CHRONIC PASSIVE HEAT EXPOSURE AND CARDIOMETABOLIC HEALTH IN OBESE WOMEN WITH POLYCYSTIC OVARY SYNDROME

by

BRETT ROMANO ELY

A DISSERTATION

Presented to the Department of Human Physiology
and the Graduate School of the University of Oregon
in partial fulfillment of the requirements
for the degree of
Doctor of Philosophy

June 2018

DISSERTATION APPROVAL PAGE

Student: Brett Romano Ely

Title: Chronic Passive Heat Exposure and Cardiometabolic Health in Obese Women with Polycystic Ovary Syndrome

This dissertation has been accepted and approved in partial fulfillment of the requirements for the Doctor of Philosophy degree in the Department of Human Physiology by:

Dr. Christoper T. Minson	Chairperson
Dr. John R. Halliwill	Core Member
Dr. Carrie E. McCurdy	Core Member
Dr. Joshua Pfeiffer	Core Member
Dr. Kirstin Sterner	Institutional Representative

and

Sara D. Hodges	Interim Vice Provost and Dean of the Graduate School

Original approval signatures are on file with the University of Oregon Graduate School.

Degree awarded June 2018

DISSERTATION ABSTRACT

Brett Romano Ely

Doctor of Philosophy

Department of Human Physiology

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Polycystic Ovary Syndrome (PCOS) is a complex endocrine disorder that increases a woman's risk of developing cardiovascular disease and diabetes. Women with PCOS have extremely high rates of obesity, insulin resistance, cardiovascular morbidity and mortality. Obese women with PCOS also tend to have elevated sympathetic nerve activity and systemic markers of inflammation, which likely contribute to cardiometabolic risk and PCOS pathogenesis. While few medication or lifestyle intervention options for women with PCOS target elevated sympathetic nerve activity, inflammation, and insulin resistance, passive heat exposure shows promise as a novel intervention for improving cardiovascular and metabolic health in this population. Therefore, the purpose of this study was to examine changes in inflammation, cardiovascular, autonomic, and metabolic health in obese women with PCOS following a 30-session, 8-10 week chronic passive heat intervention (termed 'heat therapy'). Eighteen obese women with PCOS (Age: 27±1y, BMI 41.3±1.1 $kg \cdot m^2$) were matched for age and body mass index (BMI), then divided into heat therapy (HT) or time control (CON). At the beginning (Pre), middle (Mid), and end (Post) of 8-10 weeks, subjects participated in study days to assess vascular, autonomic, and metabolic function, and additionally underwent a subcutaneous fat biopsy in Pre and Post. HT subjects took part in 30 one-

hour hot tub sessions over 8-10 weeks (3-4 per week) in 40.5˚C water, while CON subjects completed all other testing but were not exposed to heat. No change in BMI was observed over the study in HT or CON; however; HT subjects exhibited dramatically improved vascular and metabolic function, as well as reduced sympathetic nerve activity and circulating inflammatory markers. In fat biopsies, insulin signaling was improved in HT subjects, while CON subjects remained stable over time. These findings show promise for HT as a treatment option for obese women with PCOS to improve cardiovascular and metabolic risk profiles.

This dissertation includes previously published co-authored material.

CURRICULUM VITAE

NAME OF AUTHOR: Brett Romano Ely

GRADUATE AND UNDERGRADUATE SCHOOLS ATTENDED:

University of Oregon, Eugene, OR
James Madison University, Harrisonburg, VA

DEGREES AWARDED:

Doctor of Philosophy, Human Physiology, 2018, University of Oregon
Master of Science, Health Science, 2003, James Madison University
Bachelor of Science, Health Science, 2001 James Madison University

AREAS OF SPECIAL INTEREST:

Thermal Physiology
Heat Acclimation
Nutrition
Cardiometabolic Health

PROFESSIONAL EXPERIENCE:

Graduate Research Fellow. University of Oregon, Human Physiology Department, 2016-Present

Instructor, University of Oregon, Human Physiology Department, 2014

Graduate Teaching Fellow. University of Oregon, Human Physiology Department, 2012-Present

Research Coordinator/Technician. United States Army Research Institute of Environmental Medicine, Thermal & Mountain Medicine Division, 2006-2012

Program Nutritionist/Lead Nutritionist. Women, Infants, & Children (WIC) Nutrition Program, 2004-2006.

Instructor, Department of Kinesiology. James Madison University, 2003-2004

Graduate Assistant, Department of Kinesiology. James Madison University, 2002-2003

GRANTS, AWARDS, AND HONORS:

American Heart Association Predoctoral Fellowship, "Chronic passive heat exposure and cardiometabolic health in obese women", University of Oregon, 2016-2018.

Eugene & Clarissa Evonuk Fellowship in Environmental or Stress Physiology "Chronic passive heat exposure and cardiometabolic health in obese women", University of Oregon, 2015-2018.

Environmental & Exercise Physiology Nike Loren G. Myhre Predoctoral Research Award, "Heat therapy decreases adipose tissue inflammation and improves insulin signaling in polycystic ovary syndrome", American Physiological Society, 2018.

Gail E. Butterfield Nutrition Travel Award, "Eight week passive heat exposure improves cardiometabolic health in obese women." American College of Sports Medicine (ACSM), 2017.

PhD Research Award, "Eight week passive heat exposure improves cardiometabolic health in obese women." ACSM Environmental and Occupational Physiology Interest Group, 2017.

President's Cup for best graduate student research, "Chronic passive heat exposure decreases sympathetic activity and improves metabolic health in polycystic ovary syndrome" ACSM Northwest Chapter, 2017.

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ACKNOWLEDGMENTS

I am grateful for the support of my advisor, Dr. Christopher Minson, and my dissertation committee, Dr. John Halliwill, Dr. Carrie McCurdy, Dr. Joshua Pfeiffer, and Dr. Kirstin Sterner. This project would not have been possible without your guidance, collaboration, and encouragement. Thank you for your leadership and for the examples you all provide of the type of scientist and mentor I aspire to be.

I am also extremely appreciative of my Minson Lab teammates, especially Michael 'Utility Guy' Francisco, Emily 'Poster Child' Larson, Elise Wright, Vienna Brunt, Cory Miner, Jenni Miner, and our rockstar undergrads. Lindan Comrada and Samantha Bryan, your investment in this project went above and beyond expectations, and I am in awe of your commitment to see it through to completion after graduation. Karen Needham, the most competent person I know, I appreciate your calm and capable support, guidance in making my first Western blot lasagna, and skill in chiseling dry ice with a screwdriver. Zach 'Fluorescent Master Mix' Clayton, thank you for all of your help, and for being there for the many late nights of processing in the McCurdy lab.

To my friends & teammates, thank you for the early morning headlamp runs, the Friday night hikes, the cooldown therapy, for always being there and always being able to make me smile. To my parents, Susan and Sal, thank you for your encouragement and generous support through every stage of my life, but especially through this adventure. My sister Sally and brother Jack, thank you for a lifetime of building me up and (occasionally) knocking me down in the way only siblings can. You gave me the toughness and courage and curiosity I needed to meet every challenge. To Callie and Cody, thank you for always seeming to know when I needed to see your face or hear your

voice. To Matt, thank you for taking this leap with me, for your unwavering support, for the thousands of ways you helped get me here, and for the secret chocolate stash you kept on hand for hard days.

I am grateful to my subjects, who invested their time and entrusted their health in my hands, and who gave tremendous insight into the challenges of living with PCOS. This investigation was funded by the American Heart Association Predoctoral Fellowship #16PRE27780085, the Eugene & Clarissa Evonuk Memorial Fellowship, and the Ken & Kenda Singer endowment, and would not have been possible without this support.

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CHAPTER I

INTRODUCTION

Obesity and associated disease rates have reached epidemic proportions, with nearly two billion people worldwide being classified as overweight or obese (Swinburn *et al.*, 2011). For example, as of 2012, 33.7% of men and 36.5% of women in the United States were classified as obese (BMI≥30) (Ogden *et al.*, 2013), and 9.3% of the U.S. population suffered from type II diabetes, a disease closely associated with excess fat mass and a sedentary lifestyle. While all obese individuals are at an elevated risk of cardiometabolic dysfunction compared to healthy weight counterparts, there appears to be a sex difference, placing women with diabetes at an elevated risk for cardiovascular death as compared to obese, diabetic men (Huxley, 2006). Within the population of obese women, diagnosis with polycystic ovary syndrome (PCOS) additionally carries a disproportionate risk of cardiovascular disease, diabetes, and cardiovascular death (Wild *et al.*, 2010). PCOS is a complex neuroendocrine disorder characterized by clinical hyperandrogenism, menstrual dysfunction, and presence of ovarian cysts with ultrasound examination (Rotterdam ESHRE/ASRM-Sponsored PCOS consensus workshop group, 2004). Depending on the diagnostic criteria used, between 5-18% of women of child-bearing age have PCOS (Spritzer *et al.*, 2015; Dunaif, 2017), and over 50% of women with PCOS are classified as obese (Gambineri *et al.*, 2002). In addition to well-described weight and fertility issues, the metabolic, autonomic, and hormonal profiles in women with PCOS greatly increase the risk for obesity, insulin resistance, and cardiovascular disease (Luque-Ramírez & Escobar-Morreale, 2014). Sympathetic over-activity

(Lansdown & Rees, 2012; Ribiero *et al.*, 2016) and systemic, low-grade inflammation (termed 'meta-inflammation') (Shorakae *et al.*, 2015) may underlie the pathogenesis of PCOS and additionally increase risk of cardiovascular disease and diabetes, so the potential impact of a lifestyle intervention aimed at improving cardiometabolic health through reducing inflammation and sympathetic outflow would be particularly promising in this population.

Common pharmacological interventions in women with PCOS focus on managing the menstrual dysfunction (oral contraceptives), symptoms related to excess testosterone (oral contraceptives and/or Spironolactone), and insulin resistance (Metformin) that are hallmarks of the syndrome. While these medications are effective in reducing signs and symptoms of PCOS, including irregular menses, acne, and hirsutism, few have an impact on cardiovascular health, metabolic health, or weight management. Metformin can act as an insulin sensitizing agent while additionally reducing free androgen levels, but does not appear to enhance weight loss when combined with lifestyle interventions (Tang *et al.*, 2006). Oral contraceptives reduce menstrual dysfunction and androgen excess, but do not appear to improve cardiovascular risk (Orio *et al.*, 2016) and may even exacerbate metabolic dysfunction in overweight/obese women with PCOS (Legro *et al.*, 2015). Current lifestyle interventions for obese women with PCOS emphasize weight loss through dietary modification and exercise training. The most commonly reported health outcomes with exercise training interventions in PCOS are weight loss, improved insulin resistance, and improved ovulation (Harrison *et al.*, 2011*a*), with some research also indicating improved autonomic function (Giallauria *et al.*, 2008; Stener-Victorin *et al.*, 2009) and reduced systemic inflammation (Giallauria *et al.*, 2008). While changing diet

and exercise patterns can be effective strategies for reducing body mass, improving health, and managing PCOS symptoms, compliance is often low in clinical populations (Burgess *et al.*, 2017), with drop-out rates as high as 45% in some exercise intervention studies in PCOS (Harrison *et al.*, 2011*b*). Chronic, passive heat exposure (termed 'heat therapy') may offer an alternative or supplemental therapy to improve metabolic health and provide protection from cardiovascular disease in obese women with PCOS. The background literature supporting this is reviewed in Chapter II, which includes previously published co-authored material (Ely *et al.*, 2018). Previous work examining repeated heat exposure in humans has shown promising improvements in metabolic health (Hooper, 1992; Hunter *et al.*, 2013*a*), cardiovascular health (Hunter *et al.*, 2013*b*; Brunt *et al.*, 2016*b*, 2016*c*), and risk of cardiovascular death (Laukkanen *et al.*, 2015), although mechanisms are still being elucidated. From animal literature, there are a variety of potential mechanisms for the observed improvements in cardiovascular and metabolic health with heat therapy; however; heat therapy has never been examined as a lifestyle intervention or potential treatment for PCOS.

The purpose of this dissertation is to highlight the cardiometabolic decline in obese women with PCOS stemming from sympathetic over-activity and meta-inflammation, and to examine the ways in which a long-term (30 sessions over 8-10 weeks) heat therapy intervention can intersect with this decline in function to improve or restore cardiovascular and metabolic health. Our primary targets of interest include vascular health, autonomic function, and metabolic health, all of which are impaired in obese women with PCOS and will potentially be impacted by heat therapy. More

specifically, we examined a 30-session (8-10 week) heat therapy intervention in obese women with PCOS through the following specific aims:

Specific Aim 1 (Chapter IV). To examine changes in vascular function in response to 30 sessions of heat therapy. This was assessed through measurement of carotid and femoral wall thickness, measures of arterial stiffness including dynamic arterial compliance and pulse wave velocity, and flow-mediated dilation (FMD) of the brachial artery before and after a brief ischemia-reperfusion stress. We hypothesized that heat therapy would reduce wall thickness, decrease arterial stiffness, improve FMD, and attenuate the decline in FMD observed in response to ischemia-reperfusion.

Specific Aim 2 (Chapter V). To examine changes in autonomic function in response to 30 sessions of heat therapy. This was accomplished by measuring baseline muscle sympathetic nerve activity (MSNA), heart rate variability, and blood pressure, and by examining MSNA and blood pressure responses to the Valsalva maneuver. We hypothesized that blood pressure would decrease by a clinically meaningful margin (≥3mmHg, as defined by the U.S. Food & Drug Administration), baseline MSNA burst incidence would be lower, but baroreflex sensitivity during the Valsalva maneuver would not change. In addition, we hypothesized that total heart rate variability would increase, and that frequency analysis would indicate increased high-frequency and decreased low-frequency variability.

Specific Aim 3 (Chapter VI). To examine changes in inflammation, metabolic health and function in response to 30 sessions of heat therapy. This was done through a 75g, 2-hour oral glucose tolerance test (OGTT) and subcutaneous adipose tissue biopsy. Additionally, markers related to insulin sensitivity (fasting glucose, serum adiponectin) and inflammation (IL-1β, IL-6, TNFα) were examined in fasting blood samples. We hypothesized that both glucose and insulin would be reduced at all time points in the OGTT, and that isolated adipocytes would display increased insulin sensitivity. In addition, we hypothesized that increases in HSPs, increased serum adiponectin, and reductions in adipose tissue and serum markers of inflammation would potentially drive these changes in metabolic health.

If these specific aims are achieved and these hypotheses are supported, this research will be the first to provide thorough and compelling evidence that heat therapy can improve cardiometabolic health in obese women with PCOS, a population at high risk for cardiovascular and metabolic dysfunction.

CHAPTER II

REVIEW OF LITERATURE

This chapter includes previously published co-authored material (Ely *et al.*, 2018). Z.S. Clayton, C.E. McCurdy, J. Pfeiffer, and C.T. Minson provided editorial input.

Reports of PCOS in medical literature range as far back as the fifth century, with Hippocrates documenting case reports of two amenorrhoeic, hirsute women from neighboring towns on the North Aegean sea (Hippocrates, 1734). This combination of menstrual dysfunction and androgen excess was first linked to metabolic disturbances in 1921 by Archard and Thiers as "diabete des femmes a barb" (diabetes of bearded women) (Achard & Thiers, 1921). Connecting this cluster of symptoms to the condition now known as PCOS, Stein and Leventhal, in 1935, published a case study of seven women with amenorrhea, symptoms related to androgen excess, obesity, and polycystic ovaries (Stein & Leventhal, 1935). Since that report, research exploring the causal link between androgen excess, reproductive dysfunction, insulin resistance, and obesity has further characterized the PCOS phenotype. In addition to the hallmark characteristics of this syndrome (androgen excess, amenorrhea, and polycystic ovaries), women with PCOS tend to have extremely high rates of obesity (>50%, as high as 80% reported) (Dunaif, 2017), insulin resistance (75-95%) (Stepto *et al.*, 2013), hypertension (20%) (Luque-Ramírez *et al.*, 2014), and an elevated risk of cardiovascular or cerebrovascular death (Dahlgren *et al.*, 1992; Rizzo *et al.*, 2009; de Groot *et al.*, 2011). Evidence points to an increase in systemic inflammation (Shorakae *et al.*, 2015) and sympathetic over-activity (Lansdown & Rees, 2012) as primary sources of both metabolic and cardiovascular

dysfunction in obese women with PCOS (Figure 1). The following sections will discuss the role of inflammation in insulin resistance and metabolic health, the contributions and interconnections between inflammation, insulin resistance, and sympathetic overactivity to cardiovascular disease and risk in obese women with PCOS. This chapter will then highlight the potential therapeutic role of a heat intervention in reducing inflammation, insulin resistance, and sympathetic activity in obese women with PCOS, and how those factors can reduce cardiometabolic risk. Evidence from human work is included whenever possible; however, in this emerging area of research animal models are included in discussion of potential physiological underpinnings.

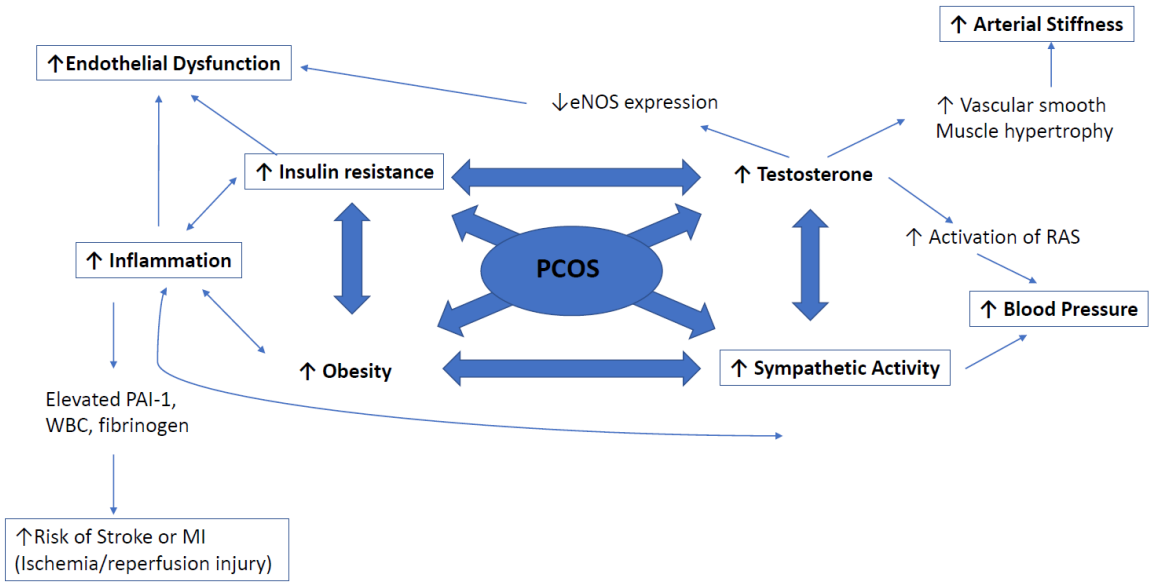


Figure 1. PCOS is associated with a cluster of conditions that increase cardiometabolic risk, including androgen excess, obesity, insulin resistance, and sympathetic overactivity. The hormonal, autonomic, and metabolic complications in PCOS manifest as elevated blood pressure, arterial stiffness, endothelial dysfunction, inflammation, and an increased risk of cardiovascular or cerebrovascular death.

Inflammation and Insulin Resistance in Obesity and PCOS

In women with PCOS, higher levels of inflammatory markers including C-reactive protein, interleukin 6 (IL-6), and tumor necrosis factor α (TNFα) have been observed, even in the absence of obesity (Shorakae *et al.*, 2015). These proteins may be secreted by adipose tissue, liver, and skeletal muscle, and have implications in both metabolic and cardiovascular dysfunction. The connection between obesity and inflammation was first clearly described in an animal model of obesity and diabetes. Hotamisligil (Hotamisligil *et al.*, 2017) demonstrated that elevated systemic and adipose tissue TNFα abundance were associated with impaired glucose tolerance and reduced adipocyte glucose uptake.

The combination of early reports of elevated inflammation in obese, diabetic individuals (Nanji *et al.*, 1985) and the finding that anti-inflammatory therapy (salicylates) reversed insulin resistance in obese rodents (Yuan *et al.*, 2001) led to the current theory that inflammation drives obesity-induced insulin resistance. This theory is further expanded in PCOS, with contributions from hyperandrogenism and autonomic dysregulation contributing to obesity, inflammation, and insulin resistance. The pathophysiological link between obesity and metabolic disease relates to increased triglyceride storage in adipocytes, driven in part by androgen excess in women with PCOS promoting adipocyte hypertrophy over hyperplasia (Diamanti-Kandarakis *et al.*, 2007), causing adipose tissue hypoxia through compression of capillary networks and inadequate blood supply relative to cell size (Trayhurn *et al.*, 2008; Ye, 2009). This initiates a cascade of adipocyte apoptosis, followed by a pro-inflammatory immune response (see figure 2). The immune response involves a variety of chemokines,

adipokines, and immune cells, which alter the profile of adipose tissue to a pro-inflammatory phenotype (Osborn & Olefsky, 2012). The change in immune cell profile includes an increase in M1 macrophages forming a crown-like structure around the adipocyte (Cinti, 2005) and releasing pro-inflammatory cytokines in the adipose tissue. Adipocytes act in a synergistic paracrine fashion with resident macrophages to increase inflammatory cytokine release by the other tissue. These cytokines are thought to disrupt insulin signaling in adipose tissue through serine phosphorylation of the insulin receptor substrate (IRS), which blocks tyrosine binding sites needed to activate the IRS within the cell and allow insulin signaling to occur.

Indeed, women with PCOS have disrupted insulin signaling in adipose tissue (Ciaraldi *et al.*, 1997; Echiburú *et al.*, 2018), resulting in impaired glucose uptake and incomplete suppression of free fatty acid release. The primary function of insulin in adipose tissue is suppression of triglyceride breakdown and fatty acid release into circulation, so the result of this impairment is increased fatty acids in the bloodstream (Zierath *et al.*, 1998). Fatty acid release is additionally driven by serum catecholamine levels, which are elevated in PCOS due to sympathetic over-activity (Yoshino *et al.*, 1991). These circulating fatty acids can accumulate in the liver and skeletal muscle and produce fatty acid intermediates such as diacylglycerol, ceramides, and long-chain fatty acid-Acyl CoA (Koves *et al.*, 2008), all of which can inhibit intracellular insulin signaling by activation of c-Jun NH_2-terminal Kinase (JNK) (Nguyen *et al.*, 2005) or Inhibitor of kappa B kinase β (IKKβ) (Yuan *et al.*, 2001; Arkan *et al.*, 2005). JNK and IKKβ similarly impair intracellular insulin signaling by serine phosphorylation of IRS (Yin *et al.*, 1998). This results in systemic insulin resistance with an impaired ability of

cells to transport glucose or suppress glucose production, creating a hyperglycemic, hyperinsulinemic profile in PCOS (Burghen *et al.*, 1980). Exercise training programs produce reliable improvements in insulin resistance, with concurrent reductions in inflammation in women with PCOS (Giallauria *et al.*, 2008; Covington *et al.*, 2016) and obese individuals without PCOS, when accompanied by weight loss (Nicoletti *et al.*, 2003), providing evidence that inflammation and obesity work in concert to drive insulin resistance in PCOS.

