain fully fasted 2 hours before testing time. Age, self-identified ethnicity, and number of days engaged in strenuous exercise per week were asked to estimate VO_2 max for each participant according to the following formula (Ainsworth et al., 1993):

Predicted VO_2 Max = 65+1.8*# times exercise-10*(1 if female, 0 if male)-0.3*age-0.6*BMI

(Body mass index (BMI) = weight (kg)/ [height (m)]^2)

2.3.2.2. Urine Measurements

Participants were asked to provide a urine sample of approximately 50mL at the beginning of testing. As per the company instructions, a Mira fertility max wand was dipped into the urine

for 10-20secs and then inserted into the Mira analyzer for 21 minutes to determine concentrations of estradiol and progesterone (Quanovate Tech Inc., San Ramon, CA).

2.3.2.3. Saliva Measurements

Participants filled a 1.5mL Eppendorf tube with saliva for storage at -80°C for future analysis. Testosterone was analyzed using a standard enzyme-linked immunosorbent assay (ELISA) kit (Salimetrics, State College, PA). The procedure involved measuring testosterone concentrations in 25uL of saliva (in duplicate) by following the instructions provided. The optical density of the completed assay was measured at 450nm by using a standard plate reader.

2.3.2.4. Microvascular Function Index (MFI)

Participants were seated on a chair in a relaxed position, with their arms over the seat's armrest and palms facing down while their feet were on the ground. A pair of piezoelectric pulse transducers (AD Instruments, Colorado Springs, USA) were wrapped around the fingertips of their left and right middle fingers to measure pulse pressure. A standard blood pressure cuff was placed around the left forearm. Participants were instructed to refrain from muscle movement throughout the test to prevent artifacts. There was a baseline period of 5 minutes, followed by 5 minutes of circulatory occlusion of the left arm, where the blood pressure cuff was inflated to 50 mmHg above resting SBP, and 5 minutes of recovery during reperfusion after cuff release. Piezoelectric signals were obtained through a PowerLab device (ADInstruments, Colorado Springs, USA) and recorded using LabChart software (ADInstruments, Colorado Springs, USA). The MFI, as described in Ramraj and colleagues (2024), was calculated with LabChart Software (Version 8.0 Pro, ADInstruments, Colorado Springs, USA) by computing the average positive area under the curve (AUC) of the pulse waveforms for 3.5 min of baseline and 10 secs around peak during reactive hyperemia in both the occluded arm (test) and the non-occluded arm (control) (Ramraj et al., 2024). Interesting, unpublished work from our lab has shown that MFI reproducibility is improved when using 30-60 secs of reactive hyperemia instead, therefore, this time frame was used for analysis. The ratio of the response in the occluded arm was then divided by the ratio of the response in the control arm to calculate MFI:

$$MFI = (AUC_{test}\ \text{post-occlusion}/\ AUC_{test}\ \text{baseline})/\ (AUC_{control}\ \text{post-occlusion}/\ AUC_{control}\ \text{baseline})$$

2.3.2.5. Heart Rate Variability

An electrocardiogram (ECG) and PowerLab device (ADInstruments, Colorado Springs, USA) were used to measure heart rate variability (HRV) as per standardized international guidelines (Malik et al., 1996) using LabChart Pro software (ADInstruments, Colorado Springs, USA). Participants laid supine for 10 mins to ensure a relaxed state, with another 5 mins of resting ECG used for HRV. The frequency, time, and non-linear domains of HRV were calculated. The frequency domain includes: 1) very low frequency (VLF; <0.04Hz) signifying long-term HR regulation mechanisms such as endocrine, 2) low frequency (LF; 0.04-0.15Hz) signifying both parasympathetic and sympathetic activity, 3) high frequency (HF; 0.15-0.4Hz) signifying parasympathetic activity, and 4) the LF/HF ratio signifying sympathetic activity. The time domain includes parameters of HRV representing PNS control, for example, SDNN or SDRR (Shaffer & Ginsberg, 2017), the standard deviation of average RR intervals for every 5 min cycle of a measurement (SDARR), the root mean square of successive differences in RR intervals (RMSSD), percentage of difference higher than 50msec in RR intervals (pRR50) (Malik et al., 1996). The non-linear domain includes variables shown in Figure 1, such as 1) the standard deviation perpendicular to the line of identity of the Poincaré scattergram plot developed when comparing RR interval against the previous RR interval (SD1), which represents PNS activity, and 2) the standard deviation along the line of identity of the Poincaré scattergram plot developed with comparing RR interval against the previous RR interval (SD2), which represents SNS activity (Malik et al., 1996).

2.3.2.6. Paced Deep Breathing

While supine participants were instructed to perform repeated cycles of deep inhalation (5 secs) and deep exhalation (5 secs) along with an auditory metronome. The participants performed 2 trials of eight breathing cycles with one minute rest between trials. The trial with the clearest changes in HR was chosen for analysis. Maximal HR during inspiration and minimal HR during expiration were determined using an ECG and the Lab Chart Pro software (ADInstruments, Colorado Springs, USA). Two measurements were calculated from this protocol, the expiration/inspiration (E/I) ratio and respiratory sinus arrhythmia (RSA). These values were computed by averaging six maximum HR and six minimum heart rates. The E/I ratio was

determined by dividing the average maximum HR by the average minimum HR. RSA was determined by subtracting average minimum HR from average maximum HR.

2.3.3. Statistical Analysis

Prior to starting the study, power calculations were conducted using 4 statistical predictors: estradiol concentration, progesterone concentration, testosterone concentration, and age. Using G Power Software, an F-test power analysis was conducted for linear multiple regression (fixed model, R^2 deviation from zero) using an expected effect size of 0.35, alpha error probability of 0.05, and power of 0.8. The total predicted sample size in all groups was 40. Outliers were determined visually within each participant group and confirmed as outliers if they were >2 standard deviations from the group mean (n=5). Outliers were removed prior to running all statistical analysis.

Age, estradiol, progesterone and testosterone, and their interactions, were entered into a multiple linear regression model (IBM SPSS Statistics, Version 29, Armonk, NY). Any significant correlations were interpreted as weak when $r < \pm 0.4$, moderate when r ranged from ± 0.4 to ± 0.7, or strong when $r > \pm 0.7$. Significant main or interaction effects were subsequently visualized in figures (Figure 4; Figure 5; Figure 6) and tables (Table 3; Table 4). Interactions between predictors were compared against variance inflation factor (VIF), where 1-5 VIF represents? low multicollinearity, 5-10 represent moderate multicollinearity, >10 represent high multicollinearity. Primary outcomes include HF, LF, LF/HF, SDRR, RMSSD, MFI, RSA, resting SBP, resting DBP, mean arterial pressure (MAP), and resting HR. Secondary outcomes include SDARR, coefficient of variance of RR (CVRR), SD rate, standard deviation of successive RR interval differences (SDSD), pRR50, total power, VLF, SD1, SD2, EI ratio.

Normality was assessed using the Shapiro-Wilks test (IBM SPSS Statistics, Version 29, Armonk, NY). Estradiol, progesterone, testosterone, age and resting HR were not normally distributed and were thus considered non-parametric. Height, body mass, SBP, DBP, MAP and predicted VO_2 max were parametric. Parametric data were presented as mean ± SD and compared using ANOVA and Tukey's HSD post hoc (IBM SPSS Statistics, Version 29, Armonk, NY). Non-parametric data were presented as median (interquartile range) and compared using a Kruskal-Wallis test (IBM SPSS Statistics, Version 29, Armonk, NY) and Dunn's post hoc (R.app, Version 4.3.1, Vienna, Austria). A p value was not reported for the average menstrual

cycle length since a definite menstrual length for PCOS could not be established, making it impossible to compare across groups. Significance was set at $p<0.05$.

Chapter 3: Results

3.1. Participant Characteristics

Between March 26th, 2024, to March 8th, 2025, a total of 38 participants were recruited and tested. Upon sex hormone analysis 5 outliers were excluded from the study, as described above. Thereafter, 33 participants were included across six groups: young males (M-Y) (n=6), females in the late follicular phase of the menstrual cycle (F-LF) (5), females in the mid luteal phase of the menstrual cycle (F-ML) (n=5), females with polycystic ovarian syndrome (F-PCOS) (n=6), postmenopausal females (F-PM) (n=6), older males (M-O) (n=6) (Table 2). Participant self-identified ethnicity was reported as: Caucasian (12), Black (2), South Asian (14), East Asian (2), Middle Eastern (2), or Hispanic (1). Estradiol was not statistically significant between groups ($p=0.752$) nor was progesterone ($p=0.308$). There was a significant difference in testosterone concentrations between groups ($p<0.001$) such that M-Y had higher testosterone than F-LF, F-ML, F-PCOS, and F-PM (all $p<0.011$), and M-O had higher testosterone than F-LF and F-PM (all $p<0.032$).

Age was significantly different between groups ($p<0.001$) such that M-O were older than F-LF, F-ML, M-Y, and F-PCOS (all $p<0.014$), and F-PM were older than F-LF, F-ML, M-Y, and F-PCOS (all $p<0.008$). There was a significant difference in height between groups ($p<0.001$) where M-Y were taller than F-LF, F-ML, F-PCOS, F-PM (all $p<0.016$), and M-O were taller than F-LF, F-ML, and F-PM (all $p<0.047$). Body mass was statistically different between groups ($p=0.011$) where M-Y had a higher body mass than F-LF and F-ML (all $p<0.013$), F-PCOS had a higher body mass than F-LF ($p=0.038$). SBP was statistically different between groups ($p=0.041$) such that F-PM had higher SBP than F-ML ($p=0.034$), and there were no other differences between groups (all $p>0.05$). DBP was statistically different between groups ($p=0.016$) such that M-O had higher DBP than F-ML ($p=0.023$), and there were no other differences between groups ($p>0.05$). MAP was statistically different between groups ($p=0.011$) such that F-PM and M-O had higher MAP than F-ML ($p<0.023$) with no other differences between groups ($p>0.05$). Resting HR was not statistically different between groups ($p=0.155$). Predicted VO_2 max was shown to be statistically different between groups ($p<0.001$) such that M-Y had higher predicted VO_2 max than F-LF, F-ML, F-PCOS, F-PM, and M-O (all, $p<0.008$), and F-LF and F-LM had higher predicted VO_2 max than F-PM (all $p<0.012$).

Table 2: Anthropometric, hemodynamic and sex hormone measures.

	M-Y	F-LF	F-ML	F-PCOS	F-PM	M-O	p
n	6	5	5	5	6	6	
Estradiol (ng/mL)	61 (45-95)	95 (57-182)	79 (53-191)	73 (55-122)	111 (46-138)	66 (54-108)	0.752
Progesterone (ng/mL)	1 (1-2)	3 (2-12)	2 (1-7)	2 (1-4)	6 (1-8)	2 (2-3)	0.308
Testosterone (pg/mL)	227 (181-244)^{bcde}	47 (45-62)^{f}	76 (59-123)	92 (70-118)	75 (55-126)^{f}	159 (125-198)	**<0.001***
Age (years)	25 (22-24)^{ef}	23 (22-27)^{ef}	24 (23-27)^{ef}	25 (20-30)^{ef}	61 (56-66)	58 (52-63)	**<0.001***
Height (cm)	177 ± 2^{bcde}	159 ± 6f	157 ± 9f	163 ± 7	158 ± 5f	171 ± 6	**<0.001***
Body Mass (kg)	85 ± 11^{bc}	56 ± 6^{d}	59 ± 10	80 ± 19	66 ± 8	78 ± 11	**0.001***
SBP (mmHg)	111 ± 9	104 ± 9	99 ± 9^{e}	110 ± 6	119 ± 17	115 ± 8	**0.041***
DBP (mmHg)	71 ± 6	73 ± 6	69 ± 4^{f}	75 ± 4	78 ± 5	80 ± 6	**0.016***
MAP (mmHg)	85 ± 6	83 ± 7	79 ± 5^{ef}	87 ± 4	92 ± 8	92 ± 5	**0.011***
Resting HR (bpm)	63 (60-67)	74 (66-82)	74 (69-84)	71 (65-85)	68 (62-71)	69 (58-83)	0.155
Predicted VO_2 max (mL/kg/min)	49 ± 4^{bcdef}	38 ± 3e	36 ± 4e	33 ± 7	25 ± 3	34 ± 6	**<0.001**

Average menstrual cycle (days)	-	28 (26-31)	29 (28-31)	27- 90^{+}	-	-	

Parametric data are presented as mean ± SD. Non-parametric data are presented as median (interquartile range). SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; Resting HR, resting heart rate. +indicates the self-identified range of the last menstrual cycle duration rather than mean (interquartile range) due to the extreme variability in PCOS. Each letter depicts a significant difference from: a = young males (M-Y), b = females late follicular (F-LF), c = females mid luteal (F-ML), d = females with polycystic ovarian syndrome (F-PCOS), e = postmenopausal females (F-PM), f = older males (M-O).

3.2. Hemodynamics and Microvascular Measures

Significant positive correlations with moderate strength, with no influence of sex hormones, were observed between age and DBP (r=0.562, p=0.034; Figure 4; Table 3) and age and MAP (r=0.581, p=0.023; Figure 5; Table 3).

The overall comparison for MFI was not significant (p=0.128), and there were no correlations among sex hormones, age, or their interactions (all p>0.08; Table 3). The overall comparison for SBP was not significant (p=0.100), and there were no correlations among sex hormones, age, or their interactions (p>0.079; Table 3). The overall comparison for DBP was significant (p=0.017) with the following relationships: 1) a weak negative correlation was found between DBP and estradiol (r=-0.076; p=0.004; Table 3), 2) a weak positive correlation was found with the two-way interaction between testosterone and estradiol (r=0.367; p=0.003; Table 3), 3) a weak negative correlation was found with the two-way interaction between estradiol and progesterone (r=-0.017; p=0.022; Table 3), and 4) a weak positive correlation was found with the three-way interaction between testosterone x estradiol x progesterone (r=0.088; p=0.009; Table 3). The two and three-way interaction effects for DBP all had VIF>16 suggesting high multicollinearity.

The overall comparison for MAP was significant (p=0.013) with the following relationships: 1) a weak negative correlation was found between MAP and estradiol (r=-0.189; p=0.005; Table 3), 2) a weak positive correlation was found with the two-way interaction between testosterone and estradiol (r=0.323; p=0.017; Table 3), 3) a weak negative correlation was found with the two-way interaction between estradiol and progesterone (r=-0.074; p=0.011; Table 3) and 4) a weak positive correlation was found with the three-way interaction between testosterone x estradiol x progesterone (r=0.203; p=0.006; Table 3). The two and three-way interaction effects for MAP all had VIF >16 indicating high multicollinearity.

The overall comparison for resting HR was not significant (p=0.23); however, subsequent regression analysis found that resting HR was weakly negatively correlated with age (r=-0.087; p=0.028; Table 3) yet moderately and positively correlated with the two-way interaction between testosterone and age (r=0.427; p=0.028; Table 3). There were no other relationships between sex hormones, their interactions, and HR (all p>0.12; Table 3).

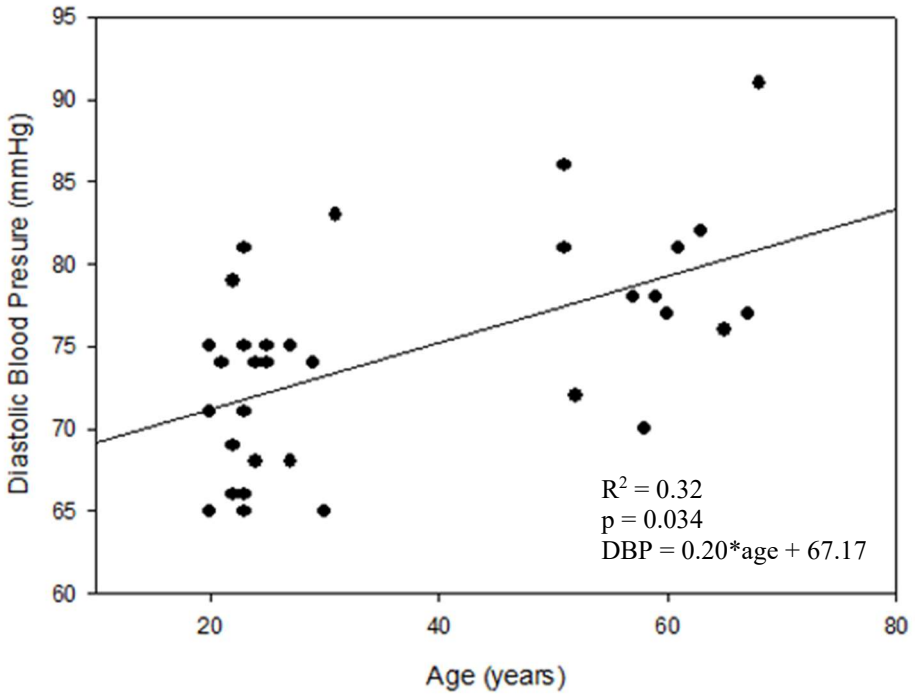

Figure 4: Scatterplot showing positive relationship between age and diastolic blood pressure (DBP) in all participants.