In addition, adipokines such as leptin and adiponectin are altered in obese women with PCOS (Spritzer *et al.*, 2015), with lower adiponectin secretion contributing to or exacerbating insulin resistance (Villa & Pratley, 2011). Adiponectin is positively correlated with insulin sensitivity (Díez & Iglesias, 2003), potentially acting by changing macrophage polarization toward an anti-inflammatory profile (Ohashi *et al.*, 2010). The end result of adipose tissue impairment is a hyper-insulinemic and meta-inflammatory profile in obese women with PCOS that vastly increases the risk of developing both metabolic and cardiovascular disease (Facchini *et al.*, 2001). The meta-inflammation in PCOS may be due in part to sympathetic nervous system dysregulation. In turn, inflammation and metabolic dysfunction contribute to elevated sympathetic activity, creating a vicious cycle, which exacerbates both metabolic and cardiovascular dysfunction Within the central nervous system, inflammation and hyperinsulinemia are associated with increased sympathetic nervous system (SNS) outflow (Smith & Minson, 2012), which is elevated in obesity and PCOS (Lambert *et al.*, 2010; Lansdown & Rees, 2012; Dag *et al.*, 2015). IL-6 receptors are present on sympathetic ganglia (Marz *et al.*, 1996; Gadient & Otten, 1996) and IL-6 infusions have been shown in to increase SNS

activity in humans (Torpy *et al.*, 2000). Further, elevated TNFα increases the expression of IL-6 receptors on sympathetic neurons (Marz *et al.*, 1996), and both cytokines are elevated in obesity (Roytblat *et al.*, 2000; Tzanavari *et al.*, 2010) and may be elevated in PCOS (Escobar-Morreale *et al.*, 2011; Gao *et al.*, 2016). Insulin also acts centrally to increase sympathetic outflow (Rowe *et al.*, 1981; Anderson *et al.*, 1992), increasing the risk of hypertension in insulin-resistant populations such as women with PCOS (Luque-Ramírez & Escobar-Morreale, 2014). High sympathetic outflow increases blood pressure through cardiac, renal, and arterial innervation, and SNS over-activity is considered an important risk factor for development of cardiovascular disease (Malpas, 2010). In addition, obesity-induced SNS over-activity contributes to end-organ damage in the kidney, blood vessels, and heart, increasing cardiovascular morbidity and mortality, even in the absence of hypertension (Lambert *et al.*, 2010). Autonomic dysfunction is also implicated in ovarian dysfunction and androgen excess in PCOS (Lara *et al.*, 1993; Sverrisdottir *et al.*, 2008; Dag *et al.*, 2015). Specific adipokines may also influence sympathetic overactivity (Smith & Minson, 2012), creating a vicious cycle of dysfunction that likely contributes to the cardiovascular and metabolic disturbances observed in obese women with PCOS (Shorakae *et al.*, 2015; Spritzer *et al.*, 2015).

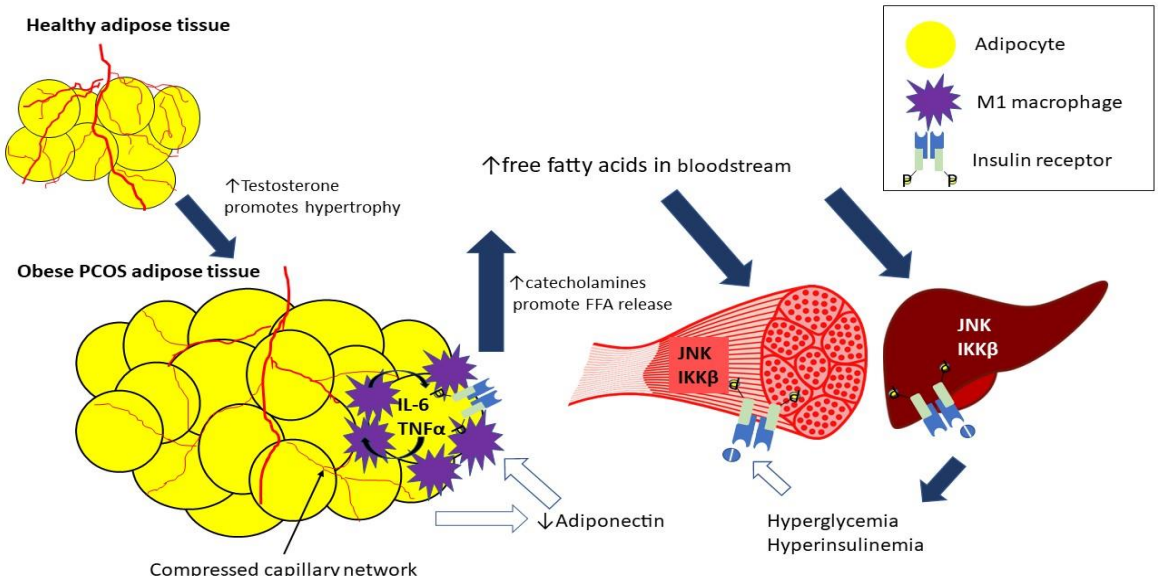


Figure 2. An overview of inflammation and ischemia in adipose tissue of obese women with PCOS. Excess fat storage, facilitated by high serum testosterone, leads to adipose tissue expansion, and the blood supply does not adequately match this tissue expansion. This adipocyte hypertrophy and inadequate blood supply causes adipocyte hypoxia, inflammatory cytokine release (IL-6, TNFα) by adipocytes and M1 macrophages, a reduction in adiponectin release, and impaired insulin action. Low adiponectin promotes the pro-inflammatory profile of macrophages, and adipocytes and macrophages act in a paracrine fashion to further increase cytokine release from neighboring adipose tissue. Insulin resistance in adipocytes causes impaired suppression of lipolysis, and elevated serum catecholamines from sympathetic overactivity in PCOS further promote lipolysis. These fatty acids are released and deposited in other tissues such as skeletal muscle and liver. Partially oxidized fatty acids increase inflammation through proteins such as JNK and IKKβ in the liver and skeletal muscle, impairing insulin signaling in these tissues. Impaired insulin action in the liver leads to an increase in glucose release (impaired suppression of glycolysis), and in skeletal muscle leads to decreased glucose uptake (impaired GLUT-4 translocation). The end result is hyperglycemia, hyperlipidemia, meta-inflammation, and insulin resistance. Filled arrows represent increased release or uptake, while unfilled arrows represent decreased release or uptake. Figure modified from Ely et al., 2017.

Inflammation and systemic insulin resistance are also associated with impaired endothelium-dependent dilation and microvascular function (Steinberg *et al.*, 1996; Scherrer & Sartori, 2000), observed in impairment of insulin's actions on blood vessels (Laakso *et al.*, 1990) as well as reduction in bioavailable nitric oxide (NO) due to the high oxidative stress seen in hyperinsulinemic individuals (Williams *et al.*, 2002). The meta-inflammatory state of obese women with PCOS additionally causes impaired vascular remodeling, resulting in increased arterial stiffness (Safar *et al.*, 2006) and intima media thickness (Dalmas *et al.*, 2013). Intima media thickening is also associated with the dyslipidemia seen in obesity and PCOS throughout the lifespan (Diamanti-Kandarakis *et al.*, 2007; Magnussen *et al.*, 2009).

While various medications are prescribed for women with PCOS to alleviate symptoms related to menstrual dysfunction, androgen excess, and hyperinsulinemia, few interventions have specifically targeted inflammation or sympathetic nervous system activity to improve cardiometabolic risk profile. Recent work has examined the imidazoline receptor agonist Moxonidine in PCOS, which acts at the rostral ventrolateral medulla to reduce sympathetic outflow. This research was based on beneficial effects on hypertension, hyperinsulinemia, dyslipidemia, and inflammation observed in other populations (Prichard & Graham, 1997; Karlafti *et al.*, 2013; Lambert *et al.*, 2017). However, no improvements in blood pressure, sympathetic nervous system activity, insulin resistance, or C-reactive protein were seen when compared to placebo in obese women with PCOS (Shorakae *et al.*, 2017). While anti-inflammatory drugs such as salicylates have been examined in other insulin resistant populations such as diabetics

(Williamson, 1901; Yin *et al.*, 1998; Yuan *et al.*, 2001), they have not been examined in PCOS related to reducing insulin resistance, vascular dysfunction, or circulating inflammatory markers.

With limited pharmacological interventions to reduce sympathetic outflow and inflammation with PCOS, lifestyle interventions appear most promising. Exercise training and low-frequency electroacupuncture both substantially reduced muscle sympathetic nerve activity with a 16-week intervention in obese women with PCOS, while the exercise training group also experienced a decrease in BMI (Stener-Victorin *et al.*, 2009). However; no change in blood pressure, fasting glucose, insulin, blood lipids, or hormones were observed with either intervention. Other exercise interventions in obese PCOS women have reported improvements in blood pressure (Vigorito *et al.*, 2007; Thomson *et al.*, 2008), microvascular function (Sprung *et al.*, 2013), metabolic health parameters (Bruner *et al.*, 2006; Vigorito *et al.*, 2007; Giallauria *et al.*, 2008; Palomba *et al.*, 2008; Thomson *et al.*, 2008), and inflammatory markers (Vigorito *et al.*, 2007; Giallauria *et al.*, 2008; Covington *et al.*, 2016) following 12-24 weeks of exercise training. The differences in health parameter improvements between studies may be due to the variety of intensity, duration, and mode of exercise training (Harrison *et al.*, 2011*a*), or the amount of weight loss accompanying the exercise training. In addition, recent work examining the efficacy of exercise training in overweight/obese women with or without PCOS found that the majority of women with PCOS (7 of 9) were classified as "non-responders" based on body mass change, and did not experience significant improvement in other metabolic health parameters including insulin sensitivity indices (Scott *et al.*, 2017). This work highlights the need for alternative or adjunctive treatments

to improve cardiometabolic health in women with PCOS, as exercise training may not be sufficient to reverse the autonomic dysregulation and inflammatory patterns that contribute to substantial cardiometabolic dysfunction and risk in this population.

How can chronic heat therapy help?

Regular heat exposure, through sauna use, hot water immersion, or combined exercise heat stress, is associated with a variety of cellular and systemic adaptations that have potential to reduce inflammation and sympathetic outflow and thus improve cardiometabolic health in obese women with PCOS. In animal work, passive heat exposure with marked elevation in core temperature is associated with changes in protein expression and abundance that lead to enhanced cardiovascular and metabolic health, as well as cellular protection from a multitude of stressors (Horowitz & Assadi, 2010; Horowitz, 2014). Long-term passive heat acclimation (30 days) has been shown in animal models to initiate cellular pathways such as Heat Shock Proteins (HSP) and Hypoxia-inducible Factor 1α (HIF1α) (Horowitz & Assadi, 2010; Horowitz, 2014) that enhance blood supply, protect cells from stressors such as ischemia (Maloyan *et al.*, 2005), and reduce inflammation (Stice & Knowlton, 2008). In addition, altered expression of adipokines such as leptin and adiponectin have been observed in animals following repeated heat exposure (Morera *et al.*, 2012). As such, there are multiple possible mechanisms by which heat therapy in humans could attenuate or prevent the development of insulin resistance, diabetes, and cardiovascular disease in obese women with PCOS. These mechanisms may work synergistically to intersect with the obesity-inflammation

cascade to potentially reduce ischemia, inflammation, insulin resistance, and vascular dysfunction.

Ischemia, Inflammation, and Insulin Resistance. Seminal work in animals using long-term heat acclimation first described heat acclimation cross-tolerance, where long-term passive heat acclimation protected cells from a multitude of stressors, including ischemia. This was first described in the rat heart, with a reduced infarct size in response to ischemia-reperfusion injury in heat-acclimated animals (Maloyan *et al.*, 2005). Since then, interest in heat/hypoxia cross-tolerance has expanded (Ely *et al.*, 2014) to include acute human studies of ischemia-reperfusion (Brunt *et al.*, 2016*d*) and human performance models (Heled *et al.*, 2012; White *et al.*, 2016). It is thought that both HSPs and HIF1α play a role in protection from ischemic injury (Horowitz & Assadi, 2010).

In obese women with PCOS, ischemia of hypertrophic adipocytes is among the first steps leading to cardiometabolic dysfunction. Chronic heat provides multiple avenues to improve blood supply (see Figure 3). For example, one downstream target of HIF-1α is vascular endothelial growth factor (VEGF) (Maloyan *et al.*, 2005), which stimulates microvascular angiogenesis. Recent human work examining acute heat exposure demonstrated increased expression of various angiogenic signals including VEGF and angiopoietin after one 90-minute leg heating session, with concomitant increases in Hsp90 expression (Kuhlenhoelter *et al.*, 2016). Hsp90 can also act through stabilizing endothelial nitric oxide synthase (Averna *et al.*, 2008), and NO acts as another angiogenic signal (Papapetropoulos *et al.*, 1997). In addition, NO production in endothelial cells is enhanced through shear stress (Thomas *et al.*, 2017), as observed during acute heating. If blood supply to adipocytes is improved through some

combination of these mechanisms, adipocytes are less likely to become ischemic, which may attenuate the inflammatory response that comes from ischemia-induced hypoxia. While acute heat exposure may result in transient increases in pro-inflammatory compounds such as IL-6 (Faulkner *et al.*, 2017) and JNK (Moon *et al.*, 2003), chronic heat treatment has been shown to decrease intracellular levels of inflammatory proteins such as JNK and IKKβ (Gupte *et al.*, 2009). Heat shock proteins have been linked with altered expression of pro- and anti-inflammatory cytokines (Cardillo & Ippolito, 2007; Dokladny *et al.*, 2010), and decreases in other inflammatory compounds such as JNK and IKKβ in skeletal muscle (Gupte *et al.*, 2009) in response to repeated heat exposure. IL-6 and TNFα, both targets of HSPs, are elevated in PCOS and associated with impaired insulin signaling in adipose tissue (Greenberg *et al.*, 1992; Hotamisligil *et al.*, 1994; Kern *et al.*, 2001). While JNK and IKKβ have not specifically been studied in adipose tissue of women with PCOS, they have both been shown in obesity to impair insulin signaling in skeletal muscle, liver, and adipose tissue in human and animal models (Hirosumi *et al.*, 2002; Arkan *et al.*, 2005; Masharani *et al.*, 2011).

HSP levels have been linked to insulin sensitivity in humans (Bruce *et al.*, 2003) through a variety of mechanisms. Individuals with type II diabetes exhibit reduced levels of HSPs in adipose tissue (Hooper & Hooper, 2009) and skeletal muscle (Bruce *et al.*, 2003). Animal work using regular heat exposure examined the relationship between various HSPs and insulin signaling in rat skeletal muscle, and found both Hsp27 and Hsp70 decreased inflammatory proteins such as JNK and IKKβ, both known to impair insulin signaling through serine phosphorylation of IRS-1 (Gupte *et al.*, 2009). In adipose tissue, Hsp70 decreases the expression of nuclear factor kappa-B, which in turn reduces

the release of pro-inflammatory cytokines such as IL-1β, IL-6, and TNFα (Dokladny *et al.*, 2010). Hsp70 is additionally involved in the protein refolding of the insulin receptor when denatured by stress (Zachayus *et al.*, 1996), providing another mechanism through which heat shock proteins can improve or maintain insulin signaling in populations with impaired metabolic health. In skeletal muscle, mild heating increases expression of genes encoding mitochondrial biogenesis (Liu & Brooks, 2012), which can increase energy flux in the cell and reduce the accumulation of the fatty acid intermediates linked to inflammation and insulin resistance. In addition, animal work has suggested that as little as five days of passive heat exposure in mice increased serum adiponectin levels (Morera *et al.*, 2012), which is associated with enhanced insulin sensitivity and reduce inflammation (Ouchi *et al.*, 2003).

In humans, hot water immersion has been examined as a therapeutic method in obese, diabetic individuals. Hooper (Hooper, 1992) examined glucose control in obese men and women with type 2 diabetes following three weeks of regular hot tub use (30 minutes per session). The subjects experienced a large decrease in fasting glucose and glycosylated hemoglobin, and the researchers postulated that this was due to the increased blood flow to skeletal muscles during heating. While no mechanisms were examined in this study, similar heating protocols have been associated with increased HSP abundance in animal models (Gupte *et al.*, 2009). Local heating of abdominal adipose tissue, when combined with mild electrical stimulation, has also been shown to reduce visceral fat storage and improve glucose tolerance in obese, diabetic individuals over a 12-week period (Kondo *et al.*, 2014). These changes were additionally associated with reductions in inflammatory markers and an increase in Hsp72 abundance in

monocytes, linking the potential benefits of heat exposure on metabolic health to increase HSP production and reduced systemic inflammation. Further supporting the potential for regular heat exposure to reduce systemic inflammation, a large prospective cohort study in Finland (2,084 men) detailing sauna use habits found an inverse relationship between serum C-reactive protein and frequency of sauna use, even after correcting for factors such as BMI, diabetes, age, and physical activity (Laukkanen & Laukkanen, 2018).

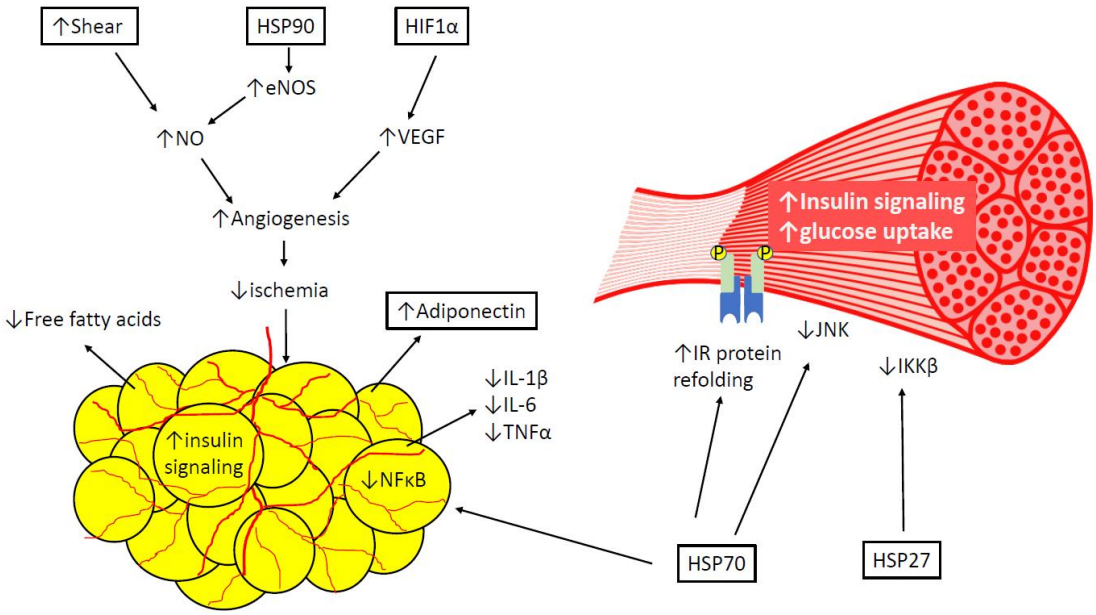


Figure 3. The potential pathways through which chronic heat exposure can reduce inflammation, improve blood flow, and reduce insulin resistance. The increases in shear, HSP90, and HIF1α all offer potential to improve blood flow and reduce adipose tissue hypoxia, through mechanisms such as increased nitric oxide (NO) and vascular endothelial growth factor (VEGF). In addition, HSP70 reduces inflammatory markers in both adipose tissue (nuclear factor kappa-B [NFκB], IL-6, and TNFα) and skeletal muscle (JNK), along with HSP27 (IKKβ). In addition, HSP70 is involved in protein refolding of the insulin receptor. In concert, these mechanisms can reduce inflammation, improve insulin signaling, increase glucose uptake and reduce fatty acid release, and increase adiponectin secretion, improving the metabolic health profile in obesity. While the changes in protein abundance and expression have been experimentally observed in human or animal models (denoted with boxes), some downstream effects have not specifically been examined in response to chronic heat. Figure from Ely et al., 2017.

Acute hot water immersion has since been studied as a means to improve glucose control. Faulkner and colleagues (Faulkner *et al.*, 2017) compared the glucose response to a meal after either a 60-min hot bath or 60-min moderate intensity exercise in lean and overweight men, and found that heat decreased peak post-prandial glucose compared to exercise, with no difference in 24-h glucose control between heat and exercise. The authors postulated that increased HSP production in response to heat drove the improved glucose control through enhanced insulin signaling.

While not a truly passive model of heat exposure, a series of studies have examined cardiometabolic risk factors after a hot yoga intervention, and reported improvements in body composition and glucose tolerance (Hunter *et al.*, 2013*a*), cholesterol, insulin, and measures of vascular health (Hunter *et al.*, 2013*b*; Guo *et al.*, 2014), and other markers of physiological and psychological well-being (Guo *et al.*, 2014). However, research is mixed on the increase in core temperature observed during a 90-min Bikram yoga class (a series of 26 postures in a room set to 40.5°C, 40% relative humidity), with values ranging from mild hyperthermia [0.6-1.0°C increase (Pate & Buono, 2014)] to changes more similar to those seen in hot water immersion [1.7-2.5°C increase (Quandt *et al.*, 2015)]. In addition, these hot yoga studies lacked a thermoneutral control group, and more recent research that employed a thermoneutral control group suggests these improvements in cardiovascular health are mediated by yoga rather than heat (Hunter *et al.*, 2018).

Vascular Dysfunction. In advance of overt cardiovascular disease, the high rates of obesity and insulin resistance in PCOS can increase cardiovascular dysfunction and blood pressure through impaired vascular remodeling (Ouchi *et al.*, 2003; Osmond *et al.*,

2009), endothelial dysfunction (Steinberg *et al.*, 1996), and elevated sympathetic nervous system activity (Smith & Minson, 2012; Canale *et al.*, 2013). While the relative contributions of PCOS, obesity, inflammation, and metabolic dysfunction are difficult to tease apart, these elements combine to create an elevated risk of cardiovascular disease and cardiovascular death (Huxley, 2006; Wild *et al.*, 2010).

Heat therapy offers potential to attenuate or reverse impairment through a variety of mechanisms (see Figure 4). First, acute heating, through hot water immersion or sauna, promotes increases in cardiac output and redistribution of blood flow to the periphery as a cooling mechanism. This increase in skin blood flow alters the shear pattern of arterial blood flow through conduit vessels to increase anterograde shear and reduce retrograde shear (Carter *et al.*, 2014; Thomas *et al.*, 2016, 2017). This altered shear pattern has been shown to enhance vascular remodeling and endothelial function following exercise training (Laughlin *et al.*, 2008; Tinken *et al.*, 2009) and passive heating (Tinken *et al.*, 2009; Carter *et al.*, 2014). Acute leg heating has also been shown in patients with symptomatic peripheral artery disease to enhance lower limb blood flow, reduce blood pressure and decrease circulating endothelin-1 (Neff *et al.*, 2016), all of which can improve vascular health and function, particularly if heat is repeatedly applied over time.

Heat shock proteins also play an important role in cardiovascular protection. In vascular remodeling, Hsp27 reduces intimal hyperplasia (Connolly *et al.*, 2003), an early step in formation of atherosclerotic plaques. Hsp72, through inhibition of Angiotensin II, reduces vascular smooth muscle hypertrophy (Zheng *et al.*, 2006). Heat exposure has additionally been associated with reductions in IL-6 (Kim *et al.*, 2005) and increases in

adiponectin (Morera *et al.*, 2012), which promote and inhibit vascular inflammation, respectively.

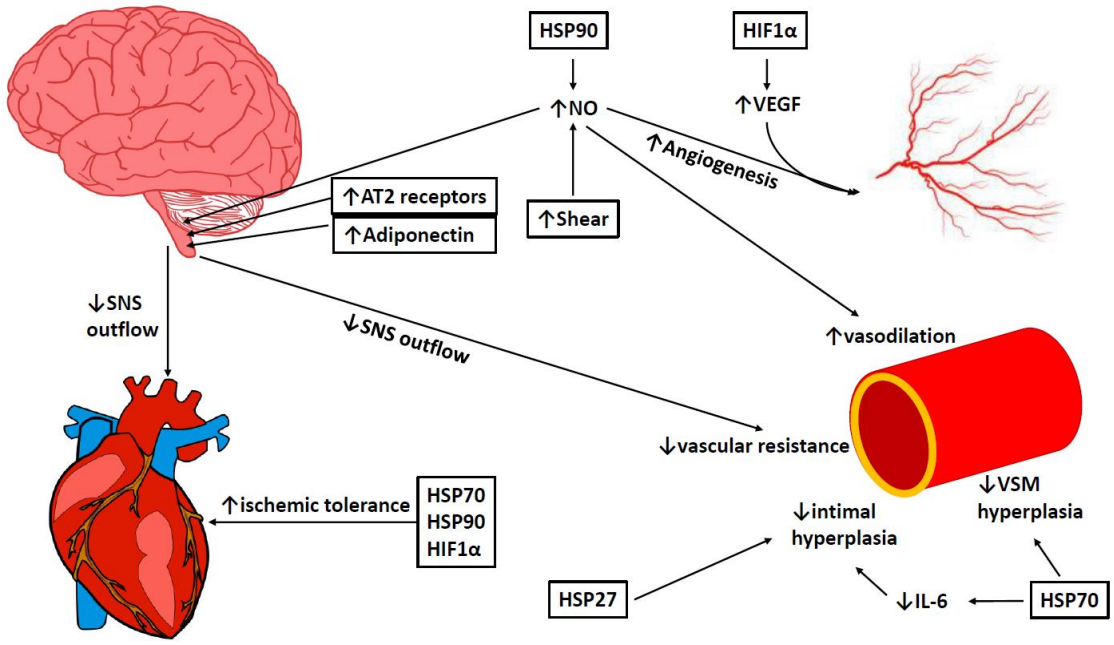


Figure 4. The potential pathways through which chronic heat exposure can improve cardiovascular health in the heart, macrovasculature, and microvasculature and autonomic activity in the brain. Increases in HSP90 and shear stress with heat therapy act to increase nitric oxide (NO), which can decrease sympathetic outflow, increase vasodilation in the microvasculature, and, along with HIF1α/VEGF, increase angiogenesis in the microvasculature. Increases in angiotensin II type 2 receptors (AT2) and adiponectin in the central nervous system can additionally reduce sympathetic outflow, which can decrease heart rate and peripheral resistance, reducing stress on the cardiovascular system. HSP27 and HSP70 decrease intimal and vascular smooth muscle (VSM) hyperplasia, and HSPs and HIF1α improve ischemic tolerance in the heart, reducing the risk or severity of cardiovascular events. Together, these mechanisms can reduce sympathetic outflow, reduce blood pressure, increase ischemic tolerance, and enhance vascular remodeling to improve the cardiovascular risk profile in obesity. Changes in shear stress, protein abundance and expression have been experimentally observed in human or animal models (indicated with boxes); however; some downstream effects have not specifically been examined in response to chronic heat. Figure from Ely et al., 2017.

Endothelial function, in healthy populations, is predominantly dependent on the production of bioavailable NO (Tousoulis *et al.*, 2012). Hsp90 is an essential cofactor for nitric oxide synthase stability (Averna *et al.*, 2008), so increases in Hsp90 expression would likely lead to an increase in endothelial NO production, as seen in animal models (Harris *et al.*, 2008; Bharati *et al.*, 2017). In human models of vascular function, this increased NO production would enhance vasodilation as assessed by techniques such as flow-mediated dilation (Brunt *et al.*, 2016*c*) and cutaneous local heating (Brunt *et al.*, 2016*b*). Since endothelial and cutaneous microcirculatory function (Steinberg *et al.*, 1996; Kraemer-Aguiar *et al.*, 2008) are impaired in obesity and PCOS (Sprung *et al.*, 2013), even in advance of overt cardiovascular disease or hyperglycemia (Kraemer-Aguiar *et al.*, 2008), improving microcirculatory function is a promising means to improve cardiovascular health in obese women with PCOS.

In humans, sauna has also been investigated as a means to improve vascular health both as an intervention in clinical populations and in prospective cohort studies. Classic Finnish saunas involve air temperatures of 80-100°C with low humidity, and individuals spend 5-30 minutes at a time in the sauna with brief breaks in a thermoneutral room between multiple bouts. A 30-min bout in an 80°C sauna quickly increases skin temperature and heart rate, and raises rectal temperature ~0.9°C (Leppaluoto, 1988). A single sauna exposure also reduces arterial stiffness and reduces blood pressure in men with at least one cardiovascular risk factor (Laukkanen *et al.*, 2017; Lee *et al.*, 2018). Two weeks of thermal therapy (60°C far-infrared sauna 6 days per week) in men with elevated cardiovascular risk significantly improved endothelial function, assessed via flow-mediated dilation (Imamura *et al.*, 2001). A study in men with congestive heart

failure underwent the same therapy and similar improvements in flow-mediated dilation were observed (Kihara *et al.*, 2002). In addition, brain natriuretic peptide (a marker of cardiac dysfunction) was significantly reduced following thermal therapy.

Vascular health and function have also been examined in response to repeated passive heat exposure in healthy, inactive men and women. Brunt and colleagues (Brunt *et al.*, 2016*c*) examined the effect of 8 weeks of hot water immersion (4-5 times per week for ~90 min per session, with core temperature increase of ~1.5˚C) and observed improvements in endothelial function, arterial stiffness, wall thickness, and blood pressure. In a companion study, this group also investigated cutaneous vasodilation in response to local heating as a model of microvascular function and specifically examined the role of NO (Brunt *et al.*, 2016*b*), and observed an increase in cutaneous vascular conductance to thermal hyperemia that was primarily mediated by NO. The evidence is currently strong for heat therapy to improve vascular function and cardiovascular risk in both healthy and clinical populations.

A large prospective cohort study (2,315 Finnish men) examined frequency and duration of sauna use and the correlation with mortality rates during a 20-year follow-up (Laukkanen *et al.*, 2015). Increased frequency and duration of sauna use were associated with substantially reduced hazard ratios for sudden cardiac death, fatal coronary heart disease, fatal cardiovascular disease, and all-cause mortality. A follow-up study by the same group showed similar results for incident hypertension (Zaccardi *et al.*, 2017). While these studies only examined men, did not include subjects that did not regularly use sauna, and did not specifically examine death related to metabolic diseases such as

diabetes, they are the largest and longest study to date on the potential long-term cardiovascular health benefits of regular passive heat exposure.

The risk of cardiovascular death has not been examined in humans in an interventional study, but the underlying injury from myocardial infarction has been examined in human and animal models. Tissue death during cardiovascular or cerebrovascular events occurs due to ischemia-reperfusion (IR) injury, a complex cellular stressor of prolonged hypoxia followed by rapid reperfusion of tissue. Promising animal work in heat-acclimated rats has examined myocardial tissue death in response to IR injury and found vastly improved tissue survival following a 30-day passive heat protocol (Maloyan *et al.*, 2005). Moreover, this improvement in tissue survival appeared to be directly related to the cytoprotective functions of HSPs and HIF1α (Horowitz & Assadi, 2010). In humans, an experimental model of IR injury in the arm using flow-mediated dilation as a model of vascular function has been used both in response to exercise (Seeger *et al.*, 2015) and passive heat exposure (Brunt *et al.*, 2016*d*), with both providing potent protection from IR stress. This is particularly important in obese women with PCOS, as obesity, metabolic dysfunction (Huxley, 2006), and PCOS (de Groot *et al.*, 2011) disproportionately increase the risk of cardiovascular or cerebrovascular death in women.

Autonomic Dysfunction. Sympathetic nervous system activity is implicated as a primary source of cardiovascular dysfunction in PCOS and strongly correlated with cardiovascular risk, and has the potential to be impacted by heat therapy. In addition, sympathetic outflow is inter-related with metabolic function in PCOS and obesity (Smith & Minson, 2012), suggesting that a reduction in sympathetic activity could have far-

reaching benefits in women with PCOS. Autonomic outflow is regulated through a variety of neurological, neurohumoral, and psychological inputs, and can be modulated by a variety of hormones and compounds including adiponectin (Tanida *et al.*, 2007), Angiotensin II (Wong *et al.*, 1992), and NO (Patel *et al.*, 2001). High circulating epinephrine suppresses insulin release from the pancreas and increases lipolysis and fatty acid release into the bloodstream (Porte, 1967). In turn, inflammation and hyperinsulinemia increase sympathetic outflow (Rowe *et al.*, 1981; Anderson *et al.*, 1992), creating a positive feedback loop for both cardiovascular and metabolic decline in obese women with PCOS. Chronic heat exposure offers the potential to reduce sympathetic outflow through reductions in inflammation and insulin resistance as previously described, and can additionally reduce sympathetic activity through increasing circulating adiponectin (Morera *et al.*, 2012) and enhancing central NO production (Pritchard *et al.*, 2001; Harris *et al.*, 2008; Bharati *et al.*, 2017). Heat acclimation has additionally been shown, in murine models, to increase Angiotensin II receptor subtype 2 (AT2) in the hypothalamus (Horowitz *et al.*, 1999), which acts to reduce sympathetic outflow (Abdulla & Johns, 2017). These alterations in circulating adipokines, vasoactive substances, and receptor density have not been examined in human or animal models of PCOS in response to heat therapy, and very little human work has been done to examine autonomic function following heat acclimation. To date, the only human data examining the potential impact of heat adaptation on resting muscle sympathetic nerve activity (MSNA) is inferred from seasonal variation studies, with an observed decrease in MSNA in summer compared with winter attributed by the authors to warmer environmental temperature (Niimi *et al.*, 1999; Cui *et al.*, 2015). In rodent models, passive heat

acclimation was associated with increased parasympathetic and decreased sympathetic influence on autorhythmic cells, as measured by infusion of atropine and propranolol (Horowitz & Meiri, 1993), although sympathetic nerve activity was not directly assessed.

Ovarian Function. In addition to potential improvements in cardiometabolic health in obese women with PCOS through reductions in inflammation and sympathetic activity, heat therapy may play a role in regulating ovarian function and potentially reducing PCOS symptoms. The possibility of reducing sympathetic outflow may not only reduce the risk of hypertension and cardiovascular disease, but high sympathetic activity is additionally implicated in cyst formation and androgen production (Lara *et al.*, 1993, 2005), so a reduction in sympathetic outflow may reduce PCOS symptomology. At the cellular level, recent work has highlighted altered expression of various HSPs in PCOS, including depressed circulating Hsp32 (also known as Heme-Oxygenase 1) (Gao *et al.*, 2014) and elevated circulating Hsp70 (Gao *et al.*, 2013), as well as depressed ovarian Hsp10 (Ling *et al.*, 2011) and elevated Hsp90B1(Li *et al.*, 2016). Hsp90B1 appears to play a pivotal role in ovarian cell development (Li *et al.*, 2016), with high levels in PCOS reducing autophagy and apoptosis, therefore promoting survival of abnormal oocytes. In contrast, women with PCOS tend to have lower Hsp10, which is involved in regulation of apoptosis in ovarian granuloma cells and contributes to ovarian dysfunction in PCOS (Ling *et al.*, 2011). While changes in ovarian tissue has not yet been examined in human or animal models of PCOS with heat therapy, altered expression and abundance of intra- and extra-cellular HSPs have been observed in heat acclimation of both animals (Horowitz & Assadi, 2010; Horowitz, 2014) and humans (Yamada *et al.*, 2007; McClung

et al., 2008; Magalhães *et al.*, 2010; Amorim *et al.*, 2015), providing a possible avenue for heat therapy to improve ovarian function in PCOS.

In combination, heat therapy offers the potential to improve cardiovascular, autonomic, and metabolic health and reduce cardiometabolic risk through a variety of mechanisms, and may additionally offer relief from symptoms of PCOS related to ovarian dysfunction and excess androgen production. To date, research in humans has been limited, and no human or animal intervention has yet explored heat therapy in PCOS models.

Summary

The multifaceted decline in cardiovascular and metabolic function and the exacerbated cardiometabolic risk in obese women with PCOS deserves further study and requires novel interventions aimed at improving health. Heat therapy offers potential as a novel or adjunctive intervention to improve cardiometabolic health, reduce risk of cardiovascular or cerebrovascular death, and to potentially reduce the medical burden of both PCOS and obesity. While not offering a direct path to weight reduction, the reductions in inflammation, improvements in glucose tolerance, and improvements in vascular function that have been observed in human and animal models with chronic heat exposure provide a variety of avenues through which cardiometabolic health can be improved in the absence of weight changes. Further, the potential for heat therapy to reduce sympathetic activity and improve ovarian function in PCOS is a promising, unexplored area of research.

CHAPTER III

EXPLANATION OF THE METHODOLOGY

Overview of Project

The research protocol involved obese women with Polycystic Ovary Syndrome undergoing a 30-session heat therapy intervention using hot water immersion (3-4 times per week over 8-10 weeks). Figure 5 shows an overview of the research timeline. An age and BMI-matched time control group did not undergo heat therapy but completed all testing days.

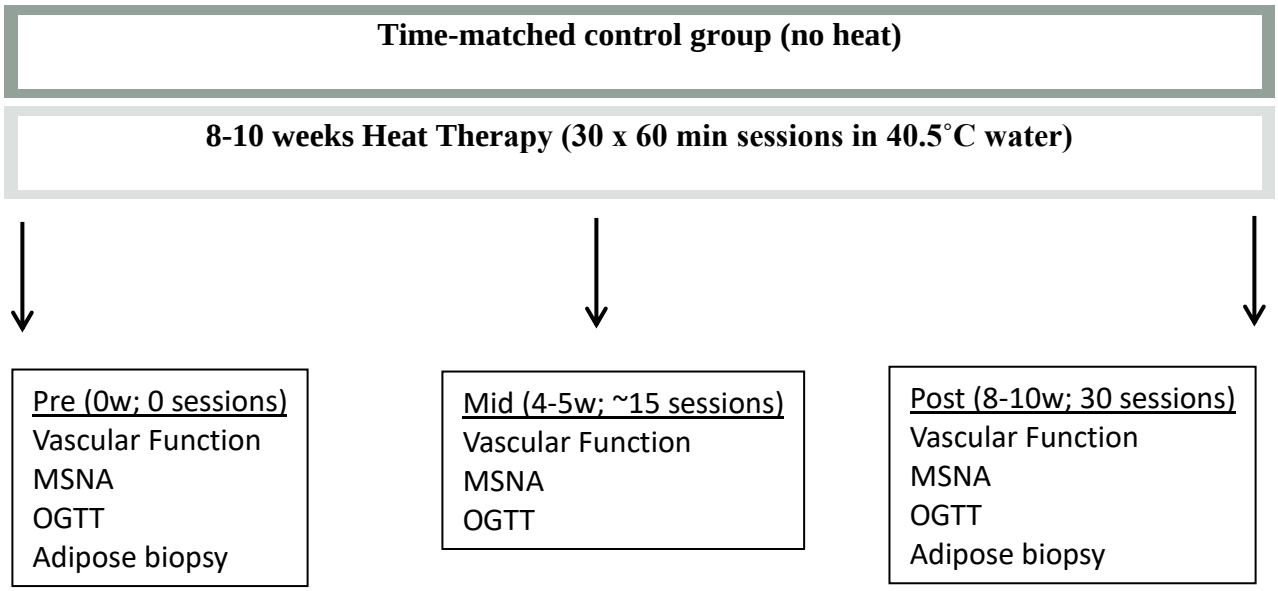


Figure 5. Overview of research timeline and vascular function, muscle sympathetic nerve activity (MSNA), oral glucose tolerance test (OGTT), and adipose biopsy study days for heat therapy and control subjects.

Subjects

Young (age 18-39) women of all races and ethnicities were eligible to be recruited for participation in this study. All subjects provided oral and written informed consent prior to participation in the study, and all experimental procedures were approved by the Institutional Review Board at the University of Oregon. Potential subjects underwent a health screening to determine eligibility and had to meet all inclusion criteria, including a BMI $\geq$30 and $\leq$45 kg/m^2, diagnosis with PCOS, non-smokers, not diagnosed with overt cardiovascular disease, and not taking prescription medications that affect insulin signaling or blood vessel function such as Metformin and Spironolactone, both of which are commonly prescribed in PCOS. Women taking oral contraceptives and anti-depression/anti-anxiety medication were included. In all, eighteen subjects volunteered to participate and completed testing, with nine subjects assigned to the experimental group and nine to the time control group. Demographic information is presented in Table 1.

Table 1. Demographics for heat therapy (HT) and control (CON) subjects

	All Subjects (n=18)	HT (n=9)	CON (n=9)
Age	27 ± 1	26 ± 2	27 ± 2
BMI	41.3 ± 1.1	41.8 ± 1.4	40.7 ± 1.9
Waist:Hip Ratio	0.85 ± 0.01	0.85 ± 0.01	0.86 ± 0.02
Medications	N=2 OCs	N=1 OC	N=1 OC
	N=4 SSRI	N=2 SSRI	N=2 SSRI

PCOS Diagnosis

All subjects had been diagnosed with PCOS by a physician, and details of diagnosis were discussed during the initial subject screening visit. PCOS diagnosis is often made with differing sets of criteria. The initial criteria were set forth by the National Institute of Health (NIH) in 1990, and included clinical hyperandrogenism, oligo- or anovulatory amenorrhea, and exclusion of all other disorders which could cause these symptoms (Zawadski & Dunaif, 1992). The presence of polycystic ovaries with ovarian ultrasound was included in the updated Rotterdam criteria in 2003 (Rotterdam ESHRE/ASRM-Sponsored PCOS consensus workshop group, 2004). The Rotterdam criteria were created by a committee sponsored in part by the European Society for Human Reproduction and Embryology and the American Society for Reproductive Medicine, and these standards for diagnosis require two of the three defined hallmarks of PCOS: 1) clinical and/or biochemical hyperandrogenism, 2) oligo- or anovulation, and 3) polycystic ovarian morphology. Guidelines have since been revised by the Androgen Excess Society (AES) (Azziz *et al.*, 2006), with androgen excess considered a mandatory component in diagnosis, which could be accompanied by either ovulatory dysfunction, polycystic ovaries, or both. While some debate remains on the optimal diagnostic standards, most physicians use more recent Rotterdam criteria for diagnosis (2 of 3 symptoms must be present). All women enrolled in the study were diagnosed with PCOS using these updated standards, and all reported bloodwork with elevated testosterone accompanied by polycystic ovarian morphology and/or oligomenorrhea.

Heat Therapy Intervention

Heat therapy occurred over an eight to ten week period, with a total of 30 x one hour sessions scheduled three to four per week in all subjects enrolled in the experimental group (control subjects were not exposed to heat). This exposure duration and timeline was selected based on previous work in our lab, where promising improvements in vascular health were seen over an 8-week, 36 session heat therapy intervention (Brunt *et al.*, 2016*c*). The acute exposure duration and total number of sessions were reduced slightly from our previous heat therapy work in order to create an intervention more similar in time commitment to exercise training protocols performed in women with PCOS (Harrison *et al.*, 2011*a*). Hot water immersion was selected as the method of passive heat stress since our goal was to significantly raise core temperature, and it is difficult to achieve large increases in core temperature without exercise in warm/hot environments. Passive hot water immersion is capable of increasing core temperature at a rate similar to moderate-intensity exercise (Kenny *et al.*, 1996), while also facilitating high skin temperature and sweating rate, all requisite components for adaptation to heat (Fox *et al.*, 1963; Buono *et al.*, 2009).

Subjects reported to the laboratory to undergo passive heating, which entailed 60 min of water immersion to the sternoclavicular line in a bath set to 40.5°C. Previous research in our laboratory suggests this temperature is optimal to raise core temperature ≥ 38.5°C within 20-30 minutes. This threshold was selected based on human heat acclimation literature using isothermic models (Fox *et al.*, 1963), and is additionally important as a threshold for induction of heat shock proteins (Taylor, 2014). Once core temperature exceeded 38.5°C, subjects sat upright in the tub (immersed to the waist) for

the remainder of the one hour session in order to maintain core temperature between 38.5-39.0°C. If temperature dropped below 38.5, subjects were asked to submerge again. After 60 minutes of exposure, subjects were asked to sit next to the tub until core temperature fell below 38.5˚C (10-15 minutes) for safety monitoring, and for monitoring the total exposure duration where core temperature was above 38.5°C. A representative tracing of core temperature during a single heat therapy session is seen in Figure 6.

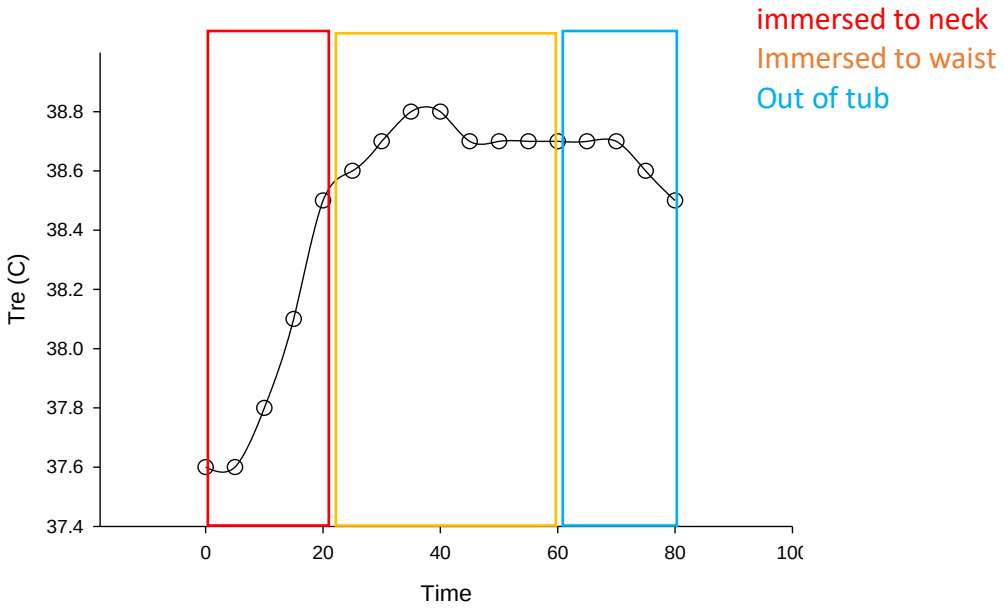


Figure 6. Representative tracing of rectal temperature (Tre) over time during a single hot tub session and recovery.

Upon arrival, subjects provided a urine sample (ideally first-void) for measurement of specific gravity (USG) to confirm euhydration (USG ≤ 1.02) prior to heat exposure. Subjects were also weighed pre- and post-heat exposure (nude, towel-dried, behind a privacy screen) and given water to drink ad libitum during heat exposure and post-heat exposure if necessary to match fluid losses. Heart rate was monitored throughout heating using commercially-available heart rate monitors (Polar), while core temperature was monitored throughout passive heating by rectal thermistor (rectal temperature, Tre). Rectal thermistors were used over less invasive methods such as aural or tympanic temperature due to safety-driven desire for a more accurate recording of deep body temperature.

Cardiovascular, Autonomic, and Metabolic Health Assessment

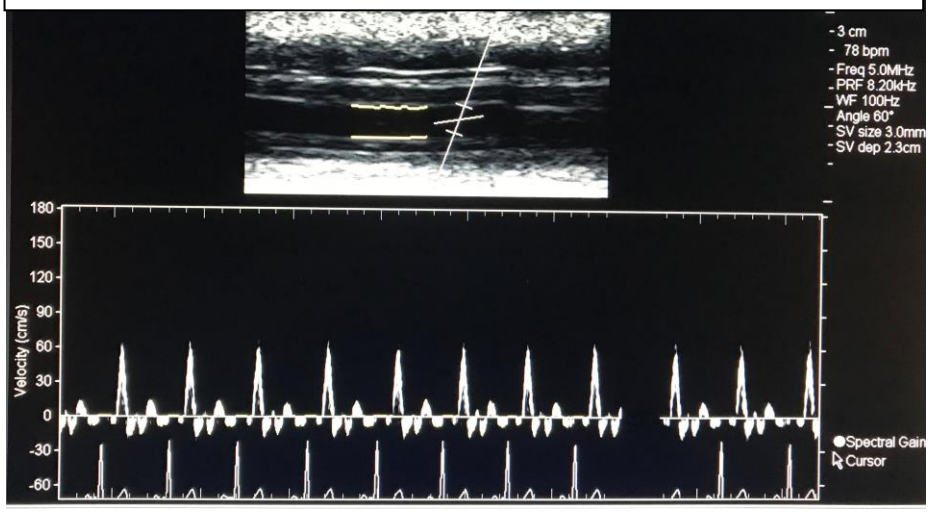
All study days took place in a thermoneutral lab environment, 24-72 hours after the most recent heat exposure in experimental subjects. While time of day varied between subjects due to work and class conflicts, time of day was held constant (within 1 hr) for each subject over time in order to minimize circadian influence. Subjects reported to the lab having refrained from food for a minimum of four hours (12 hours for OGTT studies), caffeine and alcohol for 12 hours, vitamin supplementation, all medications other than oral contraceptives, and exercise for 24 hours prior to the start of testing. Body mass and height were measured, and a urine sample was collected for confirmation that the subject was not pregnant prior to beginning testing. On one testing day at each time point, subjects were additionally assessed for waist circumference, hip circumference, and three site skinfold thickness for estimates of waist to hip ratio and body composition.