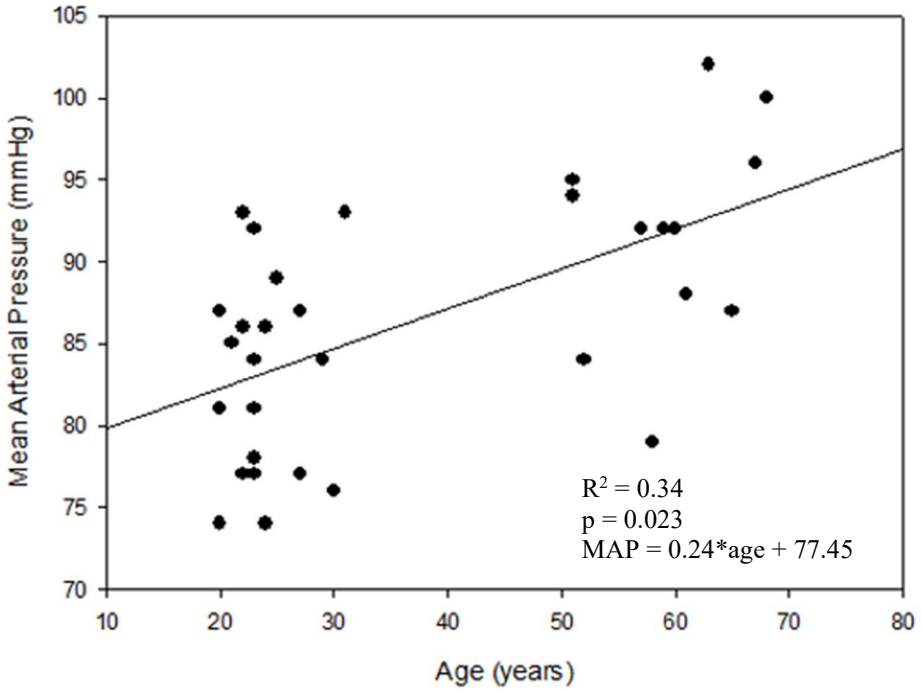

Figure 5: Scatterplot showing positive relationship between age and mean arterial pressure (MAP) in all participants.

Table 3: Correlative data between hemodynamic measures or microvascular function and estradiol, progesterone, testosterone, age, with their two-way, three-way, and four-way interactions.

	Overall ANOVA	Estradiol					Progesterone				
	p	r	p	R^2	B	VIF	r	p	R^2	B	VIF
MFI	0.128	-0.132	0.541	0.017	0.002	7.061	-0.083	0.443	0.007	0.036	5.069
SBP (mmHg)	0.100	-0.289	0.151	0.084	0.140		-0.311	0.260	0.097	-1.495	
DBP (mmHg)	**0.017***	-0.076	**0.004***	0.006	0.137		0.021	0.579	0.000	-0.319	
MAP (mmHg)	**0.013***	-0.189	**0.005***	0.036	0.151		-0.132	0.242	0.017	-0.780	
HR (bpm)	0.230	0.111	0.908	0.012	-0.009		0.163	0.088	0.027	1.972	

	Testosterone					Age				
	r	p	R^2	B	VIF	r	p	R^2	B	VIF
MFI	0.236	0.328	0.056	0.002		0.363	0.079	0.132	0.014	
SBP (mmHg)	0.292	0.244	0.085	0.077		0.400	0.195	0.160	0.275	
DBP (mmHg)	0.037	0.197	0.001	-0.037	5.142	0.562	**0.034***	0.316	0.207	3.828
MAP (mmHg)	0.154	0.317	0.024	-0.033		0.581	**0.023***	0.338	0.256	
HR (bpm)	-0.274	0.164	0.075	-0.078		-0.087	**0.028***	0.008	-0.416	

	Testosterone x Estradiol					Testosterone x Progesterone				
	r	p	R^2	B	VIF	r	p	R^2	B	VIF
MFI	0.124	0.590	0.015	0.000		0.094	0.209	0.009	0.002	
SBP (mmHg)	0.216	0.517	0.047	-0.002		0.185	0.110	0.034	0.079	
DBP (mmHg)	0.367	**0.003***	0.135	0.004	18.293	0.233	0.067	0.054	-0.040	27.831
MAP (mmHg)	0.323	**0.017***	0.104	0.004		0.217	0.209	0.047	-0.031	
HR (bpm)	0.012	0.507	0.000	-0.002		0.112	0.686	0.013	0.016	

	Testosterone x Age					Estradiol x Progesterone				
	r	p	R^2	B	VIF	r	p	R^2	B	VIF
MFI	-0.415	0.419	0.172	0.000		-0.191	0.680	0.036	0.001	
SBP (mmHg)	-0.096	0.801	0.009	-0.001		-0.111	0.054	0.012	0.078	
DBP (mmHg)	0.196	0.286	0.038	-0.002	7.137	-0.017	**0.022**	0.000	0.041	16.254
MAP (mmHg)	0.023	0.073	0.001	-0.004		-0.074	**0.011**	0.005	0.054	
HR (bpm)	0.427	**0.028**	0.182	0.010		-0.026	0.410	0.001	0.027	

	Estradiol x Age					Progesterone x Age				
	r	p	R^2	B	VIF	r	p	R^2	B	VIF
MFI	0.217	0.620	0.047	0.000		0.125	0.336	0.016	-0.003	
SBP (mmHg)	-0.076	0.430	0.006	0.005		-0.145	0.108	0.021	-0.145	
DBP (mmHg)	0.116	0.274	0.013	0.003	5.850	0.036	0.295	0.001	-0.041	6.827
MAP (mmHg)	0.064	0.201	0.004	0.004		-0.025	0.173	0.001	-0.061	
HR (bpm)	-0.054	0.747	0.003	0.002		-0.021	0.343	0.000	-0.071	

	Testosterone x Progesterone x Age					Testosterone x Estradiol x Progesterone				
	r	p	R^2	B	VIF	r	p	R^2	B	VIF
MFI	-0.139	0.318	0.019	0.000		0.237	0.521	0.056	0.000	
SBP (mmHg)	0.015	0.255	0.000	0.004		0.281	0.222	0.079	0.001	
DBP (mmHg)	-0.127	0.188	0.016	-0.002	49.087	0.088	**0.009**	0.008	0.001	27.261
MAP (mmHg)	-0.104	0.247	0.011	-0.002		0.203	**0.006**	0.041	0.001	
HR (bpm)	-0.110	0.140	0.012	0.005		-0.026	0.149	0.001	0.001	

	Estradiol x Progesterone x Age					Testosterone x Estradiol x Age				
	r	p	R^2	B	VIF	r	p	R^2	B	VIF
MFI	0.261	0.189	0.068	0.000		-0.270	0.140	0.073	0.000	
SBP (mmHg)	0.149	0.615	0.022	-0.001		-0.038	0.145	0.001	0.000	
DBP (mmHg)	0.209	0.668	0.044	0.000	17.231	-0.157	0.422	0.025	0.000	33.437
MAP (mmHg)	0.233	0.772	0.054	0.000		-0.154	0.623	0.024	0.000	
HR (bpm)	0.024	0.339	0.001	0.002		-0.020	0.119	0.000	0.000	

	Testosterone x Estradiol x Age x Progesterone				
	r	p	R^2	B	VIF
MFI	-0.268	0.574	0.072	0.000	
SBP (mmHg)	-0.043	0.815	0.002	0.000	
DBP (mmHg)	0.015	0.468	0.000	0.000	33.920
MAP (mmHg)	-0.044	0.264	0.002	0.000	
HR (bpm)	0.084	0.674	0.007	0.000	

r, Pearson's correlation; R^2, coefficient of determination; B, unstandardized regression coefficient; VIF, variance inflation factor; MFI, microvascular function index; SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; HR, heart rate. Significance is represented as bold text and * ($p<0.05$).

3.3. Autonomic Measures

The overall comparison for HF was not significant (p=0.450), and there were no correlations with sex hormones, age, or their interactions (all p>0.154; Table 4). The overall comparison for LF was not significant (p=0.531), and there were no correlations with sex hormones, age, or their interactions (all p>0.147; Table 4). For LF/HF, the overall comparison was significant (p=0.011) and there was: 1) a weak positive correlation with estradiol (r=0.023; p=0.033; Table 4), 2) a weak negative correlation with the two-way interaction between estradiol and progesterone (r=-0.087; p=0.025; Table 4), 3) a weak positive correlation with the two-way interaction between estradiol and age (r=0.184; p=0.005; Table 4), and 4) a weak positive correlation with the three-way interaction between estradiol x progesterone x age (r=0.167; p=0.036; Table 4). Other than the two-way interaction between estradiol and age, the other two and three-way interaction effects for LF/HF all had variance inflation factors (VIF) of >16 suggesting high multicollinearity. The overall comparison for RMSSD was not significant (p=0.942), and there were no correlations with sex hormones, age, or their interactions (all p>0.239; Table 4). The overall comparison for SDRR was not significant (p=0.944), and there were no correlations with sex hormones, age, or their interactions (all p>0.159; Table 4).

A significant main effect showing a strong negative correlation, without the influence of sex hormones or their interactions was observed between age and RSA (r=-0.839 p<0.001; Figure 6; Table 4).

Table 4: Correlative data between autonomic measures and estradiol, progesterone, testosterone, and age with their two-way, three-way, and four-way interactions.

	Overall ANOVA	Estradiol					Progesterone				
	p	r	p	R^2	B	VIF	r	p	R^2	B	VIF
HF (nu)	0.450	0.002	0.527	0.000	-0.097	7.061	0.035	0.940	0.001	-0.159	5.069
LF (nu)	0.531	0.016	0.574	0.000	0.094		-0.025	0.752	0.001	0.730	
LF/HF	**0.011***	0.023	**0.033***	0.001	0.017		0.027	0.367	0.001	-0.091	
RMSSD (ms)	0.942	-0.118	0.862	0.014	-0.127		-0.078	0.309	0.006	-10.441	
SDRR (ms)	0.944	-0.082	0.601	0.007	-0.258		-0.029	0.503	0.001	-4.578	
RSA (ms)	**<0.001***	0.102	0.450	0.010	-0.030		-0.024	0.454	0.001	-0.415	

	Testosterone					Age				
	r	p	R^2	B	VIF	r	p	R^2	B	VIF
HF (nu)	-0.249	0.981	0.062	0.003	5.142	-0.271	0.908	0.073	0.039	3.828
LF (nu)	0.225	0.923	0.051	-0.011		0.221	0.713	0.049	-0.135	
LF/HF (%)	0.283	0.791	0.080	0.001		0.369	0.602	0.136	-0.008	
RMSSD (ms)	-0.023	0.648	0.001	0.228		-0.106	0.579	0.011	0.892	
SDRR (ms)	-0.043	0.510	0.002	0.223		-0.145	0.744	0.021	0.352	
RSA (ms)	0.088	0.417	0.008	0.022		-0.839	**<0.001***	0.704	-0.467	

	Testosterone x Estradiol					Testosterone x Progesterone				
	r	p	R^2	B	VIF	r	p	R^2	B	VIF
HF (nu)	0.122	0.448	0.015	0.004	18.293	-0.033	0.590	0.001	-0.042	27.831
LF (nu)	-0.112	0.454	0.013	-0.004		0.054	0.569	0.003	0.048	
LF/HF (%)	0.097	0.863	0.009	0.000		0.116	0.802	0.013	0.001	
RMSSD (ms)	0.111	0.639	0.012	0.011		-0.028	0.586	0.001	-0.202	
SDRR (ms)	0.072	0.987	0.005	0.000		-0.012	0.889	0.000	-0.035	
RSA (ms)	-0.124	0.167	0.015	-0.002		-0.185	0.586	0.034	0.011	

	Testosterone x Age					Estradiol x Progesterone				
	r	p	R^2	B	VIF	r	p	R^2	B	VIF
HF (nu)	-0.251	0.438	0.063	-0.006	7.137	0.135	0.216	0.018	-0.078	16.254
LF (nu)	0.254	0.400	0.065	0.007		-0.152	0.222	0.023	0.084	
LF/HF (%)	0.327	0.354	0.107	0.000		-0.087	**0.025***	0.008	0.007	
RMSSD (ms)	0.014	0.742	0.000	-0.012		0.074	0.288	0.005	-0.318	
SDRR (ms)	-0.031	0.779	0.001	-0.007		0.044	0.199	0.002	-0.261	
RSA (ms)	-0.164	0.552	0.027	-0.001		0.016	0.330	0.000	-0.016	

	Estradiol x Age					Progesterone x Age				
	r	p	R^2	B	VIF	r	p	R^2	B	VIF
HF (nu)	-0.053	0.178	0.003	-0.013	5.850	-0.021	0.298	0.000	0.149	6.827
LF (nu)	0.036	0.258	0.001	0.012		0.031	0.349	0.001	-0.146	
LF/HF (%)	0.184	**0.005***	0.034	0.001		0.122	0.071	0.015	-0.013	
RMSSD (ms)	0.072	0.495	0.005	0.031		-0.097	0.793	0.009	-0.177	
SDRR (ms)	0.115	0.408	0.013	0.025		-0.069	0.720	0.005	-0.162	
RSA (ms)	-0.015	0.757	0.000	0.001		0.052	0.208	0.003	0.047	

	Estradiol x Progesterone x Age					Testosterone x Estradiol x Age				
	r	p	R^2	B	VIF	r	p	R^2	B	VIF
HF (nu)	-0.126	0.545	0.016	-0.002	17.231	-0.184	0.691	0.034	0.000	33.437
LF (nu)	0.122	0.532	0.015	0.002		0.192	0.633	0.037	0.000	
LF/HF (%)	0.167	**0.036***	0.028	0.000		0.268	0.970	0.072	0.000	
RMSSD (ms)	-0.157	0.691	0.025	-0.007		-0.084	0.462	0.007	0.001	
SDRR (ms)	-0.135	0.786	0.018	-0.003		-0.081	0.546	0.007	0.001	
RSA (ms)	-0.490	0.084	0.240	0.002		0.311	0.567	0.097	0.000	

	Testosterone x Progesterone x Age					Testosterone x Estradiol x Progesterone				
	r	p	R^2	B	VIF	r	p	R^2	B	VIF
HF (nu)	-0.031	0.698	0.001	-0.002	49.087	-0.319	0.154	0.102	-0.002	27.261
LF (nu)	0.032	0.636	0.001	0.003		0.323	0.147	0.104	0.002	
LF/HF (%)	0.164	0.674	0.027	0.000		0.253	0.061	0.064	0.000	
RMSSD (ms)	-0.020	0.488	0.000	-0.021		-0.166	0.239	0.028	-0.006	
SDRR (ms)	-0.039	0.613	0.002	-0.010		-0.160	0.159	0.026	-0.005	
RSA (ms)	0.384	0.542	0.147	0.001		-0.099	0.341	0.010	0.000	

	Testosterone x Estradiol x Age x Progesterone				
	r	p	R^2	B	VIF
HF (nu)	0.030	0.728	0.001	0.000	33.920
LF (nu)	-0.031	0.783	0.001	0.000	
LF/HF (%)	0.051	0.292	0.003	0.000	
RMSSD (ms)	0.111	0.941	0.012	0.000	
SDRR (ms)	0.072	0.974	0.005	0.000	
RSA (ms)	0.254	0.086	0.065	0.000	

r, Pearson's correlation; R^2, coefficient of determination; B, unstandardized regression coefficient; VIF, variance inflation factor; HF, high frequency; LF, low frequency; LF/HF, low frequency to high frequency; RSA, respiratory sinus arrhythmia; RMSSD, Root mean square of successive differences in RR intervals; SDRR, standard deviation of normal RR interval. Significance is represented as bold text ($p<0.05$).

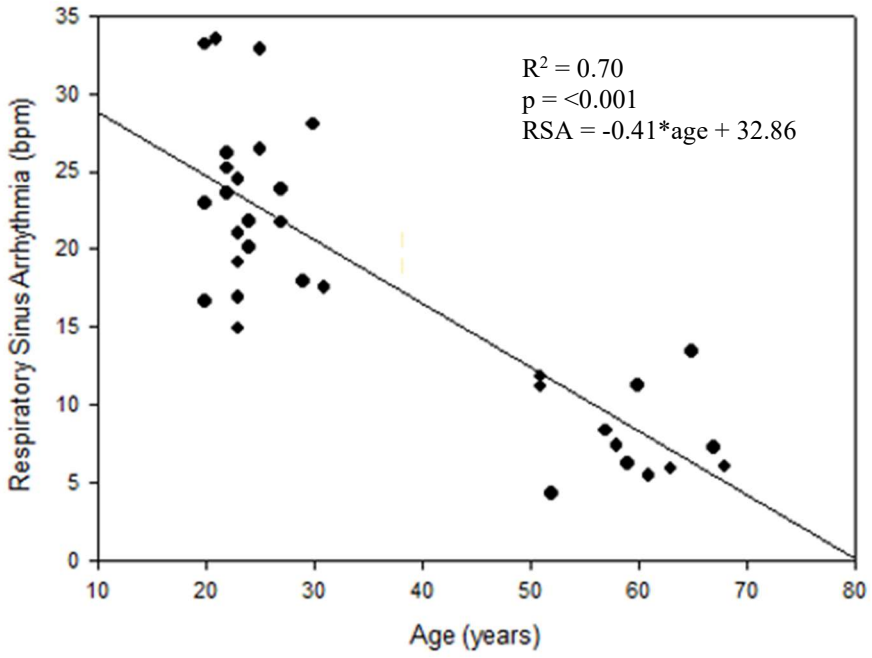

Figure 6: Scatterplot showing negative relationship between age and respiratory sinus arrhythmia (RSA) in all participants.

Chapter 4: Discussion

4.1. Summary

The current study aimed to correlate sex hormone concentrations with microvascular and autonomic function while accounting for age. There was a high level of cross-over between groups with regard to the concentrations of sex hormones present, though as expected young men had higher salivary testosterone than all the female groups. The results of this study indicate that while accounting for the concentrations of all sex hormones, aging is positively associated with DBP, MAP, and is negatively correlated with RSA. Contrary to the hypotheses, while accounting for the concentrations of all other sex hormones and age, estradiol was negatively associated with MAP and DBP and positively associated with LF/HF while progesterone and testosterone were not correlated with any microvascular or autonomic measures. We had multiple two-way and three-way interaction effects that will be discussed below, however, due to the presence of high multicollinearity, we do not consider these results to be significant. However, there was a significant two-way correlation with only moderate multicollinearity which showed a positive relationship between estradiol x age and LF/HF (Appendix 1). As only the main effect of estradiol was apparent, we interpret this interaction effect to be driven by the aforementioned positive relationship between estradiol and LF/HF rather than age.