On vascular function and MSNA days, subject rested on a padded exam table for a minimum of 20 minutes prior to beginning testing, and during this time they were instrumented with a 3-lead ECG, brachial blood pressure cuff, and beat-by-beat blood pressure monitor (Nexfin)

Vascular Function

Vascular health was measured in all subjects using a variety of assessments and techniques. Measures of arterial wall thickness, dynamic arterial compliance, and flow-mediated dilation were all measured using doppler ultrasound. Vascular ultrasound applies the principles of sound wave reflection and doppler shift to allow simultaneous measurement of arterial diameter and blood velocity. The ultrasound probe contains a piezoelectric element which converts electrical signals into mechanical vibrations, then measures sound wave reflection of these vibrations to convert sound waves into measurable electrical signals (Pellerito & Polak, 2012). The Terason t3000cv system uses a 10 MHz linear array probe to capture blood vessel diameter and velocity, and these images are video recorded for offline analysis using custom wall-tracking and velocity tracking software (DICOM, Perth, Australia). Briefly, a region of interest on the artery is selected for wall tracking (see yellow lines in arterial image in Figure 7, which track the bright vessel walls), and blood velocity was measured in a nearby location by selecting a velocity gate. DICOM traces peak blood velocity (lower panel of Figure 7), which is then used along with diameter to calculate total flow [Flow in mL/min = ½ Velocity in cm/sec*π*(radius in cm)^2*60sec/min] and shear rate [Shear rate=4*Velocity/Diameter].

Figure 7. Sample analysis of arterial diameter (upper image) and velocity (lower image) using DICOM.



Wall thickness. Wall thickness of the common carotid artery is a well-described measure of vascular health (Simova, 2015), a strong predictor of future cardiovascular events (Lorenz *et al.*, 2007), and is responsive to a passive heat therapy intervention in healthy, inactive individuals (Brunt *et al.*, 2016*c*). Further, both carotid and femoral wall thickness are increased in obesity (Dalmas *et al.*, 2013) and PCOS (Lakhani *et al.*, 2004; Meyer *et al.*, 2012; Allameh *et al.*, 2013). Wall thickness was developed as a surrogate marker for predicting atherosclerosis, and has the advantage of being less invasive than angiography, and better able to detect small changes in the early stages of wall thickening before atherosclerotic plaque development (de Groot *et al.*, 2004). The common carotid and superficial femoral artery were imaged using high-resolution Doppler ultrasound (Terason t3000cv; Teratech, Burlington, MA, USA) in B mode with 10.0 MHz linear array ultrasound transducer probe artery. The carotid artery was imaged 2 cm distal to the carotid bulb at three angles: anterior, lateral and posterior. The superficial femoral artery was imaged 2–4 cm distal to the femoral bifurcation in two planes: anterior and lateral. Clearly distinguished intimal–medial boundaries were obtained while focusing on the far

wall. Images were frozen in diastole and enlarged, and calipers were used to make three repeat measurements of the wall thickness from the lumen–intima interface to the media adventitia interface (see Figure 8). Video recording of these measurements was later reviewed offline to confirm accuracy of caliper measurement, and three measurements from each angle were averaged.

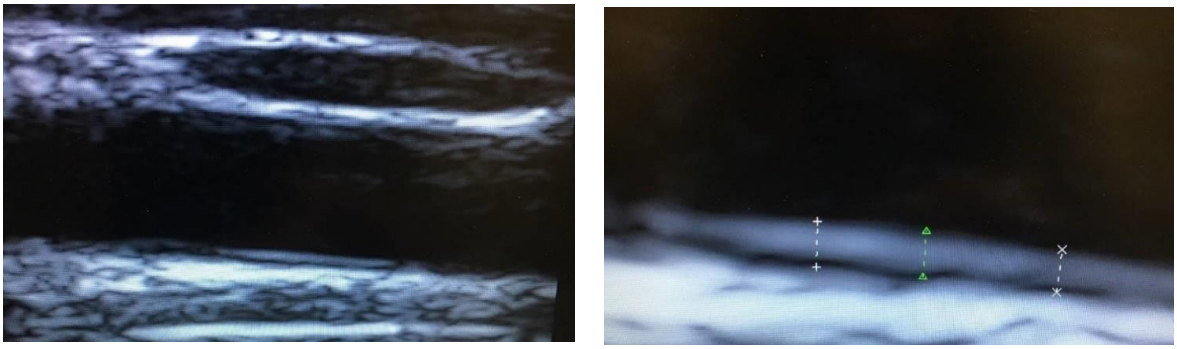


Figure 8. Image of common carotid wall (left) and zoomed image of measurement.

Arterial Stiffness. Arterial stiffness was assessed using several different methods, including carotid-femoral and brachial-ankle pulse wave velocity (PWV), as well as common carotid and superficial femoral dynamic arterial compliance (DAC). Pulse wave velocity is considered to be the simplest, most robust, and reproducible measure of arterial stiffness (O'Rourke *et al.*, 2002; Laurent *et al.*, 2006), is impaired in obese women with PCOS (Ketel *et al.*, 2010; Sasaki *et al.*, 2011), and has been shown to decrease with a chronic heat intervention in healthy, inactive individuals (Brunt *et al.*, 2016*c*). In addition, carotid-femoral PWV has been validated for predicting cardiovascular outcomes in a variety of disease conditions (Ben-Shlomo *et al.*, 2014). In obese populations where central adiposity may interfere with acquisition of clean femoral

pulse tracings, brachial-ankle PWV is a reasonable surrogate of central arterial stiffness, while providing additional information about peripheral artery stiffness (Sugawara *et al.*, 2005). DAC, while less commonly used in clinical settings, has also been reported as impaired in women with PCOS (Soares *et al.*, 2009), and to respond to repeated heat exposure in healthy and clinical populations (Brunt *et al.*, 2016*c*; Hunter *et al.*, 2017). Pulse wave velocity was assessed using applanation tonometry with pressure transducers (PCU-2000; Millar, Inc., Houston, TX, USA) placed on the carotid & femoral arteries (C-F; central PWV or aortic stiffness), as well as the brachial and dorsal pedal arteries (B-A; peripheral PWV). Tracings were recorded using data acquisition software (Windaq; Dataq Instruments, Akron, OH, USA), and the pulse upswings of a minimum of thirty simultaneously recorded pressure tracings were identified offline by a blinded investigator in order to calculate the time differential (See figure 9). Velocity was calculated as distance over time, where distance was the sum of the linear distances between the carotid probe and the sternal notch and the sternal notch to the femoral probe (carotid-femoral) or the distance differential between the brachial probe to sternal notch distance and the ankle probe to sternal notch distance (brachial-ankle).

Carotid and femoral DAC were measured using high-resolution Doppler ultrasound (Terason t3000cv; Teratech, Burlington, MA, USA) with 10.0 MHz linear array ultrasound transducer probe with concurrent applanation tonometry on the same artery on the contralateral side of the body. Ultrasound probe placement on the body, including angle of approach and internal anchors such as distance from bifurcation, were recorded on the first trial day and repeated to ensure consistency between repeated measurements over the course of the study. All ultrasound recordings were performed on

the right side of the body. Ultrasound images were recorded at 20 Hz using video recording software (Camtasia), then analyzed for diameter and blood velocity using custom-designed edge detection and wall tracking software (DICOM; Perth, Australia). Pulse pressure using applanation tonometry was simultaneously recoded via Windaq data acquisition (Dataq, Inc) at 250 Hz, and analyzed using the trough to peak pressure differential. This pressure differential (ΔP) was then analyzed relative to change in diameter (ΔD) for a minimum of 50 cardiac cycles in order to calculate cross-sectional compliance and β stiffness using the following equations:

$$\textit{Dynamic arterial compliance} = [(\Delta D/D)/2\Delta P] * \pi D^2$$

$$\textit{B-stiffness index} = Ln\ (SBP/DBP) * D/\Delta D$$

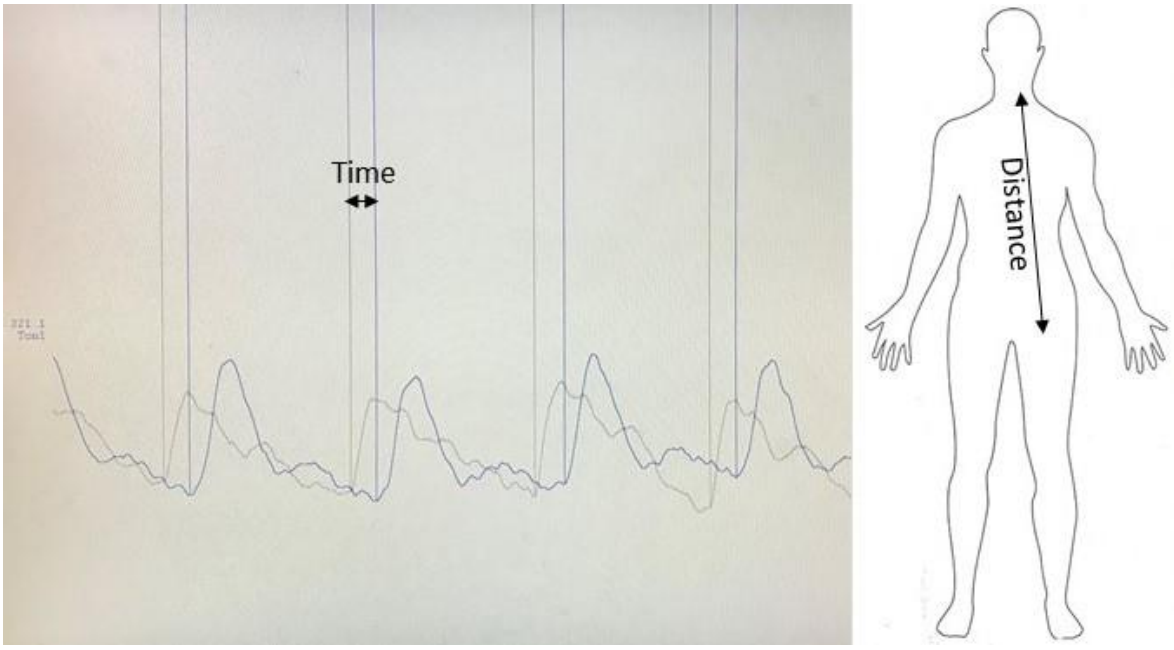


Figure 9. Sample pulse tracings with time differential (left) and distance measurement (right) used for PWV**.**

Endothelial Function. Endothelial function was assessed using flow-mediated dilation (FMD), a technique developed in 1992 by Celermajer and colleagues as a non-invasive means to assess endothelium-dependent vasodilation in children and adults (Celermajer *et al.*, 1992). FMD consists of arterial imaging by Doppler ultrasound to obtain baseline diameter and blood velocity measurement, then inflating an occlusion cuff just below the artery for a period of 5 minutes. Following release of the cuff, a large increase in blood flow occurs, creating shear stress on the blood vessel walls, and changes in velocity and diameter are captured using doppler ultrasound for three minutes after cuff release. FMD is defined as the maximal change in diameter observed in response to the 5-min occlusion of blood flow distal to the brachial artery, with a larger % change in diameter indicating greater endothelial function and reduced cardiovascular risk. FMD is a well-established predictor of cardiovascular risk and future cardiovascular events (Yeboah *et al.*, 2008; Shechter *et al.*, 2009). While dilation occurs predominantly due to the release of nitric oxide induced by shear stress acting on the endothelial cells, FMD is not exclusively NO-dependent (Wray *et al.*, 2013), and some debate exists on whether it is an optimal test of endothelial function or bioavailable NO. However, it is used in both research and clinical settings, and, importantly, FMD at the brachial artery has been shown to parallel coronary artery endothelial function (Teragawa *et al.*, 2005). Additionally, in previous work in our lab, FMD improved with heat therapy in healthy humans (Brunt *et al.*, 2016*c*).

FMD was measured at the brachial artery using Doppler ultrasonography according to established methodology (Thijssen *et al.*, 2011), with an occlusion cuff placed just distal to the elbow and inflated to 250mmHg for a period of five minutes. The

ultrasound images were recorded (Camtasia software) for one minute of baseline (prior to cuff inflation), and for three minutes after release of the cuff. The recorded images were analyzed for changes in brachial artery diameter and blood velocity using DICOM analysis software. Variables of interest included baseline diameter, peak diameter, and blood velocity at baseline and from cuff release to peak diameter. Velocity and diameter were used to calculate shear rate (blood velocity ÷ diameter) and shear area under the curve (defined as the sum of shear rate over time from cuff release to peak dilation, minus baseline shear rate) in order to assess the shear stimulus for vasodilation. FMD % dilation is commonly expressed in both absolute percentage, and shear-corrected (FMD% ÷ shear area under the curve) in order to quantify the response relative to the dose/stimulus of shear stress (Padilla *et al.*, 2008).

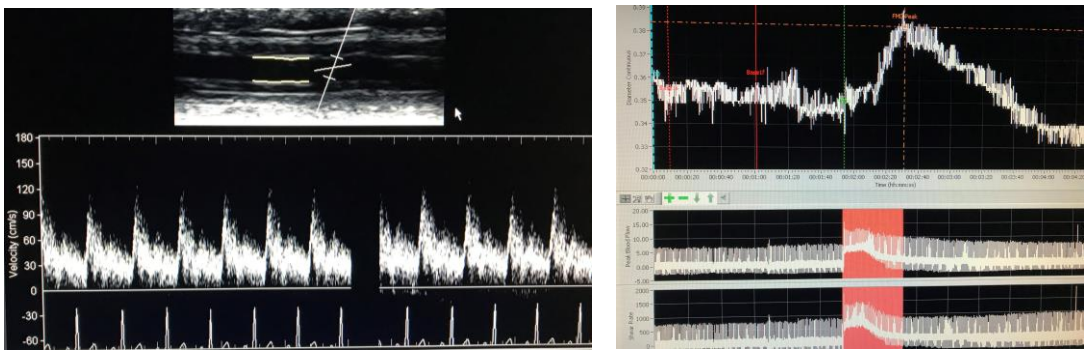


Figure 10. DICOM analysis software allows arterial diameter and velocity tracking during FMD (see yellow wall tracking of brachial artery in upper left panel). Upon release of the cuff, a large increase in velocity (lower left panel) creates a shear stimulus that leads to an increase in diameter. The right panel displays a sample analysis of baseline diameter, peak diameter (for calculation of FMD%), and shear AUC (highlighted in lower panel) for calculation of shear AUC between cuff release and peak diameter.

Ischemia/Reperfusion. In addition to being a well-established test of endothelial function, FMD is responsive to stress such as ischemia-reperfusion (IR) (Seeger *et al.*, 2015; Brunt *et al.*, 2016*d*). Since women with PCOS are at an elevated risk of coronary

or cerebrovascular events, blood vessel responsiveness to IR is an important variable to assess future risk of cardiovascular/cerebrovascular death. IR was performed by placing an occlusion cuff on the upper arm (above the point where the brachial artery was imaged for FMD) and inflating it to 250mmHg for a period of 20 minutes. Twenty minutes after release of the cuff, FMD was re-assessed. This protocol was selected as it has been used in acute exercise (Seeger *et al.*, 2015) and heat (Brunt *et al.*, 2016*d*) interventions, has been well-tolerated by subjects, and shows a short-term impairment of endothelial and microvascular function.

Autonomic Function

Muscle Sympathetic Nerve Activity. Muscle sympathetic nerve activity (MSNA) was first described in 1977 (Sundlöf & Wallin, 1977), and proposed as a global measure of sympathetic nerve activity that correlated well with plasma norepinepherine concentrations (Wallin *et al.*, 1981) and acutely tracked with changes in diastolic blood pressure (Sundlöf & Wallin, 1978). Since its inception, MSNA has been used in laboratories throughout the world to assess postganglionic sympathetic nerve traffic in clinical populations and in response to interventions. MSNA bursting rates (expressed as burst frequency; bursts/min, and burst incidence; bursts/100 heartbeats) are correlated with both the risk (Rea & Hamdan, 1990) and severity (Matsukawa *et al.*, 1993) of hypertension, and are associated with obesity-induced sub-clinical organ damage (Lambert *et al.*, 2010). In women with PCOS, this measure is particularly important as sympathetic overactivity is implicated in the pathogenesis of the disease (Lansdown & Rees, 2012), and is likely related to the elevated risk of hypertension and other cardiometabolic disturbances in this population.

MSNA was assessed in resting subjects in the peroneal nerve as previously described (Wallin & Sundlof, 1979). Subjects were instrumented with a 3-lead EKG, brachial blood pressure monitor, and beat-by-beat blood pressure monitor while resting supine on a padded exam table. The leg was slightly elevated from mid-thigh and held stable using a vacuum splint on the thigh and a raised footrest while the subject was supine, in order to easily access the regions behind the knee and near the fibular head where the nerve runs closest to the skin surface. The peroneal nerve was located using external stimulation, tracing the nerve from the fibular head through the popliteal fossa, and sites that showed strong muscle twitches were identified. Once a site was selected, a tungsten microelectrode (FHC, Bowdoin, ME) was inserted percutaneously into the peroneal nerve and manipulated until postganglionic sympathetic nerve traffic was recognized. Muscle sympathetic nerve activity was distinguished from other sources of nerve activity by the following criteria: 1) presence of spontaneous pulse synchronous bursts, peaking approximately 1.1-1.4 seconds after a QRS complex, 2) increased activity during breath hold or Valsalva straining, 3) confirmation of muscle afferent activity during light passive dorsiflexion of the foot, and 4) confirmation of a lack of skin nerve activity during light stroking of the skin on the foot and shin. Nerve recording was considered optimal with a 3:1 signal:noise ratio and a stable baseline. See Figure 11 for example of MSNA recording at rest, including stable baseline, ≥3:1 signal: noise ratio, and timing of bursts with the EKG and with blood pressure fluctuations.

Nerve traffic was continuously recorded at 250 Hz and analyzed offline by two investigators (one blinded) for burst frequency and incidence. Measures were taken at baseline (quiet supine resting), with paced breathing (resting while breathing to a

metronome set to 15 breaths/min), and during a Valsalva maneuver as an assessment of baroreflex function. While our primary autonomic outcome variables were burst frequency and incidence at rest and during pace breathing, the possibility that heat therapy would cause a large change in baseline MSNA necessitated examination of sympathetic responsiveness. This allows insight into whether heat therapy altered the set point for blood pressure, or whether baroreflex sensitivity is altered as well.

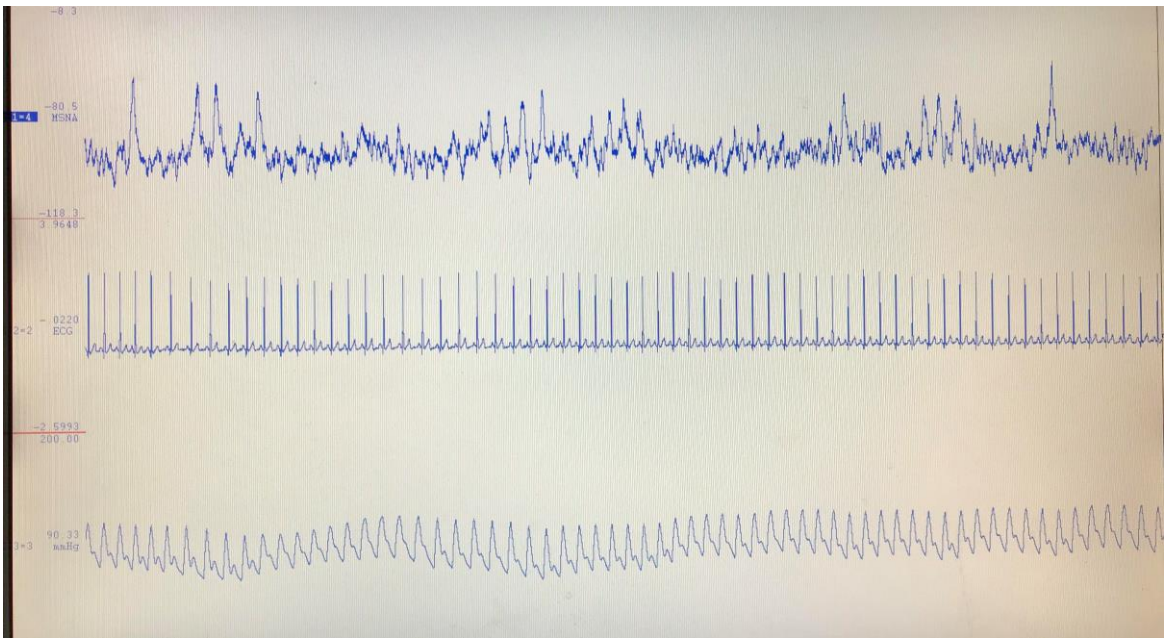


Figure 11. Sample MSNA recording during rest (upper tracing; sharp deflections represent SNS firing to muscle vascular beds), with ECG (middle) and beat-by-beat blood pressure (lower).

Valsalva Maneuver. The Valsalva maneuver, named for 17th century physician Antonio Maria Valsalva, involves forceful expiration ('bearing down') against a closed glottis. This maneuver causes dramatic changes in intrathoracic pressure, and physiological responses can be divided into four phases. Phase I begins at the onset of Valsalva straining, where a transient increase in stroke volume secondary to an increase

in intrathoracic pressure causes a temporary rise in blood pressure. However, this phase is short-lived as venous return decreases, causing blood pressure to drop and MSNA burst frequency to increase in an attempt to maintain pressure through increased vasoconstriction and heart rate. Phase III begins at the release of straining, which results in decreased intrathoracic pressure and a brief decrease in blood pressure before Phase IV (blood pressure overshoot with decreased heart rate), and resolves when blood pressure and heart rate return to baseline (see Figure 12 for sample tracing).

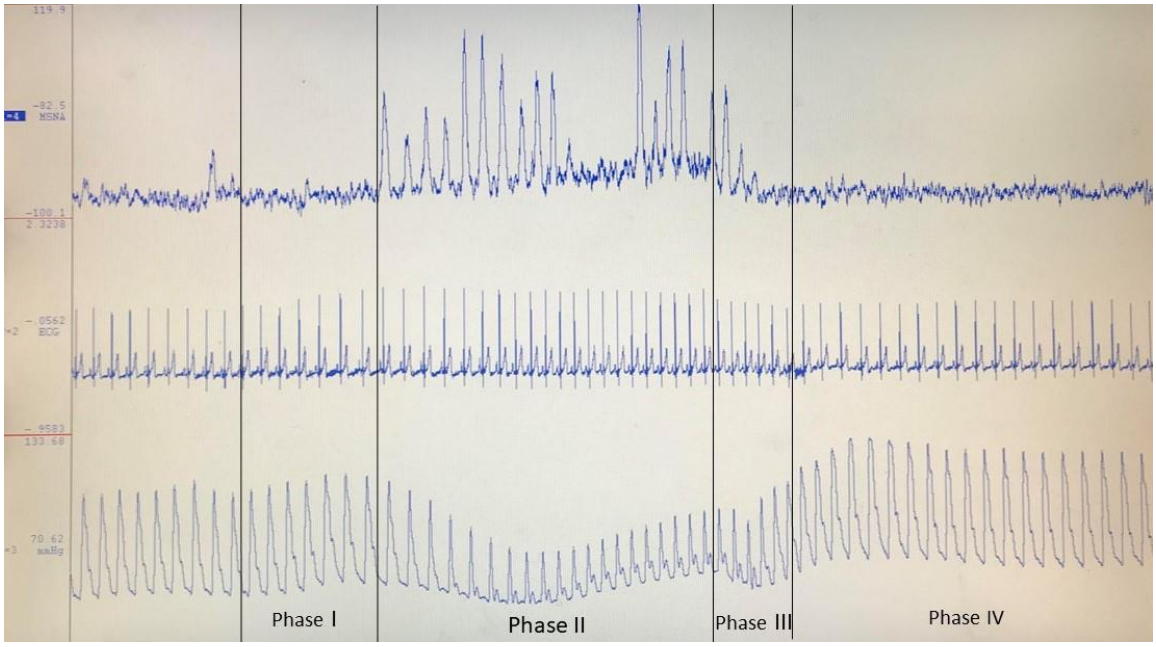


Figure 12. Sample MSNA recording during the Valsalva maneuver, with MSNA (upper panel), heart rate (middle panel), and blood pressure (lower panel) and four phases identified.