We had hypothesized that:

1. Due to the negative relationship between age and FMD, and age and HRV, we hypothesized that age would decrease microvascular function, increase BP and decrease PNS/increase SNS function – This hypothesis was partially accepted.
2. Due to the positive relationship between estradiol and FMD, and estradiol and HRV, we hypothesized that estradiol would enhance microvascular function, decrease BP and increase PNS/decrease SNS function – This hypothesis was rejected.
3. Given the negative relationship between progesterone and FMD, and progesterone and HRV, we hypothesized that progesterone would reduce microvascular function and decrease PNS/increase SNS function, yet decrease BP – This hypothesis was rejected.

4. As a result of the positive relationship between testosterone and FMD, and testosterone and HRV, we hypothesized that testosterone would improve microvascular function and increase PNS/decrease SNS function – This hypothesis was rejected.

4.2. Hemodynamics

When controlling for sex hormone concentrations, aging was associated with increases in DBP and MAP, which is consistent with previous literature that state that aging increases arterial stiffness, which reduces arterial compliance, leading to an elevation in BP (Kucharska-Newton et al., 2019). Resting HR was not statistically significant or different between groups; despite this, the two-way interaction of testosterone x age was significantly correlated to resting HR with a VIF of 7, indicating moderate multicollinearity. As only the main effect of age was apparent (not testosterone), this interaction effect was likely driven by age rather than testosterone, which is consistent with previous studies that suggest that aging decreases resting HR as a result of reduced intrinsic heart rate due to sinoatrial node remodeling (Yanni et al., 2010).

Interestingly, the results of the current study show that SBP was not correlated to age; however, previous literature suggests that SBP increases with age (Franklin et al., 1997). The difference in results could be attributed to the longitudinal study design opted by Franklin and colleagues (1997), where researchers measured SBP in the same individuals as they aged over time, allowing for within-person analysis of SBP whereas the current study only used a cross-sectional and correlational study design investigating different people across different ages at one point in time. In a cross-sectional study individual confounders such as genetics (Ehret & Caulfield, 2013) and dietary habits, (e.g. low sodium and high fiber (Abbasnezhad et al., 2020)) can reduce SBP and may have impacted underlying age-related effect on SBP. Although the current study found that estradiol increases MAP and DBP (with no effect on SBP), previous literature has shown mixed results.

A study conducted in young female mice suggests that estradiol treatment increased MAP mediated by the vasoconstrictor endothelin-1 (Subramanian et al., 2017). However, Mercuro and colleagues (1998) found that estradiol treatment decreased 24-hour DBP and SBP in hypertensive postmenopausal women and Luotola (1983) found that estradiol treatment decreased DBP and SBP in hypertensive postmenopausal women when they measured BP at a single point in time.

Unlike Mercuro and colleagues (1998) and Luotola (1983), the current study recruited younger and postmenopausal women without pre-diagnosed hypertension alongside men. Lower DBP due to estradiol in the previously conducted studies could have been observed due to the higher resting blood pressure values observed in hypertension. Furthermore, Mercuro and colleagues (1998) and Luotola (1983) only recruited hypertensive postmenopausal women and did not account for the potential confounding variables of age, sex and other sex hormones. Importantly, the current study measured endogenous estradiol in participants, whereas the aforementioned studies provided estradiol as a pharmacological treatment. Our observation of the positive relationship between estradiol and blood pressure could be from the influence of estradiol on the renin-angiotensin-aldosterone system via increasing angiotensinogen production in the liver, resulting in a greater production of angiotensin II, causing vasoconstriction and leading to a rise in BP (Yanes & Reckelhoff, 2011). Furthermore, estradiol could stimulate the release of vasopressin from the hypothalamus and cause vasoconstriction, leading to a rise in BP (Sladek & Somponpun, 2008). Changes in these vasoconstrictor pathways are more likely to influence DBP and MAP rather than SBP which correspond to our results.

Contrary to the hypothesis that progesterone decreases BP, the results of the current study found no correlation between SBP, DBP or MAP with progesterone. Shi and colleagues (2023) suggested that progesterone was associated with a decrease in DBP and MAP in healthy younger and older men, along with healthy postmenopausal women, whereas no association was observed in premenopausal women. Progesterone could lower blood pressure by promoting eNOS leading to greater NO production and vascular relaxation (You et al., 2020). Resting BP measurements were only taken once in the current study, whereas it was averaged over three measurements by Shi and colleagues (2023), providing a more reliable estimate of participants' resting BP. Future studies should aim to collect at least three BP readings to ensure accuracy and reproducibility of measurements (Özkan et al., 2024). Further, serum progesterone measurements were used by Shi and colleagues (2023), whereas urine progesterone was used in the current study. Measurements in urine are indicative of the metabolite pregnadiol glucuronide which reflects average progesterone over the previous days making it less prone to daily fluctuations, yet is still directly correlations to blood progesterone (Roos et al., 2015). Additionally, Barbagallo and colleagues (2001) suggested that progesterone therapy was associated with a decrease in SBP in rats. The authors suggested that progesterone acted as a vasodilator since it blocked L-type calcium

channels, hence reducing calcium entry, resulting in vascular smooth muscle relaxation. While animal studies are a crucial experimental model, our study used a human model which can therefore be directly applicable to the general human population.

Contrary to the hypothesis that testosterone would increase BP, the results of the current study found no correlations between testosterone and SBP, DBP or MAP; however, past literature found mixed results. For example, Huisman and colleagues (2006) found elevated levels of SBP with higher serum testosterone in younger and older black South African women, and no association between serum testosterone and blood pressure in younger and older black South African men whereas we did not observe a relationship between testosterone and blood pressure in our overall group. In the current study we used salivary testosterone measurements whereas Huisman and colleagues (2006) used serum testosterone. The salivary testosterone measured by the current study is arguably a better approach since it reflects the unbound, bioactive fraction that can readily bind to androgen receptors in tissues (Yu et al., 2010; Empen et al., 2012). Further, Huisman and colleagues (2006) recruited black South African participants, whereas the current study predominately recruited Caucasian and South Asian Canadian participants. Given that there are ethnic differences in baseline sex hormones (Kim et al., 2012), future studies should aim to include more ethnically diverse populations to be able to apply the results to wider ethnic populations. In addition, Khaw & Barrett-Connor (1988) found that elevated levels of serum testosterone decreased DBP and SBP in younger and older men. However, the current study recruited both younger and older men and women allowing for examination of the relationship independent of biological sex. Furthermore, Loh and Salleh (2017) found that testosterone treatment increased MAP in female mice, and decreased MAP in testosterone deprived male mice suggesting a sexually dimorphic effect; however, this was not observed in our human cohort study. It was previously suggested that testosterone increases BP by stimulating renin-angiotensin-aldosterone pathway leading to vasoconstriction and sodium retention (Mishra et al., 2019) thus future studies should consider these measurements.

Several interactions were observed between sex hormones for their influence on MAP and DBP including 1) a two-way interaction between testosterone and estradiol, 2) a two-way interaction between estradiol and progesterone, 3) a three-way interaction between testosterone x estradiol x progesterone. However, the VIF values were greater than 16 for these comparisons, suggesting high multicollinearity. In a regression model, multicollinearity occurs when two or

more independent variables are highly correlated to one another and provide similar information to predict the dependent variable, leading to an increase in standard errors, resulting in unreliable p-values (reviewed in Farrar & Glauber, 1967; Mason, 1987). VIF is commonly used to detect multicollinearity, where values above 10 represent high multicollinearity (reviewed in Farrar & Glauber, 1967; Mason, 1987). Therefore, although the two- and three-way interactions had statistically significant correlations, the VIF of greater than 16 suggests that the independent variables estrogen, progesterone, and testosterone are highly correlated with each other. To address this, a greater sample size is needed or a variable reduction technique called principal component analysis could be used to improve the interpretability of the model (Z. Zhang & Castelló, 2017), this is further discussed in the limitations and future direction section. However, the main effect of estradiol appears to be driving the interaction effects, as it negatively correlates with DBP and MAP with a VIF of only 7. Although the correlation coefficient was negative for DBP and MAP (Table 3), the partial regression plots showed a positive relationship for both variables (Appendix 2 & 3). The partial regression plots provide reliable results as they control for confounding variables. Therefore, the plots highlight that estradiol was positively correlated with DBP and MAP, contrary to the hypothesis. There were on observed interactions between sex hormones and SBP.

4.3. Microvascular

Contrary to our hypothesis, MFI was not correlated to age. Jujic and colleagues (2023) conducted a study on younger and older adult men and women and found that the RHI from EndoPAT was lower in participants younger than 30 years, and higher in participants older than 30 years. Jujic and colleagues (2023) suggested that younger participants might have lower RHI due to greater baseline vasodilation, resulting in a smaller increase in post-occlusion dilation compared to older participants. It is noteworthy that Jujic and colleagues (2023) had a much larger sample size (n=1812), whereas the current study had a smaller sample size (n=33). Hence, the current study may be underpowered to detect age-related effects on MFI. However, Jujic and colleagues (2023) divided the participants into only 2 groups for comparison (older and younger than 30 years old) whereas the current study kept age as a continuous variable allowing researchers to detect trends in microvascular function with aging. Unfortunately, the current study did not include participants aged 30-50, a group that was recruited by Jujic and colleagues (2023), along

with younger and older adults. Hence, the study design by Jujic and colleagues (2023) could be argued to be more effective as it captures a broader spectrum of potential age related differences in microvascular function. Further, Kang and colleagues (2018) also found that the RHI from EndoPAT was significantly lower in people older than 60 compared to the younger age groups, potentially due to increased oxidative stress with aging (Liguori et al., 2018), yet they recruited younger and older people with comorbidities such as hypertension and diabetes which contribute to endothelial dysfunction through increased oxidative stress and reduced NO bioavailability (Tooke, 1995; Zdravkovic et al., 2023). In contrast, the current study recruited a healthy group without known comorbidities.