This maneuver is used during MSNA to assess baroreflex function by examining changes in MSNA relative to changes in diastolic pressure (sympathetic baroreflex sensitivity; sBRS), and changes in the ECG R-R interval relative to systolic pressure (cardiovagal baroreflex sensitivity; cBRS). The arterial baroreflex is important in the

regulation of blood pressure during various challenges, including orthostasis and exercise (Eckberg & Sleight, 1992).

When blood pressure drops (as in Phase II of the Valsalva maneuver), sympathetic outflow increases and leads to cardiovascular adjustments including vasoconstriction of arterioles and tachycardia in an attempt to restore blood pressure. A linear regression of the change in blood pressure relative to the change in MSNA or heart rate during Phase II of the Valsalva maneuver allows for assessment of baroreflex sensitivity by examining the slope of regression line. While this can additionally be examined in Phase IV (blood pressure is elevated while heart rate is depressed), it is common to have very few sympathetic bursts during the blood pressure overshoot, making calculation of a slope difficult for sympathetic baroreflex sensitivity.

Subjects were instructed to rest for one minute while breathing normally, then perform a 20-second Valsalva maneuver by contracting their abdominals, bearing down with a closed glottis, and blowing into a tube connected to a pressure transducer (details?). The pressure gauge was connected to a CardioCap monitor, and subjects were instructed to keep pressure at 40mmHg for 20 seconds. At the end of 20 seconds, the subject resumed normal breathing, and MSNA and blood pressure were monitored for an additional 60-120 seconds, until heart rate and beat-by-beat blood pressure appeared to return to baseline. Change in MSNA, blood pressure, and heart rate during Valsalva straining and release provide insight into autonomic regulation. In general, a greater baroreflex sensitivity (steeper slope) is associated with a healthier cardiovascular risk profile (Eckberg & Sleight, 1992; Sleight, 1997).

Heart Rate Variability. Due to the difficulty in obtaining nerve tracings in some subjects, we additionally assessed autonomic function using heart rate variability at rest and during paced breathing in all subjects. Spectral analysis heart rate variability is a widely-used, non-invasive, estimate of autonomic influence on the heart. While its correlation with MSNA and cardiac NE spillover is poor (Kingwell *et al.*, 1994) in clinical populations, measures of low-frequency variability (LFnu; attributed to high sympathetic activity) do track with MSNA in healthy individuals in select conditions (DeBeck *et al.*, 2010). In addition, overall variability and high-frequency variability (HFnu; attributed to parasympathetic activity) appear to be decreased, indicating reduced parasympathetic influence, in women with PCOS (Hashim *et al.*, 2015; Ribiero *et al.*, 2016). As such, we measured HRV in all subjects during MSNA studies, and in subjects in whom we were unable to obtain adequate nerve recordings. Briefly, subjects were resting in the supine position in a dark, thermoneutral room (18-21°C) for a minimum of 20 minutes prior to testing. Heart rhythm was measured using a 3-lead EKG (CardioCap; Datex Ohmeda, Louisville, CO) while breathing to a metronome paced at 15 breaths/min for five minutes. Data were continuously recorded at 250Hz (Windaq, Dataq Instruments, Akron, OH) for offline analysis including peak detection of R waves and examination of heart rate variability using commercially available software (HRVanalysis 1.1) (Pichot *et al.*, 2016). Variables of interest within time domain analyses of total variability included the standard deviation of normal-to-normal R-R intervals (SDNN) and the square root of the mean sum of squared differences (rMSSD). Frequencial analyses were additionally employed to measure total variability (Ptot) and to estimate the relative contribution of parasympathetic and sympathetic

modulation using low frequency (LF, LFnu; defined as 0.04-0.15Hz), high frequency (HF, HFnu; defined as 0.15-0.4Hz), and the ratio of low to high frequency (LF/HF).

Alternative Techniques. While MSNA is the most direct assessment of sympathetic nerve traffic available in humans, there are several alternative approaches to measure or estimate sympathetic activity. These include serum catecholamines, urinary excretion of catecholamine metabolites, whole-body (Straznicky *et al.*, 2016) or organ-specific norepinephrine spillover (Esler *et al.*, 1984). Serum and urine catecholamine levels are a global estimate of sympathetic activity; however; there are a variety of situations in which these measures do not adequately reflect changes in sympathetic outflow (Young *et al.*, 1984). Cardiac norepinephrine spillover allows for acute examination of cardiac sympathetic activity, which is well-correlated with MSNA. While this technique more specifically measures sympathetic activity to the heart as compared to muscle vascular beds, it is expensive and invasive, requiring radiotracers and cardiac catheterization. Global norepinephrine spillover, while less organ-specific, tracks with changes in sympathetic activity (Straznicky *et al.*, 2010, 2016), but similarly requires radiotracers and arterial catheterization.

For assessment of baroreflex sensitivity, the gold standard assessment is the Modified Oxford technique, which involves sequential intravenous infusion of nitroprusside and phenylephrine to create systemic vasodilation and vasoconstriction, respectively (Rudas *et al.*, 1999). These changes in vascular tone cause dramatic fluctuations in blood pressure, which are sensed by baroreceptors that alter sympathetic and parasympathetic outflow in order to maintain pressure. The change in MSNA and

heart rate during these vasoactive drug infusions allow for assessment of sBRS and cBRS in a tightly controlled research environment through a wide range of pressures that allow for consistent, reliable baroreflex slopes (Hart *et al.*, 2010). The one limitation of this technique is that nitroprusside is not exclusively a vasoactive substance (Hogan *et al.*, 1999; Kienbaum & Peters, 2004). In addition, the technique is relatively invasive and drug infusions can interfere with other tests performed after the Modified Oxford, therefore simpler approaches such as baroreflex threshold analysis (calculated during rest) have been examined as non-pharmacological assessments of baroreflex sensitivity during MSNA (Hart *et al.*, 2010). Spontaneous baroreflex sensitivity also correlates well with measures made during the Valsalva maneuver (Yang & Carter, 2013). Provided that a reasonable range of blood pressures are present during recording, all measures are valid assessments of the baroreflex.

Metabolic Function

Oral Glucose Tolerance Test (OGTT). An OGTT is a commonly used clinical tool to assess glucose tolerance and estimate insulin sensitivity. Selection of a 2-hr, 75 gram OGTT was based on its wide clinical utility, well-established thresholds for impaired fasting glucose and impaired glucose tolerance, and its inclusion as part of recommended screening for all obese women diagnosed with PCOS (Azziz *et al.*, 2006). The standard glucose dose (75 grams in a 10-oz beverage) and blood sampling points allow examination of blood glucose and insulin over time in order to build a curve for each over a two hour period. Optimal values include a fasting blood glucose <100mg/dL, and a 2-hr glucose under 140 mg/dL. Values that exceed this range indicate impaired fasting glucose

or impaired glucose uptake. In addition, as the slope of the early rise (0-30 min) in blood glucose and insulin are indicative of hepatic glucose sensitivity (suppression of endogenous glucose production), and the slope of decrease in glucose from 60 to 120 minutes are indicative of peripheral tissue (primarily skeletal muscle) glucose uptake, the area under the curve (AUC) for glucose and insulin provide insight into whole-body insulin sensitivity (Abdul-Ghani *et al.*, 2007).

Subjects arrived at the laboratory following an overnight fast (≥12hr) and having refrained from caffeine and alcohol for 12 hours, vitamin supplements and medications for 24 hours, and heavy exercise or heat exposure for >24 hours. In addition, diet was recorded using a 24-hour food recall to ensure similar macronutrient composition between tests for each subject. After 15 minutes of seated rest, a venous catheter was inserted into a vein in the antecubital space or hand and baseline samples were drawn into syringes, then placed into appropriate tubes. After baseline (fasted) sampling, a 75-g glucose drink was ingested in a 2-5 minute period, and blood samples were taken at 15, 30, 45, 60, 90, and 120 minutes for blood glucose, and insulin. The resulting glucose and insulin curves provide an indication of glucose tolerance and insulin sensitivity through measurement of the area under the curve for both glucose and insulin. In addition, commonly used fasting markers of insulin resistance were calculated as assessments of insulin resistance (Piché *et al.*, 2007). These included the homeostatic model assessment for insulin resistance (HOMA-IR=[glucose mg/dL*insulin mU/L]/405) and quantitative insulin sensitivity check index (QUICKI=1/[Log(glucose) +Log(insulin)]), both calculated based on values obtained after a 12-hr overnight fast.

Samples were placed in EDTA tubes for analysis of glucose using the glucose oxidase method (YSI 2300 Stat Plus, Yellow Springs, OH) and serum separator tubes (SST) for analysis of insulin. The EDTA tubes were immediately placed on ice and centrifuged, while the SSTs were allowed to clot at room temperature for 30 minutes prior to centrifugation. All samples were centrifuged at 1500 x *g* for 10 minutes at 4°C, and plasma or serum was aliquoted into cryovials and placed in -80°C freezer.

Alternative Techniques. While the 2-hr, 75g OGTT is a widely used clinical measure to assess glucose tolerance and screen for Type II diabetes, the "gold standard" assessment for insulin sensitivity in research is the Euglycemic-Hyperinsulinemic clamp technique. This technique was developed in 1979 by Defronzo and colleagues (DeFronzo *et al.*, 1979). The procedure involves infusing a standard insulin dose, and then measuring the glucose infusion rate required to maintain euglycemia in order to calculate whole-body glucose disposal rate as a measure of insulin sensitivity. While this technique is more commonly used in research rather than clinical settings, thresholds for insulin resistance have been established (Tam *et al.*, 2012). Correlation between results obtained with this technique and with indices obtained from an OGTT are good (Stumvoll *et al.*, 2000; Piché *et al.*, 2007), with the strongest correlations in those with some degree of insulin resistance (Piché *et al.*, 2007). The drawbacks of this assessment include the assumption of complete suppression of hepatic glucose production, a standard insulin infusion that may not be able to detect differences between extremely insulin-resistant populations, and the insulin infusion representing a non-physiological concentration for many populations (Muniyappa *et al.*, 2008). Additional considerations include time, cost, and highly specialized supervision requirements.

Adipose Tissue Biopsies. Adipose tissue inflammation and dysfunction are common in obesity (Hodson *et al.*, 2013), insulin resistance (Hotamisligil *et al.*, 2017), and PCOS (de Zegher *et al.*, 2009; Villa & Pratley, 2011; Echiburú *et al.*, 2018), and improvements in systemic glucose tolerance or insulin resistance in response to an intervention are often apparent in adipose tissue biopsies in human (Bruun *et al.*, 2006; Albers *et al.*, 2015*a*) and animal studies (Rogers *et al.*, 2015). Therefore, we examined subcutaneous adipose tissue biopsies before and after heat therapy or time control in order to examine tissue-specific mechansisms to support possible changes in whole-body measures of glucose tolerance, insulin sensitivity, and inflammation. The selection of subcutaneous fat sampling from the umbilical region was driven by ease of sampling and comfort of participants. The abdominal region was selected over the gluteal region due to greater central obesity in PCOS. While visceral fat appears to have a stronger relationship with metabolic dysfunction (Wajchenberg *et al.*, 2002) and insulin resistance (Preis *et al.*, 2010) in obesity, obese women with PCOS exhibit dysfunction in both visceral and subcutaneous depots in the abdominal region (Echiburú *et al.*, 2018). Further, due to the location of visceral fat depots, sampling is only performed in humans undergoing abdominal surgery (Camastra *et al.*, 2017), such as gastric bypass or gall bladder removal.

Subcutaneous white adipose tissue samples were obtained after a 4-hour fast from the peri-umbilical area. The sampling area was cleaned with antiseptic (Chloraprep) and a local anesthesia was induced by intracutaenous and subcutaneous injection of 5ml of 1% lidocaine near the site of incision in the side of the umbilicus. Following local anesthesia, a 3mm wide skin incision was made with a sterile scalpel at the edge of the umbilicus,

and 15 cm long, 16 gauge infiltration cannula (Millenium Surgical, Narbeth, PA) was advanced through the incision. This cannula was connected to a 60-ml syringe containing a mixture of 50 ml of 0.9% sterile saline and 7.5mL of 1% lidocaine. This fluid was injected in the subcutaneous fat in a fan-like fashion from the incision site, covering a total area of ~5x5cm. Next, an 11 gauge aspiration cannula (Millenium Surgical, Narbeth, PA) connected to a 20mL sterile syringe was advanced in the same fan-like fashion while gentle suction was applied. A total sample of 10-12mL of fluid and fat was taken, resulting in an adipose tissue sample of 4-7mL. This sample was collected and washed three times in sterile saline before a portion of whole adipose tissue was snap-frozen in liquid nitrogen for later analysis of HSPs and inflammatory markers. The remaining sample was placed in phosphate-buffered saline (PBS) for adipocyte isolation.

Adipocytes were isolated by digesting tissue with collagenase (1mg/mL in KRH buffer) at 37°C. When tissue chunks were no longer visible, the sample was passed through a 250um Pierce tissue strainer (Thermofisher Scientific, Rockford, IL) and washed with an equal volume of wash buffer (KRH buffer with 10uL adenosine and 2% BSA) before centrifugation at 500 x *g* for 5 minutes. The wash buffer and centrifugation process was repeated, then 3 x 100uL cell samples were each placed in wash buffer for a two hour serum-starve incubation at 37°C.

After incubation, one sample was left untreated and insulin (Humulin-R; Lilly Pharmaceuticals) was added to the second and third samples in physiological (1.2ng/mL) and supra-physiological (12ng/mL) concentrations. These samples were placed in a 37°C incubator for five minutes before adipocytes were extracted and snap-frozen in liquid

nitrogen for later analysis of insulin signaling by measuring phosphorylated AKT (p-AKT).

Whole adipose tissue samples were thawed and combined with a cocktail of 10X RIPA (AbCam, Cambridge, MA) and protease inhibitors in a 1:1 sample:cocktail mixture before homogenization. Homogenized samples were spun at 1500 x *g* for 10 minutes, and protein extract was isolated from beneath the lipid layer of each sample. Samples were analyzed for Hsp27 (Santa Cruz Biotecnology, Santa Cruz, CA), Hsp70 (AbCam, Cambridge, MA), Hsp90 (AbCam, Cambridge, MA) and inflammatory markers JNK, p-JNK, and IKKβ (Santa Cruz Biotechnology, Santa Cruz, CA) using Western Blotting, with α-Vinculin (AbCam, Cambridge, MA) used as a loading control.

Isolated adipocytes were thawed and combined with a 1:1 volume of protease inhibitor cocktail for tissue homogenization. Homogenized samples were rotated at 4°C for one hour, then spun at 1200 x *g* for 15 minutes prior to protein subnatant extraction and quantification. Analysis of p-AKT was completed using Wes (ProteinSimple, San Jose, CA) as previously described (Harris, 2015).

The examination of specific HSPs and inflammatory markers was based primarily on previous literature linking these HSPs to inflammatory targets and metabolic dysfunction (Gupte *et al.*, 2009; Rogers *et al.*, 2015). Insulin signaling can be assessed by a variety of downstream targets, and functional outcomes such as GLUT-4 translocation or radiolabeled glucose uptake can also be performed. Phosphorylation of AKT was selected because it is a critical step in the insulin signaling process, and because the PI3K-Akt signaling pathway is known to be disrupted in women with PCOS (Li *et al.*, 2017).

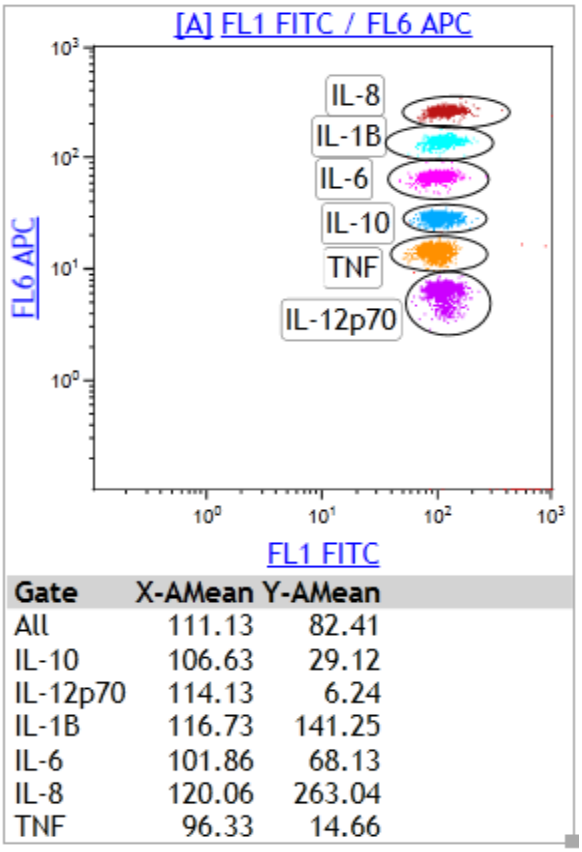
Blood analysis

On the morning of each OGTT, additional blood samples were collected for baseline analysis of hormonal changes and inflammatory markers. Plasma samples for cytokine analysis were collected into EDTA tubes and immediately placed on ice prior to centrifugation at 1500 g for 10 minutes at 4°C. Serum samples were collected into serum separator tubes and allowed to clot at room temperature prior to following the same centrifugation protocol.

Serum samples were frozen at -80°C for batch analysis of C-reactive protein (Enzo Life Sciences High-Sensitivity Human C-Reactive Protein), total testosterone (Enzo Life Sciences High-Sensitivity Testosterone), and Estradiol (Enzo Life Sciences High-Sensitivity 17β Estradiol) using commercial enzyme-linked immunosorbent assay (ELISA) kits. Samples were diluted 1:4 as suggested and run in duplicate, then calculated based on a atandard curve.

Plasma samples were frozen at -80°C for batch analysis of inflammatory cytokines using a commercial microbead array kit (BD Biosciences Cytometric Bead Array, Human Inflammation Panel). Cytometric bead array kits provide antibody or antigen-speccific binding sites on beads of varying sizes and/or flourescence, which allows for multiplexing assays with a single sample. This kit contained microbead specific to IL-1β, IL-6, IL-8, IL-10, IL-12p70, and TNF. Plasma samples were incubated overnight with these microbeads to facilitate binding, then read using flow cytometry (Beckman-Coulter Gallios). Flow cytometry separates these beads by size, and produces a scatter based on the flouresence emitted from bound microbeads. These scatters are

then compared to a standard curve of known concentrations of each cytokine, and values are calcuated using linear regression from the standard curve from 0 to 5,000pg/mL.



Gate	X-AMean	Y-AMean
All	111.13	82.41
IL-10	106.63	29.12
IL-12p70	114.13	6.24
IL-1B	116.73	141.25
IL-6	101.86	68.13
IL-8	120.06	263.04
TNF	96.33	14.66

Figure 13 (left). Sample cytokine array plot from flow cytometer. Cytokine beads are separated by size (Y axis) and the scatter along the X axis for each analyte is used to calculate concentration based on the standard curve.

In total, these tests of cardiometabolic health, sympathetic activity, and inflammation provide a detailed picture of cardiovascular, autonomic, and metabolic function, and provide insight into possible mechanisms through which heat therapy could improve cardiometabolic health in obese women with PCOS. The techniques and tests selected represent a balance between gold-standard measurement techniques and subject comfort and time commitment.

CHAPTER IV

HEAT THERAPY AND VASCULAR HEALTH IN OBESE WOMEN WITH PCOS

Heat exposure has been used for centuries in various populations for purported therapeutic benefit, including Scandinavian sauna use, Japanese Waon therapy, Turkish baths, and Native American sweat lodges. More recently, repeated passive heat exposure (termed 'heat therapy') has received renewed interest for improving cardiovascular risk profile in healthy populations (Brunt *et al.*, 2016*b*, 2016*c*) and those with overt cardiovascular disease (Imamura *et al.*, 2001; Kihara *et al.*, 2002; Thomas *et al.*, 2017). However, a large spectrum of cardiovascular risk exists between health and disease, and populations at an elevated risk of developing cardiovascular disease may have the greatest potential benefit of such an intervention.

Polycystic ovary syndrome (PCOS), an endocrine disorder characterized by menstrual dysfunction, clinical hyperandrogenism, and polycystic ovarian morphology (Azziz *et al.*, 2006), affects 6-15% of women of child-bearing age and is often accompanied by extremely high rates of obesity, insulin resistance, autonomic dysfunction (Lansdown & Rees, 2012; Li *et al.*, 2014; Ribiero *et al.*, 2016), and elevated markers of inflammation (Escobar-Morreale *et al.*, 2011; Shorakae *et al.*, 2015; Spritzer *et al.*, 2015). In combination, these factors substantially elevate cardiovascular risk in women with PCOS (Wild *et al.*, 2010). The cardiovascular risk profile includes impaired endothelial function (Sprung *et al.*, 2013), increased arterial stiffness (Soares *et al.*, 2009; Sasaki *et al.*, 2011), increased wall thickness (Lakhani *et al.*, 2004), and an overall increased mortality due to cardiovascular and cerebrovascular events (Rizzo *et al.*, 2009;

Wild *et al.*, 2010). Limited pharmaceutical therapies or lifestyle interventions for PCOS are specifically aimed at improving cardiovascular health in this population. While regular exercise training can improve elements of health including BMI, endocrine function, and insulin resistance (Tang *et al.*, 2006; Palomba *et al.*, 2008; Harrison *et al.*, 2011*a*) in obese women with PCOS, effects of exercise training on cardiovascular risk are less consistent (Harrison *et al.*, 2011*a*).

Repeated passive heat exposure ('heat therapy'), through sauna bathing or regular hot tub use, has shown promise in acute and chronic intervention studies as well as prospective cohort studies to improve cardiovascular health and reduce risk of mortality in various populations. Acute hot water immersion is associated with redistribution of blood flow due to cutaneous vasodilation, creating beneficial vascular shear patterns (Thomas *et al.*, 2016, 2017) similar to those experienced during exercise (Thomas *et al.*, 2016), which can promote endothelial cell streaming (Vanbavel, 2007) and reduce plaque deposition (Chappell *et al.*, 1998). Acute heat exposure, through hot tub or sauna use, has additionally been shown to reduce blood pressure (Boone *et al.*, 1999; Laukkanen *et al.*, 2017) and improve arterial compliance (Laukkanen *et al.*, 2017; Lee *et al.*, 2018). These acute cardiovascular adjustments and functional improvements, with repeated heat exposure, may drive the chronic changes observed in long-term heat therapy interventions in humans, including reduced arterial blood pressure, reduced wall thickness, decreased arterial stiffness, and enhanced endothelial function in the brachial artery (Brunt *et al.*, 2016*c*) and cutaneous microvasculature (Brunt *et al.*, 2016*b*).

While changes in vascular function with heat therapy collectively reduce cardiovascular risk profile, emerging evidence from large prospective studies indicates

that increased frequency and duration of heat (sauna) exposure reduces cardiovascular morbidity (risk of incident hypertension (Zaccardi *et al.*, 2017)) and mortality (Laukkanen *et al.*, 2015). Fatal cardiovascular events involve blockages to coronary (myocardial infarction) or cerebral vasculature (stroke), which lead to tissue ischemia/reperfusion (IR) injury. In murine models, 30-day heat acclimation affords protection from IR injury, such that cardiac myocytes are better able to survive IR stress (Maloyan *et al.*, 2005). In humans, acute hot tub use appears to temporarily protect tissue from IR stress (Brunt *et al.*, 2016*d*), but this effect has not been examined in a chronic heat intervention. This protection would be particularly powerful in obese women with PCOS, a population with increased risk of cardiovascular or cerebrovascular death.