There were no correlations between MFI, sex hormones, or their interactions. Previous literature produced similar results to the current study in terms of estradiol and progesterone, where a combination of estradiol and progesterone treatment was not associated with a change in RHI measured by EndoPAT in menopausal women, thus not affecting endothelial function (Kling et al., 2015). However, Miner and colleagues (2011) found lower FMD with estradiol and progesterone therapy in premenopausal women. A potential reason why the current study and the study conducted by Kling and colleagues (2015) were unable to find correlations between estradiol, progesterone, and endothelial function might be methodological where MFI and EndoPAT measure blood pressure or volume in the microvasculature of the fingertips, whereas FMD measured endothelial function of the larger brachial artery, a conduit vessel. Indeed, Shufelt and colleagues (2023) also found no correlation between estradiol and RHI measured by EndoPAT in young women with functional hypothalamic amenorrhea, further suggesting that estradiol does not affect microvascular function.

Contrary to the results of the current study that found no correlation between testosterone and endothelial function, Corrigan and colleagues (2015) found that serum testosterone is positively correlated with RHI measured by EndoPAT in healthy younger and older men, suggesting that testosterone improves endothelial function. Similarly, Kumagai and colleagues (2021) found a positive correlation between serum testosterone and RHI measured by EndoPAT in Japanese men, suggesting that testosterone increases microvascular function. Notably, both previous studies only recruited men. In contrast, the current study recruited both men and women, allowing for the assessment of the relationship in humans regardless of biological sex. In addition, Kumagai and colleagues (2021) and Corrigan and colleagues (2015) measured serum testosterone,

whereas the current study measured salivary testosterone. Regarding microvascular function, salivary testosterone which contains unbound testosterone, and is thus bioactive, potentially serves greater value since it can readily bind to androgen receptors in tissues (Yu et al., 2010; Empen et al., 2012).

4.4. Autonomic Function

As hypothesized, aging was associated with lower RSA, which is consistent with previous literature. For example, De Meersman and colleagues (1993) found that RSA decreases with age due to decreased in parasympathetic activity. Indeed, Hellman & Stacy (1976) found a strong negative correlation between age and computed percent of the variation of heart rate from average heart rate with breathing, which indicated the amount of RSA, suggesting that RSA decreases with age. In addition, (Masi et al., 2007) found an inverse relationship between RSA and age (Craft & Schwartz, 1995). Together, these results suggest that aging is independently associated with lower PNS control of HR.

Despite the aforementioned influence of age on RSA, unexpectedly, we did not observe an influence of age on HRV variables such as LF, HF, LF/HF, RMSSD or SDNN. This is in contrast to previous work by Zhang and colleagues (2007) who similarly conducted a 5 minute HRV recording and observed an inverse relationship between age and LF or HF, indicating lower PNS control with age. Zhang and colleagues (2007) included 470 participants between the ages of 10-80 whereas the current study recruited 33 subjects, between the ages of 18-30 and ages 50-70. The study design by Zhang and colleagues (2007) allowed researchers to capture a wider range of age-related differences in HRV variables; however, they didn't concurrently investigate the influence of any interacting sex hormones. Yeragani and colleagues (1997) found that age was negatively correlated with LF and HF and positively correlated with LF/HF ratio in using a 24-hr Holter ECG recording in healthy younger and older men and women, suggesting a decrease in PNS control and an increase in SNS control. Yeragani and colleagues (1997) used a 24-hr Holter ECG recording, whereas we only used a supine resting 5-min ECG recording. The 24-hr Holter monitoring captures cardiac activity across a full day, reflecting the heart's response to real-life conditions, compared to a brief 5-min ECG recording. However, Min and colleagues (2008) found correlations between 24-hr and 5-min HRV measurements such that a moderate correlation was observed for LF and a strong correlation for HF, suggesting that these methodological factors are unlikely to explain the

differences. The participants in the current study were adults between the ages of 20 and 68, whereas Yeragani and colleagues (1997) recruited people between the ages of 6 and 61, thus including children who have not yet fully developed. The inclusion of children might have skewed the HRV measurements, contributing to differences between studies since HRV changes non-linearly from ages 1-10 (Silvetti et al., 2001).

We found no relationships between RSA and sex hormones or their interactions, which is consistent with the previous literature on estradiol and progesterone. For example, Lüthi and colleagues (2008) found no change in RSA across the different phases of the menstrual cycle (early follicular, ovulation, mid-luteal) in premenopausal women, along with no changes in RSA in postmenopausal women taking hormonal therapy (estradiol and progesterone), indicating that variations of estradiol and progesterone do not influence parasympathetic control. Contrarily, with regard to testosterone, Porges and colleagues (2015) found that higher testosterone levels are associated with greater RSA, indicating that testosterone increases PNS function. Its notable that Porges and colleagues (2015) and Lüthi and colleagues (2008) only recruited one sex whereas we recruited younger and older men and women. Other parameters of HRV such as RMSSD, SDRR, and HF, each primarily representing parasympathetic activity, along with LF, which represents both parasympathetic and sympathetic activity (Malik et al., 1996) or baroreceptor sensitivity (Goldstein et al., 2011), were not correlated to sex hormones, age, or their interactions. However, previous literature has shown that sex hormones are associated with these HRV variables. For example, Sato and colleagues (1995) used a 5 minutes HRV recording and observed findings where LF was higher while HF was lower in the late luteal phase of the menstrual cycle compared to the late follicular phase, potentially suggesting greater SNS activity in the luteal phase due to the presence of progesterone. In addition, Rosano and colleagues (1997) used a 24-hour HRV recording and found an increase in SDRR, RMSSD, HF and LF upon estradiol therapy in postmenopausal women, indicating that estradiol enhances PNS activity. Further, Ermis and colleagues (2010) used a 24-hour HRV recording and observed a positive correlation between testosterone and SDNN, LF, and HF, indicating that higher testosterone levels increased PNS function. As explained earlier, some of the potential discrepancies could be due to methodological differences where both Rosano et al. and Ermis et al. used 24-hr Holter monitoring; however, this is unlikely due to the work of Min et al. who found similar results using these two methods. Importantly, Rosano and colleagues (1997) only recruited postmenopausal women and Sato and

colleagues (1995) only recruited premenopausal women. In contrast, the current study recruited younger and older women and men to account for changes in multiple sex hormones, including testosterone, and age.

LF/HF was our only indicator of SNS activity. Significant correlations were observed between LF/HF, sex hormones, and age interactions including 1) a weak positive correlation was found with estradiol, 2) a weak negative correlation was found with the two-way interaction between estradiol and progesterone, 3) a weak positive correlation was found with the two-way interaction between estradiol and age, and 4) a weak positive correlation was found with the three-way interaction between estradiol x progesterone x age. All interaction effects, but one, had VIF >16 suggesting high multicollinearity. Only the two-way interaction between estradiol and age has acceptable multicollinearity with a VIF of 6. When investigating this relationship, we noted that only an effect of estradiol was apparent, thus the estradiol x age interaction effect was likely driven by estradiol rather than age, suggesting that estradiol increased the LF/HF ratio and thus SNS activity, contrary to our hypothesis. However, previous studies have found that conjugated estrogen therapy decreases LF/HF in postmenopausal women, indicating reduced SNS activity (Neves and colleagues (2007)**)** . Similarly, Yang and colleagues (2013) also found a decrease in LF/HF with conjugated equine estrogen therapy in postmenopausal women. A notable difference between our study and these previous studies is that the current study cross-sectionally measured endogenous estradiol, whereas the previous literature provided conjugated equine estrogen therapy and measured changes longitudinally. It is also important to note that Neves and colleagues (2007) and Yang and colleagues (2013) only recruited postmenopausal women and did not include younger women and men, as we did. As mentioned previously, estradiol treatment allows researchers to examine the longitudinal effect of estradiol on LF/HF to determine causal effects in participants; however, these studies have demonstrated pharmacological effects in older women only. Estradiol may increase SNS activity by reducing the release of inhibitory GABAergic neurotransmitters in the insular cortex (involved in autonomic regulation), leading to an increase in SNS activity (Saleh et al., 2005).

Contrary to the results of the current study that found no correlation between LF/HF and serum testosterone, Yildirir and colleagues (2006) used a 5-min HRV analysis and found a positive correlation between testosterone and LF/HF in PCOS women, reflecting high SNS activity. In addition, Doğru and colleagues (2010) showed that using 24-hour Holter monitoring, serum

testosterone levels in younger and older men with cardiac disease were negatively correlated with LF/HF, indicating that testosterone decreases SNS function. Yildirir and colleagues (2006) recruited only PCOS women, whereas the current study recruited younger and older men and women along with PCOS women, to account for the potential confounding variables of age and other sex hormones. Doğru and colleagues (2010) recruited younger and older men with cardiac disease, whereas the current study recruited healthy younger and older men and women, allowing the study of testosterone without comorbid conditions, regardless of biological sex. Further, Yildirir and colleagues (2006) and Doğru and colleagues (2010) both measured serum testosterone, whereas the current study measured salivary testosterone. Salivary testosterone which only contains unbound testosterone, and is thus bioactive, is arguably more valuable in physiological studies since it can readily bind to androgen receptors in target tissues (Yu et al., 2010; Empen et al., 2012). As mentioned previously, some of the potential discrepancies could also be due to HRV methodological differences where Doğru and colleagues (2010) used 24-hour Holter monitoring; however, since Min and colleagues (2008) found similar results using 5 min recordings vs 24-hr Holter monitoring differences between the studies is unlikely due to the HRV methodology.

4.5 Limitations and Future Directions

This study aimed to correlate sex hormone concentrations with microvascular and autonomic function while accounting for age. However, this study had limitations which need to be discussed. Since this study had a correlational design, causality could not be determined. Hence, future studies should aim for a randomized control design. For example, in an animal model, researchers could select mice with ovariectomy and orchiectomy, then randomly assign them to receive either hormone replacement therapy, including estrogen, progesterone, testosterone, or placebo. In a human model, researchers could administer a gonadotropin releasing hormone antagonist to suppress endogenous sex hormones in participants then, randomly assign them to receive either hormone replacement therapy, including estrogen, progesterone, testosterone, or a placebo (Pierce et al., 2000). Both animal and human study designs would allow for observing of causal relationships between sex hormone concentrations, microvascular function, autonomic function and age.

The current study was further limited by its small sample size. The *a priori* predicted overall sample size was 40; however, due to sample size restrictions for ELISA kits, only 38

participants were tested. After removing outliers based on sex hormone concentrations (as described above), the final sample size was 33. Future studies should recruit a larger sample size to increase statistical power. Further, it would be important to recruit people in their 30s and 40s to consider the hormonal transitions in women undergoing perimenopause, a period where women experience fluctuations in estrogen and progesterone and a gradual decline in testosterone (Fitzgerald et al., 1998). In addition, the current study only measured younger women in the late follicular and mid-luteal phases of the menstrual cycle; however, future studies should also consider examining the early follicular phase, when there are low levels of estrogen and progesterone, and ovulation when this is high estrogen, high testosterone and high gonadotropins (luteinizing hormone (LH) and follicular stimulating hormone (FSH)). Young men have similar estrogen and progesterone concentrations in comparison to young women during the early follicular phase (Sherman & Korenman, 1975). Therefore, the inclusion of these additional phases would allow for a complete representation of the hormonal fluctuations across menstrual cycle. Moreover, with these additional menstrual phases in mind, future studies should consider including gonadotropins such as LH and FSH, which are released from the pituitary gland and regulate the production of estrogen, progesterone and testosterone through a negative feedback mechanism (Schally, 1970). Notably, the decline in estrogen and progesterone levels during the menopausal transition reduces the negative feedback to the hypothalamus which suppresses release of LH and FSH from the pituitary gland, resulting in elevated LH and FSH levels in menopausal women (Fitzgerald et al., 1998). Thus, measuring LH and FSH in future studies could provide insight into whether these hormonal changes also contribute to changes in hemodynamics, microvascular function, or autonomic function.

The age gap in the current study (people aged 30-49) may pose a statistical limitation as the model assumes a linear relationship between age and outcomes; however, previous literature has reported a linear decline of HRV indices of PNS function, such as SDNN, with age (Almeida-Santos et al., 2016) along with a linear decline of RSA with age (De Meersman, 1993). Another statistical issue was high multicollinearity for multiple comparisons, where a VIF greater than 16 was observed for most of the two- and three-way interactions for sex hormones. To address this in future studies, a technique called principal component analysis, which uses variable reduction, could improve the interpretability of the model (Z. Zhang & Castelló, 2017). It transforms correlated variables combining them into a smaller number of new variables, known as principal

components, that are uncorrelated with each other and contain the same variance from the original dataset, allowing for stable analysis (Z. Zhang & Castelló, 2017). For example, estrogen and progesterone could be combined in a single principal component, and compared against microvascular and autonomic measures, reducing multicollinearity and improving model stability. However, this approach would not show the different influences of each hormone which was the *a priori* purpose of our study. As a result, while principal component analysis may offer statistical benefits, it would limit the interpretability of the unique contribution of each hormone. Therefore, a better approach would be to increase the sample size, include participants between ages 30-50, and include more timepoints when natural hormone concentrations are varied (e.g. throughout the day, ovulation, early follicular phase, after exercise)

Estradiol is the predominant form of estrogen in premenopausal women, and is the focus of the current study, yet estrone is the predominant form in postmenopausal women (Cui et al., 2013). Direct measurements of estrone could have provided more insight into estrogenic activity in postmenopausal women. In future studies, estrone could be measured with ELISA or with mass spectrometry. However, previous literature suggests that estrone has a lower affinity for estrogen receptors than estradiol, along with reduced biological activity (Kuiper et al., 1997). Therefore, while not measuring estrone is certainly a limitation, estradiol arguably has a greater relevance for outcomes assessed in this study.

The use of HRV for cardiac autonomic testing serves as a limitation for several reasons. Firstly, HF is commonly presented as the cardiac parasympathetic modulation; however, this relationship is dependent on the respiratory rate. For example, when respiratory rate falls outside the HF band, such as <0.15 Hz during slow breathing, and >0.4 Hz during rapid breathing, HF is unable to accurately represent parasympathetic modulation (reviewed in Shaffer & Ginsberg, 2017). For the current study, respiratory rate was not controlled; however, participants were instructed to lie supine for 10 minutes before assessing HRV, which should have normalized breathing patterns within the standardized HF range. Secondly, the current study measured 5 minutes of HRV as it can track dynamic changes in autonomic function while being performed in a controlled environment to minimize external influences; however, it can be disadvantageous due to its inability to monitor fluctuations of heartbeat intervals over a longer period of time (Li et al., 2019). For example, 24-hour long-term HRV monitoring could be more beneficial as it is more stable than short-term analysis and can examine changes over a longer period of time and during

regular life-events (Li et al., 2019). Indeed, HRV was measured only in the morning in the current study, yet some HRV variables can change throughout the day. For example, LF and LF/HF have higher values during early morning and lower values during the night (Bilan et al., 2005). However, while we didn't include changes in HRV due to circadian rhythm, the inclusion of data from the morning only would have minimized variability.

The MFI technique is a novel method introduced by Ramraj (2024) which has shown to correlate with EndoPAT results. Reproducibility studies are currently ongoing in the Edgell lab and moderate day-to-day reproducibility (ICC=0.60) has been found using the 30-60s timepoint (Edgell, personal communication). Notably, both Ramraj et al. (2024) and the current study investigated small without cardiovascular disease. Hence, the results may not be generalizable to larger diverse populations with comorbidities. For future studies, the reproducibility and accuracy of MFI from day-to-day should be conducted in larger and more diverse populations with and without chronic conditions in order to explore variations related to ethnicity, age, sex, and health status.

While we expected higher testosterone levels in PCOS compared to women without PCOS, testosterone levels were similar to those of all groups except for Y-M. A potential reason could be a result of how well the PCOS group were managing their condition, which may reduce symptoms, leading to lower testosterone levels. While the PCOS participants were not taking medications, some of the PCOS women who were recruited engaged in strenuous exercise 3-4 times a week, and exercise can reduce the symptoms of PCOS (Benham et al., 2018). In addition, another possible limitation is that the current study did not track the dietary habits of PCOS women. Low-carbohydrate diets with low glycemic index can lessen the symptoms associated with PCOS by improving reproductive health such as regulating menstruation (Shang et al., 2021) and consumption of high-fat diary may protect against anovulation (Rajaeieh et al., 2014). Further, daily dietary habits could impact estradiol concentrations. For example, a Mediterranean diet, including fruits, vegetables, and legumes, is negatively associated with estradiol levels (Carruba et al., 2006), a higher intake of dairy products is positively associated with estradiol (Brinkman et al., 2010). And a higher intake of dairy products, desserts, bread and pastries has been associated with lower testosterone levels and increased risk of hypogonadism (Hu et al., 2018).

Despite the literature stating that men produce ~20 times more testosterone than women, testosterone was similar between groups (reviewed in Saez et al., 1972; Southren et al., 1968). It

is therefore important to highlight that saliva testosterone was collected in the current study rather than plasma testosterone. Plasma testosterone in plasma reflects total testosterone including that bound to circulating proteins such as sex hormone binding globulin and albumin, along with free, unbound testosterone (Dunn et al., 1981). Meanwhile, salivary testosterone only contains free, unbound testosterone (Rilling et al., 1996). Importantly, in terms of microvascular and autonomic function, the unbound testosterone is arguably more important as it can readily bind to androgen receptors in tissues (Yu et al., 2010; Empen et al., 2012). Additionally, the hydration level of participants could affect both urinary and salivary concentrations of sex hormones where dehydrated participants could have overestimated values. For future studies, hydration levels could be controlled for by instructing the participants to drink 1 L of water before sleep and in the morning before coming to the lab.