Therefore, the purpose of this study was to examine the effect of a 30-session heat therapy intervention on arterial stiffness, arterial wall thickness, endothelial function, and vascular tolerance to ischemia-reperfusion in obese women with PCOS. Based on the results of Brunt et al. (Brunt *et al.*, 2016*c*), we hypothesized that a 30-session heat therapy intervention in obese women with PCOS would decrease arterial wall thickness and stiffness, increase endothelial function (assessed by flow-mediated dilation; FMD), and reduce the impairment in FMD following IR stress.

Methods

Subjects. Eighteen obese women (defined as a BMI ≥30 and ≤45) volunteered to participate in this study. All subjects were non-smokers and had not been diagnosed with overt cardiovascular disease or diabetes. All women had to be diagnosed with PCOS by a physician and based on the Rotterdam criteria (clinical hyperandrogenism, menstrual

dysfunction, and/or presence of ovarian cysts upon ultrasound examination) qualify for inclusion. Women were matched for age and BMI and placed in either the heat therapy intervention (HT) or time control (CON; no heat exposure) group. A summary of physical characteristics is listed in Table 2. Using the 2017 American Heart Association guidelines for hypertension, seven subjects (3 HT, 4 CON) were classified as "elevated blood pressure", and seven were classified as Stage 1 Hypertensive (4 HT, 3 CON). Two subjects in each group were classified as normotensive (systolic [SBP] <120 and diastolic [DBP] <80 mmHg).

Table 2. Summary of physical characteristics and baseline blood pressure in all vascular function subjects, heat therapy (HT) and control (CON).

	All Subjects (n=18)	HT (n=9)	CON (n=9)
Age (years)	27 ± 1	26 ± 2	27 ± 2
BMI	41.3 ± 1.1	41.8 ± 1.4	40.7 ± 1.9
Waist:Hip Ratio	0.85 ± 0.01	0.85 ± 0.01	0.86 ± 0.02
Blood Pressure (mmHg)			
SBP	123 ± 2	124 ± 2	123 ± 3
DBP	76 ± 1	77 ± 2	75 ± 2
MAP	92 ± 1	93 ± 1	91 ± 2

Heat Therapy Intervention. Heat therapy occurred over an eight to ten week period, with a total of 30 x one hour sessions scheduled three to four per week in all

subjects enrolled in the HT group (control subjects were not exposed to heat, but completed all testing at matched timepoints [Pre, Mid, Post]). This exposure duration and timeline was selected based on previous work in our lab, where promising improvements in vascular health were seen over an 8-week, 36 session heat therapy intervention (Brunt *et al.*, 2016*c*). The acute exposure duration and total number of sessions were reduced slightly from our previous heat therapy work in order to create an intervention more similar in time commitment to exercise training protocols performed in women with PCOS (Harrison *et al.*, 2011*a*). Passive hot water immersion was selected as the method of heat stress because it is capable of increasing core temperature at a rate similar to moderate-intensity exercise (Kenny *et al.*, 1996), while also producing high skin temperature and sweating rate, all requisite components for adaptation to heat (Fox *et al.*, 1963; Buono *et al.*, 2009).

Subjects reported to the laboratory to undergo passive heating, which entailed 60 min of water immersion in a bath set to 40.5°C. Previous research in our laboratory suggests this water temperature will raise core temperature ≥ 38.5°C within 20-30 minutes. This threshold was selected based on human heat acclimation literature using isothermic models (Fox *et al.*, 1963), and is additionally important as a threshold for induction of heat shock proteins (Taylor, 2014). Once core temperature rose to 38.5°C, subjects sat upright (immersed to the waist) for the remainder of the one hour session in order to maintain core temperature between 38.5-39.0°C. If temperature dropped below 38.5, subjects were asked to submerge again. After 60 minutes of exposure, subjects were asked to sit next to the tub until core temperature fell below 38.5˚C (10-15 minutes) for

safety monitoring, and for monitoring the total exposure duration where core temperature was above 38.5°C.

Upon arrival, subjects provided a urine sample for measurement of specific gravity (USG) to confirm euhydration (USG ≤ 1.02) prior to heat exposure. Subjects were also weighed pre- and post-heat exposure (nude, towel-dried, behind a privacy screen) and given water to drink ad libitum during heat exposure and post-heat exposure if necessary to match fluid losses. Heart rate was monitored throughout heating using commercially-available heart rate monitors (Polar Electro, New York, NY), while rectal temperature (Tre) was monitored throughout passive heating by rectal thermistor (YSI, Yellow Springs, OH).

Vascular Function. Vascular function study days took place at the start of testing (Pre), after 4-5 weeks (Mid; 15 heat therapy sessions or matched time control), and at the conclusion of heat therapy (Post; 8-10 weeks, 30 sessions, or equivalent time control), 24-72 hours after the most recent heat exposure in HT subjects. All subjects reported to a thermoneutral lab environment, having refrained from food for a minimum of four hours, caffeine and alcohol for 12 hours, vitamin supplementation, medications (other than oral contraceptive) and exercise for at least 24 hours. Time of day was held constant (within 1 hr) for each subject over time in order to minimize circadian influence. Body mass and height were measured, and a urine sample was collected for confirmation that the subject was not pregnant prior to beginning testing. Subjects rested on a padded exam table for a minimum of 20 minutes prior to beginning testing, and during this time they were instrumented with a 3-lead ECG, brachial blood pressure cuff, and beat-by-beat blood

pressure monitor (Nexfin, Edwards Life Sciences, Irvine, CA). Testing order was held constant, beginning with assessment of common carotid wall thickness and dynamic arterial compliance (DAC), followed by superficial femoral wall thickness and DAC, Carotid-Femoral and Brachial-Ankle pulse wave velocity, and flow-mediated dilation (FMD) before and after ischemia-reperfusion.

Wall thickness. Wall thickness of the common carotid and superficial femoral artery were imaged using high-resolution Doppler ultrasound (Terason t3000cv; Teratech, Burlington, MA, USA) in B mode with 10.0 MHz linear array ultrasound transducer probe artery. The carotid artery was imaged 2 cm distal to the carotid bulb at three angles: anterior, lateral and posterior. The superficial femoral artery was imaged 2–4 cm distal to the femoral bifurcation in two planes: anterior and lateral. Clearly distinguished intimal–medial boundaries were obtained on the far wall. Images were frozen in diastole and enlarged, and calipers were used to make three repeat measurements of the wall thickness from the lumen–intima interface to the media–adventitia interface. Video recording of these measurements was later reviewed offline to confirm accuracy of caliper measurement, and three measurements from each angle were averaged.

Arterial Stiffness. Arterial stiffness was assessed using several different methods, including carotid and superficial femoral dynamic arterial compliance (DAC), and carotid-femoral and brachial-ankle pulse wave velocity (PWV). Carotid and femoral DAC were measured using high-resolution Doppler ultrasound (Terason t3000cv; Teratech, Burlington, MA, USA) with 10.0 MHz linear array ultrasound transducer probe with concurrent applanation tonometry (PCU-2000; Millar, Inc., Houston, TX, USA) on

the same artery on the contralateral side of the body. Ultrasound probe placement on the body, including angle of approach and internal anchors such as distance from bifurcation, were recorded on the first trial day and repeated to ensure consistency between repeated measurements over the course of the study. All ultrasound recordings were performed on the right side of the body. Ultrasound images were recorded at 20 Hz using video recording software (Camtasia), then analyzed for diameter and blood velocity using custom-designed edge detection and wall tracking software (DICOM; Perth, Australia). Pulse pressure using applanation tonometry was simultaneously recoded via WinDaq data acquisition (Dataq, Inc) at 250 Hz, and analyzed using the trough to peak pressure differential. This pressure differential (ΔP) was then analyzed relative to change in diameter (ΔD) for a minimum of 50 cardiac cycles in order to calculate cross-sectional compliance and β stiffness using the following equations:

$$\textit{Dynamic arterial compliance} = [(\Delta D/D)/2\Delta P] * \pi D^2$$

$$\textit{B-stiffness index} = Ln\ (SBP/DBP) * D/\Delta D$$

Pulse wave velocity was assessed using tonometry probes placed on the carotid & femoral arteries (central PWV or aortic stiffness), as well as the brachial and dorsal pedal arteries (peripheral PWV). Tracings were recorded using data acquisition software (WinDaq; Dataq Instruments, Akron, OH, USA), and the pulse upswings of a minimum of thirty simultaneously recorded pressure tracings were identified offline by a blinded investigator in order to calculate the time differential. Velocity was calculated as distance over time, where distance was the sum of the linear distances between the carotid probe and the sternal notch and the sternal notch to the femoral probe, or the distance

differential between the brachial probe to sternal notch and the ankle probe to sternal notch.

Flow-mediated Dilation with Ischemia/Reperfusion. Endothelial function was assessed using flow-mediated dilation (FMD) at the brachial artery using Doppler ultrasonography. On each study day, FMD was measured at baseline and again after a 20 minute occlusion-20 minute reperfusion as a model of endothelial function in response to an acute IR episode. FMD consists of imaging of the brachial artery by Doppler ultrasound to obtain baseline diameter and blood velocity measurement, then inflating an occlusion cuff (set to 250 mmHg) just below the elbow for a period of 5 minutes. Velocity and diameter were captured using Doppler ultrasound, and the ultrasound images were recorded (Camtasia software) and analyzed for changes in brachial artery diameter and blood velocity after cuff release. FMD at the brachial artery has been shown to parallel coronary artery endothelial function (Teragawa *et al.*, 2005), is a well-established predictor of cardiovascular risk and future cardiovascular events (Yeboah *et al.*, 2008; Shechter *et al.*, 2009), and has improved with heat therapy in healthy humans (Brunt *et al.*, 2016*c*). In addition, FMD is impaired by IR (Seeger *et al.*, 2015; Brunt *et al.*, 2016*d*). IR was performed by placing an occlusion cuff on the upper arm (above the point where the brachial artery was imaged for FMD) and inflating it to 250mmHg for a period of 20 minutes. Twenty minutes after release of the cuff, FMD was re-assessed. This protocol was selected as it has been used in acute exercise (Seeger *et al.*, 2015) and heat (Brunt *et al.*, 2016*d*) interventions, has been well-tolerated by subjects, and shows a short-term impairment of endothelial and microvascular function.

Fasting Blood Lipids and C-Reactive Protein. On a separate day at each timepoint (Pre-Mid-Post), subjects reported to the lab after a 12-hr overnight fast, having refrained from medications, exercise, and heat exposure for ≥24 hours, for a venous blood draw. Blood was drawn from the antecubital space into serum separator tubes, then allowed to clot at room temperature for 30 minutes prior to centrifugation (10 minutes at 1500 x *g* at 4°C). Serum was frozen at -80°C and later thawed for analysis of C-reactive protein (Enzo Life Sciences) and cholesterol panels (OHSU lipid lab, Portland, OR).

Statistics. All data are presented as mean ± SEM. Results were analyzed using mixed-model ANOVA in GraphPad Prism 6, with repeated measures within HT or CON groups for each subject, and non-repeated measures comparison between groups of subjects. If a significant main effect was observed, Holm-Sidak post-hoc analysis was utilized to examine within- or between-group effects.

Results

Nine HT subjects completed the heat intervention, and eight subjects completed the time control (one CON subject withdrew after completing Pre testing, and was therefore excluded from analysis). All experimental subject completed 30 heat therapy sessions over 8-10 weeks. Sessions were scheduled at the subject's convenience, with the first (session 1) and last session (session 30) scheduled at the same time of day in order to examine variables related to heat adaptation that are sensitive to circadian influence. A significant reduction in baseline heart rate and core temperature (measured seated in thermoneutral room, prior to hot water immersion) and increase in sweating rate during heating were evident in the final session (Table 3).

Table 3. Baseline heart rate, core temperature, and sweating rate in the first (session 1) and last (session 30) heat therapy day.

	Session 1	Session 30	P value
Baseline Heart Rate (BPM)	113 ± 2	98 ± 3	0.003
Baseline Tre (°C)	37.6 ± 0.1	37.2 ± 0.1	0.0003
Sweating Rate (L/hr)	0.71 ± 0.06	1.21 ± 0.06	<0.0001

Wall Thickness. As seen in figure 14, both common carotid and superficial femoral wall thickness decreased, with femoral artery wall thickness decreasing by the mid timepoint, and carotid decreasing at the end of 8-10 weeks (30 heat sessions; Post).

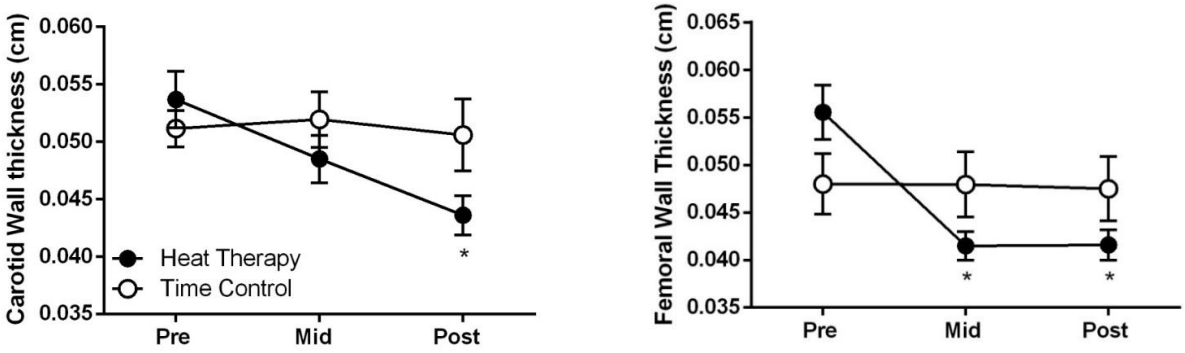


Figure 14. Common carotid and superficial femoral wall thickness over time in heat therapy (HT) and control (CON) subjects. * Denotes significant different from Pre timepoint.

Arterial Stiffness. Cross-sectional compliance and β-stiffness for the common carotid and superficial femoral artery are shown in Figure 15. Beta stiffness did not change in either the carotid or femoral artery; however; a decrease in carotid compliance occurred at the end of HT in experimental subjects. Brachial-ankle PWV decreased at the Post timepoint in experimental subjects, while carotid-femoral PWV did not change (Table 4). Systolic, diastolic, and mean arterial pressure decreased in experimental subjects throughout the 8 weeks, with an average decrease of 10mmHg (Table 4).

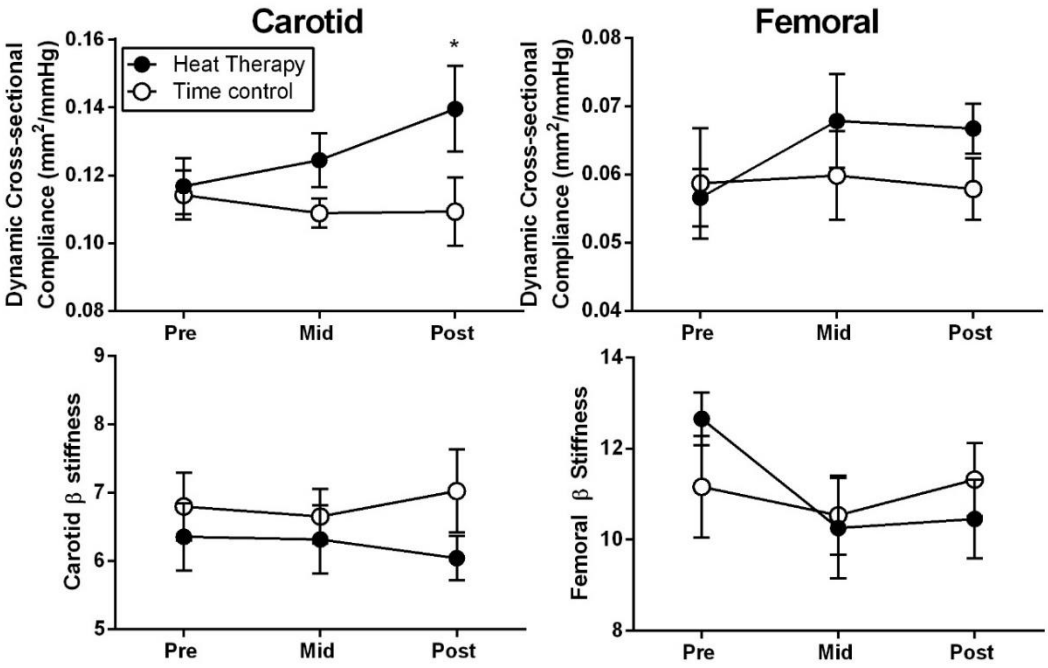


Figure 15. Common carotid and superficial femoral DAC and β stiffness index over time in heat therapy (HT) and control (CON) subjects. * Denotes significant different from Pre timepoint.

Blood lipids and C-reactive protein. C-reactive protein levels were classified as 'moderate risk' in both groups, and significantly decreased in HT subjects in Mid ($p=0.033$) and Post testing ($p=0.038$), with no change in CON over time (Table 4).

Table 4. BMI, C-reactive protein, blood pressure, PWV, and brachial diameter over time in heat therapy (HT) and control (CON) subjects. * Denotes significantly different from Pre timepoint.

		Pre	Mid	Post
BMI	HT	41.8 ± 1.4	41.9 ± 1.4	41.8 ± 1.5
(kg/m^2)	CON	39.9 ± 1.9	39.8 ± 1.8	39.5 ± 1.8
C-Reactive Protein	HT	2.03 ± 0.49	1.55 ± 0.35*	1.63 ± 0.45*
(mg/L)	CON	1.92 ± 0.53	2.16 ± 0.67	2.13 ± 0.67
SBP	HT	124 ± 2	119 ± 2	114 ± 2*
(mmHg)	CON	122 ± 3	124 ± 2	120 ± 2
DBP	HT	77 ± 2	69 ± 4*	68 ± 1*
(mmHg)	CON	74 ± 2	73 ± 2	75 ± 2
MAP	HT	93 ± 1	86 ± 3*	83 ± 1*
(mmHg)	CON	90 ± 2	90 ± 2	90 ± 2
Carotid-Femoral PWV	HT	698 ± 23	711 ± 43	689 ± 15
(cm/sec)	CON	697± 59	690 ± 42	680 ± 38
Brachial-Ankle PWV	HT	870 ± 27	844 ± 42	798 ± 24*
(cm/sec)	CON	869 ± 43	841 ± 54	845 ± 41
Brachial Artery	HT	0.315 ± 0.025	0.326 ± 0.024*	0.337 ± 0.022*
Diameter (cm)	CON	0.302 ± 0.029	0.304 ± 0.030	0.306 ± 0.026

Pre-IR FMD. FMD, expressed as a percent change in brachial artery diameter, did not change in HT or CON subjects over time (Figure 16). However, HT subjects experienced a significant increase in baseline diameter at Mid and Post (Table 4), so that when FMD was corrected for shear rate, an increase in FMD was seen at the Mid and Post timepoint. No changes in FMD were seen in CON subjects in absolute % dilation or shear-corrected FMD.

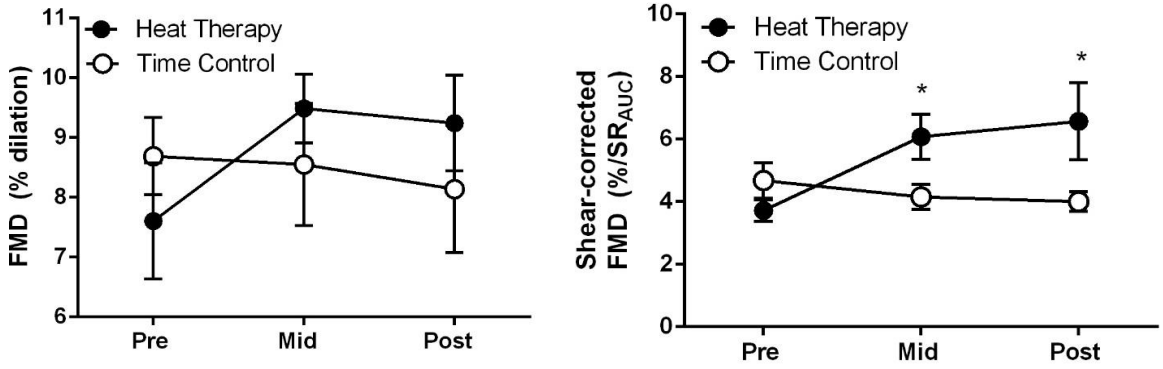


Figure 16. FMD (%) and shear-corrected FMD over time in heat therapy and control subjects.

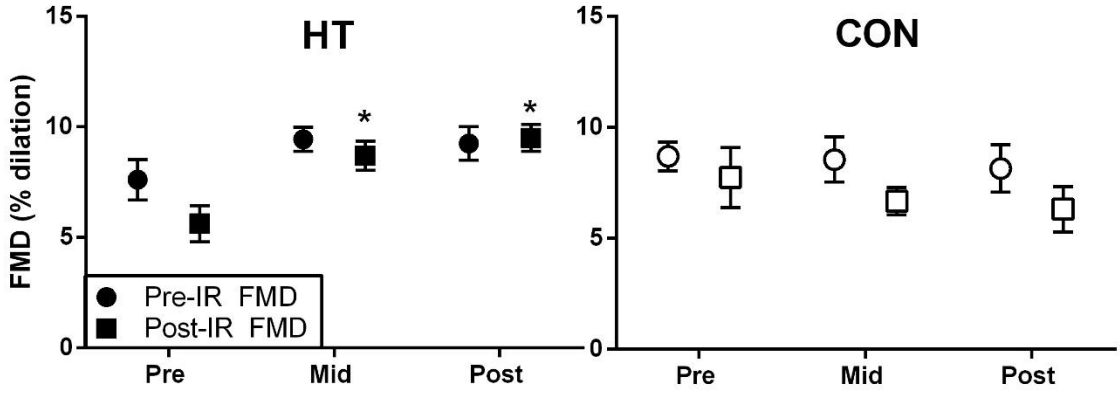


Figure 17. FMD before (Pre-IR) and after (Post-IR) IR in heat therapy (HT) and control (CON) subjects. * Denotes significant difference from Post-IR at Pre timepoint.

Post-IR FMD. FMD was significantly decreased, albeit variably, by IR at the Pre timepoint in both groups (Figure 17). However, in HT subjects, post-IR FMD significantly increased at the Mid and Post timepoints so that FMD was preserved (no impairment from IR) in Post testing.

Discussion

The cardiovascular risk profile of obese women with PCOS has been well-described; however; relatively few pharmaceutical or lifestyle interventions for women with PCOS address cardiovascular risk reduction. Based on our results, heat therapy appears to be a promising intervention to reduce cardiovascular risk in this population. Specifically, our primary findings indicate that a 30-session heat therapy intervention: 1) promoted reductions in carotid and femoral wall thickness 2) reduced serum C-reactive protein 3) increased carotid compliance and BA-PWV 3) improved endothelial function as assessed by FMD and 4) protected endothelial function from IR-related impairments in obese women with PCOS. As described in previous work (Brunt *et al.*, 2016*c*) and recent reviews (Ely *et al.*, 2018), there are multiple possible mechanisms for the observed improvements in vascular function, including hemodynamic adjustments during acute heat, increased abundance of heat shock proteins, and potential neurovascular changes.

PCOS, especially when accompanied by obesity, is associated with a meta-inflammatory state (Shorakae *et al.*, 2015) and dyslipidemia (Diamanti-Kandarakis *et al.*, 2007). In combination, these factors can lead to accelerated plaque deposition on arterial walls, and increased wall thickness observed in obesity and PCOS (Meyer *et al.*, 2012;

Allameh *et al.*, 2013). In PCOS, hyperinsulinemia, elevated blood pressure, and high serum testosterone can additionally influence plaque deposition and arterial wall thickening. Exercise training protocols produce similar changes in carotid wall thickness in obese women with PCOS (Orio *et al.*, 2016), and the magnitude of change in carotid wall thickness is on par with what has been observed in similar heat therapy interventions in healthy, sedentary humans (Brunt *et al.*, 2016*c*). However, the large and clinically relevant change in superficial femoral wall thickness has not been observed in healthy humans undergoing heat therapy (Brunt *et al.*, 2016*c*), and has not been assessed in obese women with PCOS following exercise training. Since arterial thickening can be related to inflammation (Chang *et al.*, 2017), oxidative stress, and blood pressure (Oren *et al.*, 2003), it is possible that the higher level of baseline inflammation and dysfunction in obese women with PCOS, combined with a dramatic decrease in blood pressure, allowed for a larger effect of heat therapy to be observed compared to healthy, inactive humans (Brunt *et al.*, 2016*c*). It is also possible that healthy humans have a 'basement effect' on some cardiovascular parameters, making further improvement in already healthy blood vessels difficult to achieve. In support of this, superficial femoral wall thickness measures at the end of heat therapy were still higher in obese women with PCOS than those observed at the start of heat therapy in healthy individuals (Brunt *et al.*, 2016*c*).

Arterial wall thickening and plaque deposition is a process driven by hyperlipidemia, inflammation, and oxidative stress. C-reactive protein is released by the liver in response to pro-inflammatory cytokines and adipokines (Yudkin *et al.*, 1999), and is considered a global inflammatory marker that is highly associated with various markers of cardiovascular health (Wilson *et al.*, 2008). C-reactive protein levels are stratified as

<1 mg/L (low risk), 1-3 mg/L (average risk), and >3mg/L (high risk) (Ridker *et al.*, 2002). Subjects in this study displayed serum C-reactive protein in the moderate range, which was expected based on previous work in obese women with PCOS (Escobar-Morreale *et al.*, 2011; Spritzer *et al.*, 2015). C-reactive protein has not previously been examined in heat therapy interventions; however; frequency of sauna use was inversely associated with C-reactive protein in a prospective cohort study in men (Laukkanen & Laukkanen, 2018). In addition, animal models have observed reductions in inflammatory cytokines including IL-6 and TNFα in response to heat (Kim *et al.*, 2005), and C-reactive protein production is influenced by changes in systemic inflammation, so a change in circulating pro-inflammatory cytokines in response to heat could explain the HT-induced reduction in C-reactive protein in obese women with PCOS. The magnitude of improvement we observed in HT was not large enough to alter risk category; however; it indicates that heat therapy is reducing systemic inflammation, which likely contributes to observed reductions in wall thickness, arterial stiffness, and enhanced endothelial function.

In healthy humans, chronic heat therapy reduced measures of arterial stiffness, including superficial femoral compliance and β-stiffness, and aortic (carotid-femoral) pulse wave velocity (Brunt *et al.*, 2016*c*). In obese women with PCOS, heat therapy increased common carotid compliance and reduced brachial-ankle PWV, with no significant impact on femoral compliance, β-stiffness, or aortic PWV. As the β-stiffness index is less influenced by blood pressure changes than cross sectional compliance measures, it is likely that a decrease in blood pressure, rather than a change in structural properties of the artery, explained the increase in common carotid artery compliance.

Similarly, due to the inclusion of resistance vessels, brachial-ankle PWV is more sensitive to changes in autonomic influence and vascular tone than carotid-femoral PWV, so these results may again indicate an overall decrease in blood pressure and baseline vascular tone rather than structural arterial remodeling. It is worth noting that BMI and waist circumference (central obesity) are both associated with increased aortic stiffness (Safar *et al.*, 2006; Ketel *et al.*, 2010; Strasser *et al.*, 2015; van den Munckhof *et al.*, 2017), and the high BMI and central obesity that persisted through the HT intervention could be driving central arterial stiffness in this subject population. It is also possible that 30 1-hr heat therapy sessions over 8-10 weeks are not sufficient to create significant arterial remodeling in a population with multiple contributing risk factors (obesity, hyperinsulinemia, androgen excess, elevated blood pressure). It is also possible that with the large variation in magnitude of response and the spectrum of baseline dysfunction in obese women with PCOS did not allow for consistent, significant, and clinically meaningful changes in arterial stiffness in this subject population and sample size.

Clinically meaningful changes in endothelial function were present by the mid-point of HT in experimental subjects, and further increased by the end of heat therapy. FMD is a well-described measure of endothelial function (Atkinson *et al.*, 2013) and strong clinical correlate of cardiovascular risk (Yeboah *et al.*, 2008; Shechter *et al.*, 2009), with a 2% improvement in FMD representing a 15% improvement in cardiovascular risk (Shechter *et al.*, 2009). The ~3% change in shear-corrected FMD over the heat therapy intervention is smaller than observed in healthy humans undergoing a similar protocol (Brunt *et al.*, 2016*c*), but more robust than the change in FMD observed in overweight women with PCOS following 10-26 weeks of exercise training

(Almenning *et al.*, 2015; Orio *et al.*, 2016). While FMD is not exclusively dependent upon nitric oxide (Wray *et al.*, 2013), heat therapy-mediated increases in NO bioavailability as observed over the course of heat therapy in previous work (Brunt *et al.*, 2016*b*) are likely predominantly responsible for increases in FMD in obese women with PCOS over HT.

The effect of heat therapy on FMD after IR stress was even more pronounced than at baseline (Pre-IR), approaching a 4% increase, and, more importantly, heat therapy preserved FMD following IR stress by the end of 8-10 weeks. While this experimental model may be difficult to translate to true IR tissue injury, these data support the effect observed in isolated rat hearts following a 30 day heat acclimation protocol (Maloyan *et al.*, 2005), and offer a potential explanation for the reduction in fatal cardiovascular events seen with increased sauna use in men (Laukkanen *et al.*, 2015). It is possible that this protection is related to increased abundance of cytoprotective heat shock proteins, as seen in isolated rat heart models (Maloyan *et al.*, 2005; Horowitz & Assadi, 2010). It is also possible, given that IR injury is primarily due to high oxidative stress during reperfusion (Kaminski *et al.*, 2002), that heat therapy confers protection from oxidative stress as seen in human exercise-heat acclimation (Kaldur *et al.*, 2014).

In summary, a 30-session heat therapy intervention leads to robust improvements in cardiovascular risk in obese women with PCOS. The reductions in blood pressure, wall thickness, arterial stiffness, and improved endothelial function are similar to or greater than those seen with exercise training and/or diet interventions in women with PCOS. Moreover, these changes occurred without any change in BMI (Table 4), indicating that heat therapy used in combination with diet or exercise interventions that lead to weight

loss may provide an additive benefit through mechanisms unrelated to changes in body mass. These data support previous work examining heat therapy and cardiovascular health (Imamura *et al.*, 2001; Brunt *et al.*, 2016*c*), and additionally indicate that heat therapy can reduce inflammation and provide protection from IR injury in populations with an elevated cardiovascular morbidity and mortality. In combination, the magnitude and breadth of changes observed in this study dramatically reduce the cardiovascular risk profile in obese women with PCOS.

CHAPTER V

HEAT THERAPY AND AUTONOMIC ACTIVITY IN OBESE WOMEN WITH PCOS

Polycystic ovary syndrome (PCOS) is a complex endocrine disorder characterized by androgen excess, menstrual dysfunction, and the appearance of cysts on the ovaries. While this syndrome affects 5-18% of women of child-bearing age and has various associated health risks including obesity, insulin resistance, and hypertension (Luque-Ramírez & Escobar-Morreale, 2014), the pathophysiology of PCOS is still poorly understood and potential treatments to ameliorate the health risk profile in these women deserve further study. One particular target requiring further study is the sympathetic nervous system, both as a potential driver of ovarian dysfunction (Aguado, 2002) and associated cardiometabolic dysfunction (Di Domenico *et al.*, 2013) in PCOS, and as a potential therapeutic target (Lansdown & Rees, 2012). Women with PCOS have elevated muscle sympathetic nerve activity (MSNA) (Sverrisdottir *et al.*, 2008; Li *et al.*, 2014). Similarly, measurement of heart rate variability (HRV) as an estimate of cardiac parasympathetic and sympathetic influence suggests elevated sympathetic tone and low parasympathetic tone in PCOS (De Sá *et al.*, 2011; Di Domenico *et al.*, 2013; Hashim *et al.*, 2015; Ribiero *et al.*, 2016). Limited research has examined treatments targeting the sympathetic nervous system in PCOS, with pharmacological treatment using Moxonidine, a central inhibitor of sympathetic outflow acting in the rostral ventrolateral medulla showing no effect on MSNA, HRV, or endothelial function in PCOS women (Shorakae *et al.*, 2017). The only effective interventions for reducing MSNA in PCOS have been exercise training and electroacupuncture (Stener-Victorin *et al.*, 2009), both of

which reduced MSNA but, somewhat surprisingly, did not contribute to a healthier cardiovascular risk profile. Chronic passive heat exposure (termed 'heat therapy') offers promise as a novel therapy to alter autonomic outflow, potentially reducing MSNA and increasing HRV in women with PCOS.

To date, research on heat therapy and autonomic function is extremely limited in humans. The only evidence in humans on heat adaptation influencing resting MSNA is inferred from seasonal variation studies, with an observed decrease in MSNA in summer compared with winter potentially attributed by the authors to warmer environmental temperature (Niimi *et al.*, 1999; Cui *et al.*, 2015). In rodent models, passive heat acclimation was associated with increased parasympathic and decreased sympathetic influence on autorhythmic cells, as measured by infusion of atropine and propranolol (Horowitz & Meiri, 1993), although sympathetic nerve activity was not directly assessed. Sympathetic nerve activity in obesity and PCOS is influenced by a multitude of factors, including hyperinsulinemia, inflammatory cytokines, adipokines, hormonal factors, and other bioactive molecules such as nitric oxide. Nitric oxide (NO) production or bioavailability in cutaneous microcirculation increases in human heat therapy (Brunt *et al.*, 2016*b*), and if heat therapy results in similar increases in neuronal NO release or bioavailability in the central nervous system, this would lead to a reduction in central sympathetic outflow. While there is some evidence of altered insulin sensitivity (Gupte *et al.*, 2009), inflammatory cytokine production (Kim *et al.*, 2005), and adipokine secretion in animal models of heat exposure (Morera *et al.*, 2012), most other mediators of sympathetic outflow have not been examined in human heat therapy models.

Heat acclimation studies in humans have examined plasma (Nielsen *et al.*, 1993, 1997; Febbraio *et al.*, 1994) or urinary (Maher *et al.*, 1972) norepinephrine concentrations immediately after acute exercise-heat stress and noted a decline following an exercise-heat acclimation protocol, indicating reduced sympathetic activity during acute exercise-heat exposure as a result of heat acclimation. However; this response may be specific to the stress (acute exercise) and the acclimation model (exercise-heat exposure) employed. Similarly, heart rate variability has been assessed following exercise-heat acclimation in healthy individuals (Frank *et al.*, 2001; Epstein *et al.*, 2010; Flouris *et al.*, 2014) but again measurements were only made in the heat and/or during exercise, and the studies achieved disparate results, with two reporting increased variability (increased parasympathetic activity) (Epstein *et al.*, 2010; Flouris *et al.*, 2014) and one reporting an increase in sympathetic tone (Frank *et al.*, 2001) following heat acclimation. No study to date, in the human or animal literature, has examined resting muscle sympathetic nerve activity after a long-term heat intervention. This is particularly important to examine in a clinical population with high sympathetic activity, such as obese women with PCOS.

Therefore, the purpose of this study was to examine muscle sympathetic nerve activity in obese women with PCOS over the course of 30 session, 8-10 week heat therapy intervention, and to examine blood parameters including serum insulin, testosterone, inflammatory cytokines, and adipokines that might contribute to changes in MSNA. A secondary purpose was to examine heart rate variability over the same time as a supplemental index of autonomic function. We hypothesized that baseline MSNA would decline following the chronic heat intervention, and that indices of heart rate

variability would increase, suggesting a decrease in sympathetic and increase in parasympathetic tone.

Methods

Eighteen obese women (body mass index; BMI 30-45) were enrolled in this study. All subjects completed a health screening visit prior to enrollment to ensure they met all inclusion criteria. All subjects were diagnosed with Polycystic Ovary Syndrome by their primary care physician and met the Rotterdam Criteria (menstrual dysfunction, hyperandrogenism, and/or ovarian cysts)(Rotterdam ESHRE/ASRM-Sponsored PCOS consensus workshop group, 2004). Subjects were non-smokers and did not have any overt cardiovascular disease or diabetes. These women were matched for age and BMI, and assigned to either heat therapy (HT) or time control (CON) groups. The goal was to recruit subjects who were not taking any medications; however; the extremely high prescription rates in PCOS made this difficult. In total, one subject in each group was taking oral contraceptives (OC), and two subjects in each group were taking selective serotonin reuptake inhibitors (SSRI) for treatment of depression or anxiety, both common in PCOS (Deeks *et al.*, 2010). While these medications can potentially influence autonomic outflow, medication rates were matched between groups, and subjects were taking the medication at a consistent dose and time of day throughout the study. Demographic data for all subjects are presented in Table 5.

Table 5. A summary of demographic characteristics, including age, BMI, blood pressure, and medications in HT and CON subjects.

	All Subjects (n=18)	HT (n=9)	CON (n=9)
Age	27 ± 1	26 ± 2	27 ± 2
BMI	41.3 ± 1.1	41.8 ± 1.4	40.7 ± 1.9
Waist:Hip Ratio	0.85 ± 0.01	0.85 ± 0.01	0.86 ± 0.02
Blood Pressure			
SBP	123 ± 2	124 ± 2	123 ± 3
DBP	76 ± 1	77 ± 2	75 ± 2
MAP	92 ± 1	93 ± 1	91 ± 2
Medications			
	N=2 OCs	N=1 OC	N=1 OC
	N=4 SSRI	N=2 SSRI	N=2 SSRI

Heat Therapy Intervention. Heat therapy occurred over an eight to ten week period, with a total of 30 x one hour sessions scheduled three to four per week in all subjects enrolled in the experimental group (control subjects were not exposed to heat). The acute exposure duration and total number of sessions was selected because it is similar in time commitment to exercise training protocols performed in women with PCOS (Harrison *et al.*, 2011*a*). For each session, subjects reported to the laboratory to undergo 60 min of water immersion in a bath set to 40.5°C. Previous research in our laboratory suggests this temperature is optimal to raise core temperature ≥ 38.5°C within 20-30 minutes. Once core temperature rose to 38.5°C, subjects sat upright (immersed to

the waist) for the remainder of the one hour session in order to maintain core temperature between 38.5-39.0°C. If temperature dropped below 38.5, subjects were asked to submerge again. After 60 minutes of exposure, subjects were asked to sit next to the tub until core temperature fell below 38.5˚C (10-15 minutes) for safety monitoring, and for monitoring the total exposure duration where core temperature was above 38.5°C. This threshold was selected based on human heat acclimation literature using isothermic models (Fox *et al.*, 1963), and is additionally important as a threshold for induction of heat shock proteins (Taylor, 2014), which may contribute to changes in sympathetic activity through their role in enhancing NO signaling (Pritchard *et al.*, 2001; Bharati *et al.*, 2017), reducing inflammatory cytokines (Dokladny *et al.*, 2010), and increasing insulin sensitivity (Gupte *et al.*, 2009).

Upon arrival, subjects provided a urine sample for measurement of specific gravity (USG) to confirm euhydration (USG ≤ 1.02) prior to heat exposure. Subjects were also weighed pre- and post-heat exposure (nude, towel-dried, behind a privacy screen) and given water to drink ad libitum during heat exposure and post-heat exposure if necessary to match fluid losses. Heart rate was monitored throughout heating using commercially-available heart rate monitors (Polar Electro, New York, NY), while rectal temperature (Tre) was monitored throughout passive heating by rectal thermistor (YSI, Yellow Springs, OH).

Muscle sympathetic nerve activity (MSNA). MSNA was recorded via microneurography of the peroneal nerve (Sundlöf & Wallin, 1977; Wallin & Sundlof, 1979) at the beginning (0 heating sessions), mid-point (after 14-16 heating sessions, or a similar 4-5 week time interval in control subjects) and end (after 30 heat sessions or

equivalent time control). All testing took place in a thermoneutral (18-21°C) room, 24-72 hours after the most recent heat session in an attempt to isolate chronic, rather than acute, effects of heat. Briefly, the nerve was located using external stimulation in the region behind the knee and below the fibular head, and sites that showed strong muscle twitches were marked for reference. Once a site was selected, post-ganglionic MSNA was recorded through a tungsten microelectrode inserted percutaneously into the peroneal nerve. Nerve traffic was recorded continuously at 250 Hz and analyzed using standard techniques as described previously (Halliwill, 2000) at baseline (quiet supine resting with and without paced breathing to a metronome). MSNA was recorded using Windaq data acquisition software with simultaneous recording of EKG and beat-by-beat blood pressure (Nexfin). Sympathetic nerve bursts were identified offline by two investigators (one blinded) with a minimum 3:1 signal: noise ratio and confirmed by measuring pulse synchronicity. Primary variables of interest for MSNA were burst frequency (bursts/min; measured over a minimum of five minutes) and burst incidence (bursts/100 heartbeats; measured over a minimum of 5 minutes).

Sympathetic baroreflex sensitivity was assessed by having subjects perform a Valsalva maneuver during MSNA. This was accomplished using an expiratory pressure gauge with an on-screen display of pressure. Subjects were encouraged to maintain pressure at 40mmHg for 20 seconds while contracting their abdominals and avoiding muscle contraction in other parts of the body. Sympathetic baroreflex sensitivity was examined by locating the diastolic pressure nadir (5 consecutive cardiac cycles with pressure dropping during Phase II) and examining change in diastolic pressure and burst incidence during this period. By plotting diastolic pressure and burst incidence at rest

compared with during Phase II nadir, a slope (ΔBI/ΔDBP) was calculated as a measure of baroreflex sensitivity. This assessment of sympathetic baroreflex sensitivity during Valsalva is well correlated with measures of spontaneous baroreflex sensitivity (Yang & Carter, 2013).

Heart Rate Variability. Due to difficulty in obtaining nerve tracings in some subjects, we additionally assessed autonomic function using heart rate variability at rest and during paced breathing in all subjects. Spectral analysis heart rate variability is a widely-used, non-invasive, estimate of autonomic influence on the heart. While its correlation with MSNA and cardiac NE spillover is poor (Kingwell *et al.*, 1994) in clinical populations, measures of low-frequency variability (LFnu; attributed to high sympathetic activity) do track with MSNA in healthy individuals in select conditions (DeBeck *et al.*, 2010). In addition, overall variability and high-frequency variability (HFnu; attributed to parasympathetic activity) appear to be decreased, indicating sympathetic dominance, in women with PCOS (Hashim *et al.*, 2015; Ribiero *et al.*, 2016). As such, we measured HRV in all subjects during pace breathing in MSNA studies, and with paced breathing in subjects in whom we were unable to obtain adequate nerve recordings. Subjects were resting in the supine position in a dark, thermoneutral room (18-21°C) for a minimum of 20 minutes prior to testing. Heart rhythm was measured using a 3-lead EKG (CardioCap; Datex Ohmeda, Louisville, CO) while breathing to a metronome paced at 15 breaths/min for five minutes. Data were continuously recorded at 250Hz (Windaq, Dataq Instruments, Akron, OH) for offline analysis including peak detection of R waves and examination of heart rate variability using commercially available software (HRVanalysis 1.1) (Pichot *et al.*, 2016). Variables

of interest within time domain analyses of total variability included the standard deviation of normal-to-normal R-R intervals (SDNN) and the square root of the mean sum of squared differences (rMSSD). Frequential analyses were additionally employed to measure total variability (Ptot) and to estimate the relative contribution of parasympathetic and sympathetic modulation using low frequency (LF, LFnu; defined as 0.04-0.15Hz), high frequency (HF, HFnu; defined as 0.15-0.4Hz), and the ratio of low to high frequency (LF/HF).

Blood analysis. On a separate day, blood samples were drawn from a venous catheter in the antecubital space for analysis of insulin, testosterone, adipokines, and various inflammatory cytokines. Blood was collected in serum separator tubes and allowed to clot for 30 minutes at room temperature prior to centrifugation. Samples were then centrifuged at 1500 x *g* for 10 minutes at 4°C, then placed in a -80°C freezer. Thawed samples were batch-analyzed at the end of the study for total testosterone using a commercial ELISA kit (Enzo life sciences), while inflammatory markers IL-6, IL-1β, TNFα were examined in EDTA-treated plasma with a commercial cytometric bead-based array (BD Biosciences Human Inflammatory Panel) using flow cytometry (Beckman-Coulter Gallios). Serum insulin was analyzed by Oregon Clinical and Translational Research Institute (OCTRI).

Statistics. All results are reported as mean ± SEM. Results were compared using a two-way mixed model ANOVA with Graphpad Prism 6, and significant main effects were examined using Holm-Sidak post-hoc analysis. Based on results from an exercise intervention in PCOS women, a power analysis for our primary variable of interest

(MSNA burst frequency) using conventional α=0.05 and β=0.80 determined a minimum sample size of six subjects/group.

Results

Of the eighteen women who participated in the study, successful nerve recordings were obtained on 14 individuals (7 HT, 7 CON), with one HT subject missing mid-point MSNA. Demographic data are presented in Table 5. All subjects were classified as obese and diagnosed with PCOS using the Rotterdam criteria. Using the most recent guidelines for hypertension, seven subjects (3 HT, 4 CON) were classified as "elevated blood pressure", and seven were classified as Stage 1 Hypertensive (4 HT, 3 CON). Two subjects in each group were classified as normotensive (<120/<80 mmHg). HT

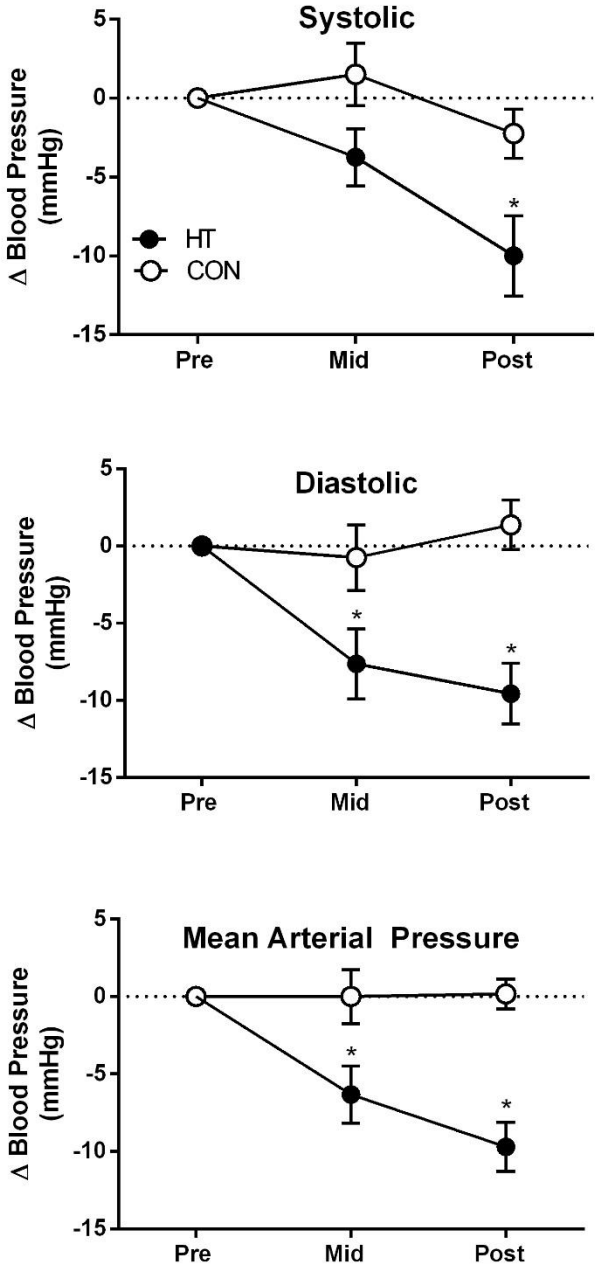


Figure 18. Change in blood pressure over time in HT and CON. * Denotes significant difference from Pre (time within group) and CON (between group at matched timepoint)

subjects experienced a large, clinically significant decrease in blood pressure (10mmHg decrease in MAP; see Figure 18) with no change over time in CON subjects (Figure 18). By the end of testing, only one HT subject had not changed classification (elevated blood pressure), despite experiencing a 3mmHg decrease in MAP. HT subjects also experienced a significant decrease in resting heart rate over time (Pre: 73 ± 4; Mid: 68 ± 3; Post: 64 ± 3 beats/min, p=0.003 Pre vs Post), while HR in CON subjects did not change (Pre: 74 ± 5; Mid: 74 ± 4; Post: 72 ± 4 beats/min, p=0.53 Pre vs Post).