Unexpectedly, we noted that women in the late follicular and mid-luteal phases had similar concentrations of urinary progesterone. The presence of lower progesterone in the mid-luteal phase could have resulted from some participants not ovulating this cycle, since anovulation occurs in more than one-third of women with a regular menstrual cycle (Prior et al., 2015). The presence of higher progesterone in the late follicular phase could have resulted from being tested between 7 - 12 days of their menstrual cycle. Due to the variable length of monthly cycles per person, they may have reached ovulation earlier than the expected day 14. Other reasons for variations in the concentrations of estradiol and progesterone could be from the participant providing inaccurate dates about their menstrual cycle, recall can be imprecise. Furthermore, although day 14 is considered the average day of ovulation, it has been shown that women may begin to ovulate on day 8 (Wilcox et al., 2000). Lastly, while unlikely, the presence of high progesterone in the late follicular phase could be due to pregnancy which was not tested for.

Another potential limitation is the reliability of the commercially available MIRA device used to measure estradiol and progesterone from urine samples. Although MIRA lacks the accuracy and sensitivity provided by serum testing in terms of predicting the fertile window around ovulation (Usala et al., 2024), the rise in urinary progesterone after ovulation has been shown to reliably identify the post-ovulatory luteal phase, when assessed through an area under the curve algorithm (Usala et al., 2024). Further, a study by Nakhuda and colleagues (2023) found a strong correlation between urine estradiol as measured by MIRA and serum estradiol levels, suggesting that MIRA is a feasible alternative to serum testing.

Lastly, several participants wore lipstick to the lab which was inadequately removed and contaminated the edge of the saliva collection tube used for testosterone analysis. Preservatives found in lipstick, such as phthalates, have been suggested to alter sex hormone concentrations, specifically decreasing testosterone levels, even following acute exposure (Zhu et al., 2022). For future studies, participants should be instructed to refrain from using any cosmetic products on their lips to avoid contamination.

4.6 Conclusions

In conclusion, the study examined the correlations between multiple sex hormone concentrations and microvascular or autonomic function, while accounting for the influence of age. The findings suggest that while controlling for all sex hormone concentrations, aging decreases RSA and increases DBP and MAP, indicating that aging reduces parasympathetic activity and increases blood pressure, potentially from changes in heart rate and/or cardiac output (which was not measured). In addition, while controlling for multiple sex hormone concentrations and age, estradiol was not correlated with microvascular function; however, it was positively correlated with LF/HF, DBP and MAP, indicating that estradiol enhances sympathetic activity, resulting in greater blood pressure. Progesterone and testosterone were not correlated with any microvascular, hemodynamic, or autonomic measures. The results of the current study may differ from previous studies due to the differences in: 1) methodology, 2) cohort characteristics, such as the inclusion of participants with no chronic conditions compared to those with chronic conditions, and 3) a previous lack of control for multiple sex hormones and age as covariates. These findings have meaningful implications for both society and future clinical research. Specifically, the observed association between increased estradiol concentration and higher BP challenged the cardioprotective findings of estradiol from past literature. Therefore, after future studies which have investigated the roles of pharmaceutical hormones (as opposed to the current study of endogenous hormones), our findings will be able to influence the decision of individuals planning to use hormonal replacement therapy, hormonal contraceptives, or gender affirming hormone therapy. The potential risk of developing hypertension from hormonal treatment is an important consideration and will support safer, more personalized healthcare across diverse populations.

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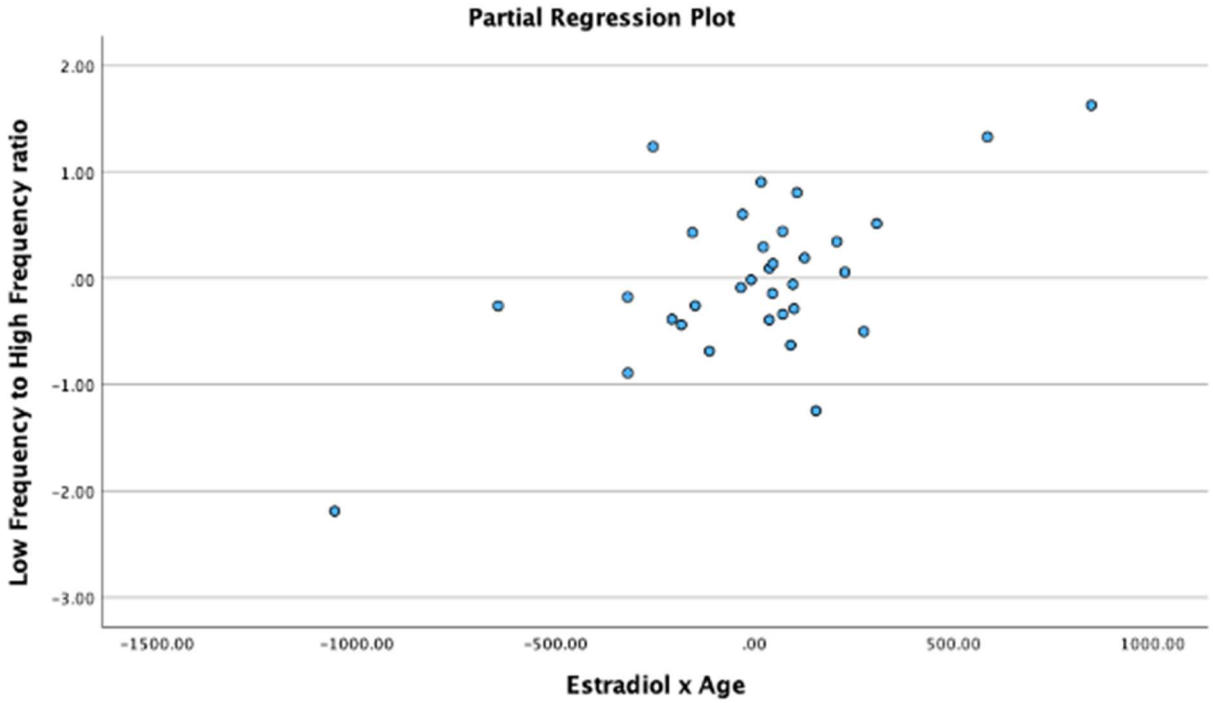
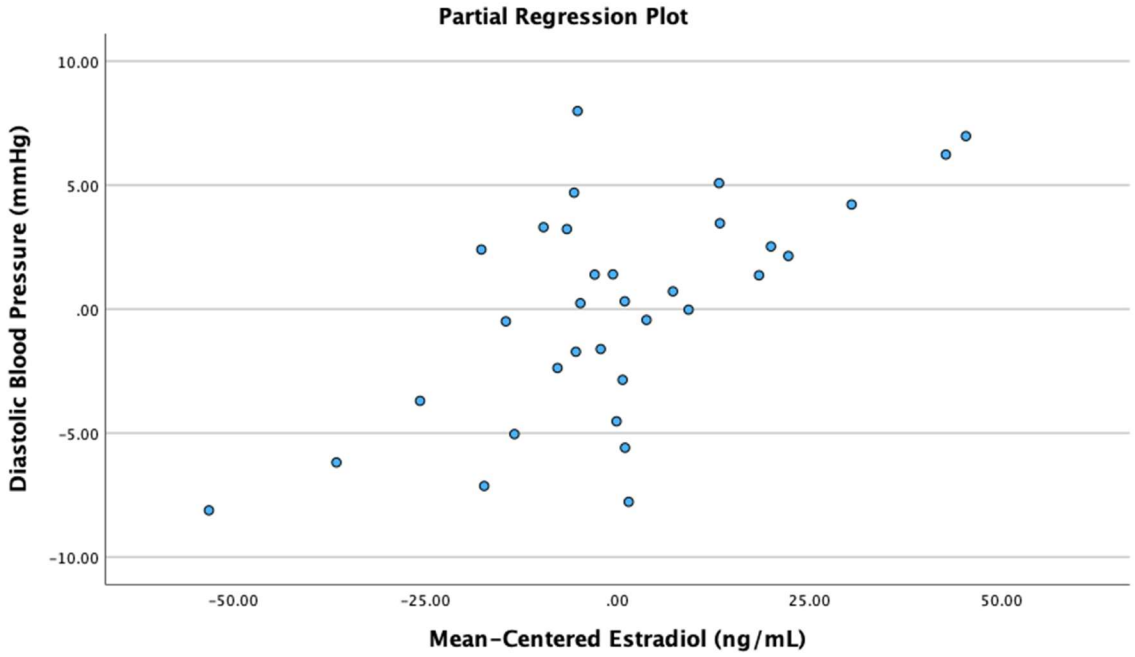
Appendix

Appendix 1: Partial regression plot showing positive relationship between low frequency to high frequency ratio (LF/HF) and interaction between estradiol and age in all participants.



Appendix 2: Partial regression plot showing positive relationship between diastolic blood pressure (DBP) and mean-centered estrogen in all participants.



Appendix 3: Partial regression plot showing positive relationship between mean arterial pressure (MAP) and mean-centered estradiol in all participants.