MSNA.

MSNA significantly declined over the course of HT, with an approximately ~40% decline in burst frequency evident at the mid-point, and no change in CON subjects. Individual data for all subjects for both burst frequency and incidence are shown in Figure 19. All HT subjects exhibited a decrease in baseline MSNA by the mid time point, which was maintained in post testing, and no changes over time were observed in CON subjects.

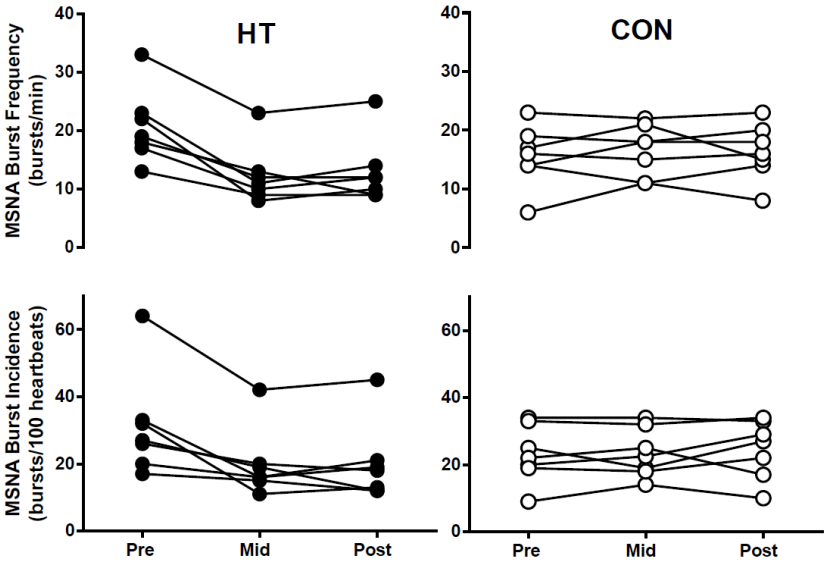


Figure 19. Individual MSNA burst frequency (BF) and incidence (BI) in HT and CON. HT subjects experienced a significant decrease in BF and BI in Mid and Post compared with Pre.

Baroreflex Sensitivity. Baroreflex sensitivity was assessed during the Valsalva maneuver by comparing baseline burst incidence (BI) and diastolic blood pressure (DBP) to BI during the five lowest consecutive DBPs during Phase II in order to calculate slope (ΔBI/ΔDBP). Due to the significant decrease in DBP, HT subjects exhibited a shift in intercept to a lower set point at Mid and Post, with no change in CON (Figure 20). BI

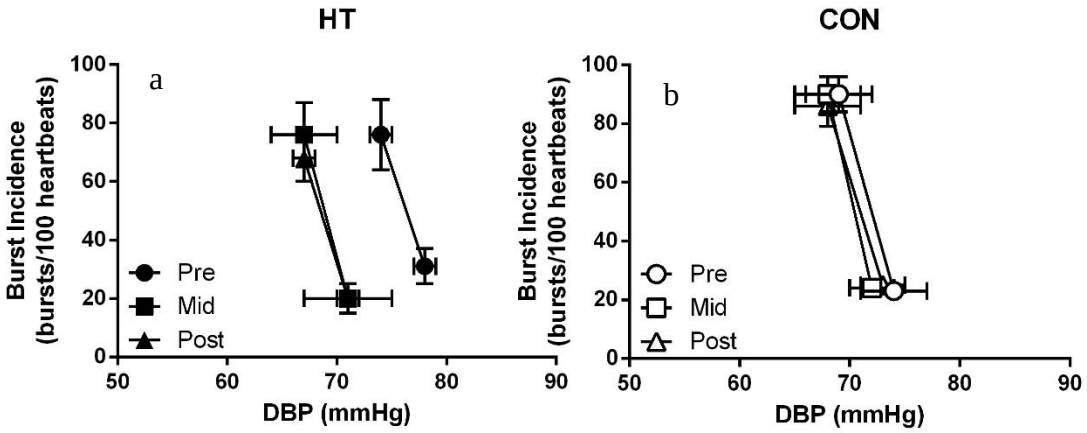
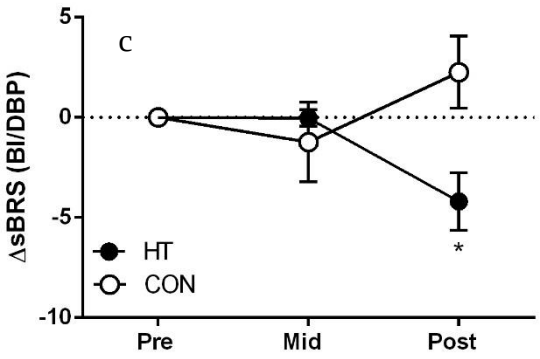


Figure 20. sBRS slopes in HT (a) and CON (b). HT subjects experienced a decrease in set point and increase in sensitivity (more negative slope) compared to CON (c). *Denotes significant difference from CON in Post.



slope (ΔBI/ΔDBP) significantly decreased, indicating increased sBRS sensitivity, in HT subjects as compared to CON (interaction effect p=0.003; Figure 20c).

Heart Rate Variability. Measures of total variability (rMSSD, SDNN, Ptot) are displayed in Table 6. While measures of total variability tended to increase throughout testing in HT subjects and remain stable in CON, only rMSSD significantly increased over time in HT subjects (p=0.043). In the frequency domain, both LF and HF tended to increase over time, resulting in no change in LFnu, HFnu, or LF/HF in either group.

Table 6. Heart rate variability over time in HT and CON. *denotes significant difference from Pre within group.

		Pre	Mid	Post
SDNN	HT	47 ± 6	53 ± 9	62 ± 7
	CON	53 ± 7	55 ± 8	57 ± 8
rMSSD	HT	44 ± 6	45 ± 11	66 ± 9
	CON	53 ± 9	48 ± 12	54 ± 13
Ptot	HT	2365 ± 704	2666 ± 704	3757 ± 1317*
	CON	2275 ± 615	2906 ± 803	3213 ± 873
LFnu	HT	37 ± 9	54 ± 6	37 ± 6
	CON	30 ± 6	43 ± 10	35 ± 6
HFnu	HT	50 ± 8	40 ± 6	54 ± 8
	CON	64 ± 6	50 ± 10	48 ± 11
LF/HF	HT	1.16 ± 0.44	1.61 ± 0.31	1.01 ± 0.35
	CON	0.55 ± 0.16	1.69 ± 0.76	1.11 ± 0.44

Blood parameters. Most subjects had elevated total testosterone at Pre (>30 ng/mL), excluding the one subject in each group taking oral contraceptives (Table 7). Of the remaining six subjects who underwent HT and were not taking oral contraceptives, five reported beginning regular menstrual cycles by the mid-point of the study. In these five women, serum testosterone decreased from 65 ± 9 to 38 ± 7 ng/mL (mean change 27 ± 9 ng/mL) over HT. Total testosterone significantly decreased over time in HT subjects ($p=0.03$), with no change in CON ($p=0.21$). Serum Insulin was elevated and did not change in either HT or CON. For cytokine analysis, one HT and one CON subject fell below the detectable limits for all analytes, so data represent n=8 experimental and n=7 control. IL-6, IL-1β, and TNF were variable in control subjects, but tended to decrease over time in HT. IL-1β significantly decreased over time in HT as compared with CON (interaction effect $p=0.0404$), and TNF tended to decrease over time in HT compared with CON (interaction effect $p=0.0689$). While IL-6 decreased in all HT subjects, this change was not statistically significant ($p=0.17$).

Correlation Analysis. As seen in Figure 21, change in total testosterone (Calculated as Mid – Pre and Post – Pre in HT and CON) was significantly correlated with change in MSNA BF ($r=0.64$), BI ($r=0.65$), and baroreflex slope ($r=0.54$). Change in insulin over time was not significantly correlated with change in MSNA BF ($r=0.22$), BI ($r=0.14$), or baroreflex slope ($r=0.07$). Similarly, change in IL-6 and TNFα were not significantly correlated with change in MNSA BF ($r=0.25$ and 0.11, respectively), BI ($r=0.29$ and 0.04, respectively), or baroreflex slope ($r=0.03$ and 0.27, respectively).

Table 7. Blood parameters over time in HT and CON. *denotes significant difference from Pre within group. #denotes significant difference from CON.

		Pre	Mid	Post
Total Testosterone	HT	51 ± 8	42 ± 3	34 ± 4*
(ng/mL)	CON	42 ± 4	39 ± 8	46 ± 7
Serum Insulin	HT	23 ± 4	22 ± 5	23 ± 4
(mU/L)	CON	26 ± 2	25 ± 2	22 ± 2
IL-1β	HT	23 ± 7	12 ± 5	12 ± 6#
(pg/mL)	CON	15 ± 4	28 ± 10	28 ± 9
IL-6	HT	19 ± 10	10 ± 8	1 ± 1
(pg/mL)	CON	14 ± 3	20 ± 7	16 ± 5
TNFα	HT	24 ± 7	13 ± 6	9 ± 6
(pg/mL)	CON	13 ± 3	17 ± 2	12 ± 3

Discussion

This study was the first to examine changes in MSNA and HRV at rest in response to a chronic heat intervention. The large reduction in baseline MSNA, clinically important reduction in resting blood pressure, increased sBRS sensitivity, and improvement in inflammatory profile work in concert to reduce cardiometabolic risk and provide evidence of significant clinical benefits of heat therapy to women with PCOS. The elevated MSNA in obese women with PCOS not only contributes to cardiometabolic dysfunction, but also contributes to ovarian dysfunction, therefore the observed reduction in MSNA may be related to reductions in serum testosterone and improvements in PCOS symptoms observed in this investigation.

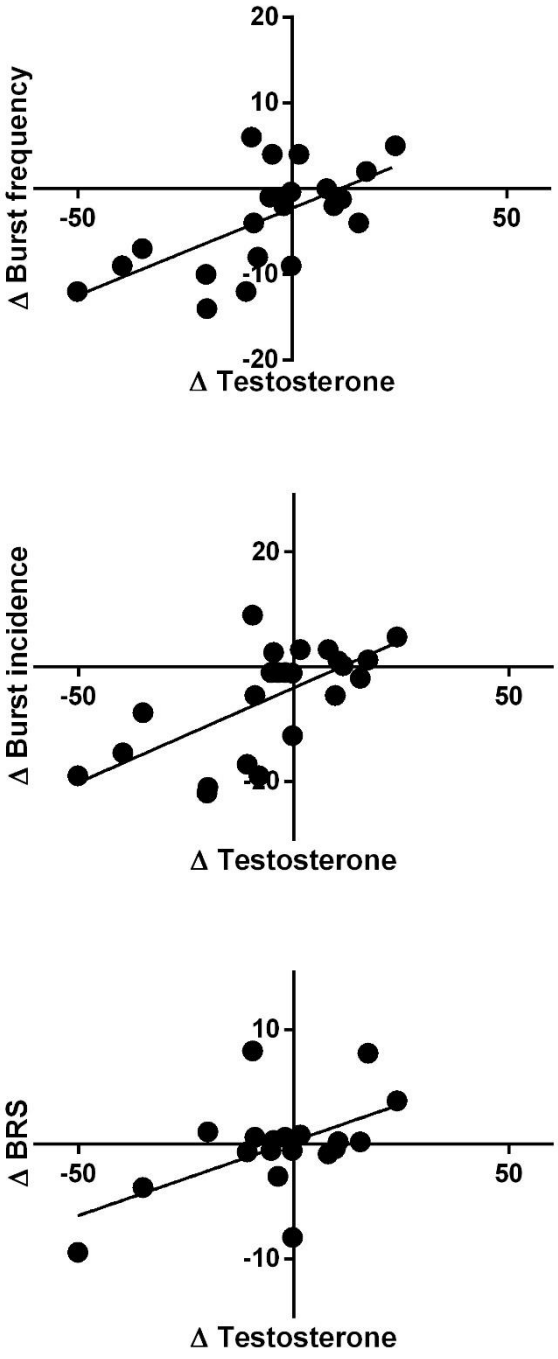


Figure 21. Correlation analysis of change in total testosterone and MSNA variables. Change in BF, BI, and sBRS were all significantly correlated with change in testosterone.

The ~40% reduction in burst frequency and incidence after 4-5 weeks of heat therapy in this study is similar in magnitude to changes observed in response to 16 weeks of exercise training or electroacupuncture in overweight women with PCOS (Stener-Victorin *et al.*, 2009). This decrease persisted through post HT testing, and was accompanied by clinically significant decreases in SBP, DBP, MAP, and heart rate. Together, this shift in baseline MSNA firing rate and lower blood pressure indicate a change in baroreflex set point with heat therapy. This change was also evident when examining sBRS slope, with HT subjects exhibiting a shift to

a lower firing rate and pressure in Mid and Post, and a steeper slope in Post. Since greater baroreflex sensitivity is associated with healthier cardiovascular profiles (Eckberg & Sleight, 1992), this change in slope further contributes to the improved cardiovascular risk profile with heat therapy.

In obese women with PCOS, there are multiple factors associated with elevated MSNA, including obesity (Narkiewicz *et al.*, 1998), hyperinsulinemia (Monroe *et al.*, 2000), inflammatory cytokines (Zhang *et al.*, 2003; Helwig *et al.*, 2008), and androgen excess (Sverrisdottir *et al.*, 2008). BMI and fasting insulin did not change over the course of the study and are therefore unlikely responsible for changes in MSNA. However, HT subjects experienced a decrease in circulating TNFα. TNFα tends to be elevated in obese women with PCOS (Escobar-Morreale *et al.*, 2011; Shorakae *et al.*, 2015; Spritzer *et al.*, 2015) and increases sympathetic outflow at the rostral ventrolateral medulla (Marz *et al.*, 1996; Zhang *et al.*, 2003). While changes in circulating inflammatory cytokines may modulate sympathetic activity, the changes in IL-6 and TNFα in this study did not significantly correlate with changes in MSNA variables. Finally, women in the HT intervention experienced a decrease in testosterone over time. Elevated serum testosterone has been associated with sympathetic activity in PCOS, although there is some debate whether testosterone drives sympathetic activity (Sverrisdottir *et al.*, 2008) or if high sympathetic activity to the ovary drives androgen production (Lara *et al.*, 1993). We observed a moderate correlation between in changes in MSNA and changes in testosterone in the women in this study. However, it remains unclear whether this is a correlative or causative relationship, as we did not independently manipulate either variable in isolation.

Alterations in baroreflex sensitivity were also correlated with changes in total testosterone. While estrogen and progesterone have been shown to alter sympathetic and/or cardiovagal baroreflex sensitivity in women (Mohamed *et al.*, 1999; Minson *et al.*, 2000*a*, 2000*b*; Brunt *et al.*, 2013) and testosterone appears to increase baroreflex sensitivity in older men with heart failure (Caminiti *et al.*, 2009), no studies have assessed the impact of testosterone on baroreflex sensitivity in women. However, it is possible that changes in sBRS are unrelated to changes in testosterone and are driven by other unknown mechanisms with heat therapy. For example, baroreflex sensitivity is positively associated with endothelial nitric oxide synthase (eNOS) and NO bioavailability (Paton *et al.*, 2001), and heat acclimation or heat therapy is associated with enhanced NO production (Pritchard *et al.*, 2001; Harris *et al.*, 2008; Bharati *et al.*, 2017) . In addition, Angiotensin II receptor subtype 2 (AT2) increase baroreflex sensitivity via NO-dependent mechanisms, and AT2 receptor density increased in the rat hypothalamus with heat acclimation (Horowitz *et al.*, 1999). If a similar change in NO bioavailability and AT2 receptor density occurred in the women in this study, these mechanisms could be responsible for the observed changes in baroreflex sensitivity. As NO and AT2 receptors both act centrally to reduce baseline sympathetic outflow (Wong *et al.*, 1992; Schwarz *et al.*, 1995; Abdulla & Johns, 2017), these mechanisms could additionally explain the reduction in baseline MSNA.

The change in MSNA, total testosterone, and reported changes in menstrual regularity deserve further comment. While attributing causation to any one variable is difficult, the theory that high sympathetic activity to the ovary drives ovarian dysfunction (Lara *et al.*, 1993) and androgen production matches the time course of observed

decreases in MSNA (evident by Mid testing) and total testosterone (not significantly decreased until Post). However, insulin resistance, inflammation, and many other variables may contribute to ovarian dysfunction and elevated MSNA, so it is possible that changes in inflammation, insulin sensitivity (Chapter V), or myriad unknown factors decreased MSNA and improved ovarian function.

As an attempt to examine autonomic function in subjects we were unable to obtain nerve tracings, we measured heart rate variability (HWV) during supine rest. While measures of total variability tended to increase, individual responses did not match the timecourse or magnitude of changes in MSNA during HT. Further, we did not observe a shift in LF/HF despite clear changes in MSNA with HT. Use of the LF/HF ratio to asses sympathetic and parasympathetic influence of heart rate involves a series of assumptions, and, while widely used, some researchers convincingly argue it does not accurately reflect sympathovagal balance (Billman, 2013). While muscle sympathetic nerve activity is distinct from cardiac sympathetic nerve activity, the significant decrease in systolic blood pressure and heart rate indicated an effect of heat therapy on sympathovagal control of cardiac function that was not evident in measures of HRV.

In summary, heat therapy resulted in profound decreases in blood pressure and MSNA in obese women with PCOS, a population with well-described sympathetic overactivity and elevated risk of hypertension. These changes not only resulted in a reduction in hypertensive risk category for HT subjects, they were accompanied by changes in serum testosterone and self-reported menstrual function. Heat therapy holds great promise as a lifestyle intervention in obese women with PCOS to reduce MSNA and improve ovarian function, although mechanisms behind these improvements are

unclear. The inter-relationships between testosterone, inflammation, other neurohumoral or hormonal changes and changes in MSNA in obese women with PCOS require further investigation.

CHAPTER VI

HEAT THERAPY AND METABOLIC FUNCTION IN OBESE WOMEN WITH PCOS

Polycystic ovary syndrome (PCOS) is a complex neuroendocrine disorder that affects 5-18% of women (Spritzer *et al.*, 2015; Dunaif, 2017) and is characterized by clinical hyperandrogenism, menstrual dysfunction, and presence of ovarian cysts (Rotterdam ESHRE/ASRM-Sponsored PCOS consensus workshop group, 2004). In addition to well-described reproductive health issues, the metabolic and hormonal profiles in women with PCOS greatly increase the risk for obesity, insulin resistance, and diabetes (Luque-Ramírez & Escobar-Morreale, 2014). The PCOS phenotype displays insulin resistance through a spectrum of BMI from lean to obese (Stovall *et al.*, 2011), but with over 50% of women with PCOS classified as obese (Gambineri *et al.*, 2002), obese women with PCOS exhibits marked insulin resistance and an elevated risk of developing Type II diabetes.

Insulin signaling is disrupted in adipose tissue (Ciaraldi *et al.*, 1997; Echiburú *et al.*, 2018) in obese PCOS women, resulting in impaired glucose uptake and incomplete suppression of free fatty acid release (Zierath *et al.*, 1998). These circulating fatty acids can accumulate in the liver and skeletal muscle and produce fatty acid intermediates such as diacylglycerol, ceramides, and long-chain fatty acid-Acyl CoA (Koves *et al.*, 2008), all of which can inhibit intracellular insulin signaling by activation of c-Jun NH_2-terminal Kinase (JNK) (Nguyen *et al.*, 2005) or Inhibitor of kappa B kinase β (IKKβ) (Yuan *et al.*, 2001; Arkan *et al.*, 2005). JNK and IKKβ impair intracellular insulin signaling by serine phosphorylation of the insulin receptor substrate, preventing downstream signaling (Yin

et al., 1998). This results in systemic insulin resistance with an impaired ability of cells to transport glucose or suppress glucose production, creating a hyperglycemic, hyperinsulinemic profile in PCOS (Burghen *et al.*, 1980). This insulin resistance is exacerbated by factors such as low serum adiponectin, elevated systemic inflammation, and disruptions in the PI3K-Akt signaling pathway in PCOS (Li *et al.*, 2017). While medications such as Metformin (Tang *et al.*, 2006) and lifestyle interventions such as diet and exercise (Giallauria *et al.*, 2008; Covington *et al.*, 2016) can improve this metabolic dysfunction, most research suggests that they are unable to fully restore insulin sensitivity, therefore warranting investigation of alternative or adjunctive therapies

Repeated heat exposure, through hot water immersion, has been investigated in human and animal models of diabetes and insulin resistance. Promising reductions in fasting blood glucose were seen in Type 2 diabetics after two weeks of hot tub use (Hooper, 1992), although no measures of insulin were taken. In animals exposed to repeated heat shock, improvements in glucose tolerance and skeletal muscle glucose uptake were observed (Gupte *et al.*, 2009). Similar results were seen in select white adipose tissue depots, with improved insulin signaling following a single bout of heat treatment (Rogers *et al.*, 2015). These improvements were associated with increased heat shock protein abundance in muscle and adipose tissue, which acted to inhibit accumulation and activation of inflammatory proteins such as JNK and IKKβ. Specifically, Hsp27 is associated with inhibition of the inflammatory compound IKKβ (Park *et al.*, 2003), while Hsp70 inhibits the activation of JNK (Park *et al.*, 2001) and additionally acts to repair damaged insulin receptors (Zachayus *et al.*, 1996) through its well-described roles in molecular chaperoning and protein refolding (Kregel, 2002).

Hsp90 does not appear to play a role in insulin signaling, but is involved in adipocyte differentiation and fat mass accumulation (Desarzens *et al.*, 2014). In support of the relationship between HSP abundance and metabolic health in humans, low levels of HSPs in adipose tissue have been observed in patients with Type II diabetes (Hooper & Hooper, 2009). In concert, these studies show great potential for heat therapy to improve insulin signaling and glucose tolerance in insulin-resistant humans, but no study has yet investigated the effect of heat therapy on markers of metabolic health and adipose tissue specific insulin signaling and inflammation.

Therefore, the purpose of this study was to observe the impact of heat therapy on metabolic health in obese women with PCOS. Assessment of metabolic health included body mass index, circumference and skinfold thickness measures, glucose and insulin responses to an OGTT, and changes in inflammation, HSP expression, and insulin signaling in subcutaneous adipose tissue in order to examine potential mechanisms behind changes in glucose tolerance. We hypothesized that heat therapy would improve glucose tolerance and enhance insulin signaling in adipocytes, and that these changes would occur in the absence of changes in BMI or body composition. Based on previous work in animals exposed to heat treatment (Gupte *et al.*, 2009; Rogers *et al.*, 2015), we additionally hypothesized that HSP abundance would increase and inflammatory markers would decrease in subcutaneous adipose tissue.

Methods

Subjects. Eighteen obese (body mass index; BMI 30-45) women with PCOS participated in this study (Age: 27 ± 2 years; BMI: 40.9 ± 1.1 kg/m^2), with nine subjects assigned to a heat therapy intervention (HT) and nine assigned to a time control group (CON). Subjects were matched for BMI and age between groups. All subjects were non-smokers, not diagnosed with overt cardiovascular disease or diabetes, and were not taking medications known to impact insulin sensitivity (Metformin, Spironolactone), with the exception of one subject in each group taking oral contraceptives. Of these eighteen subjects, adipose tissue biopsies were performed on sixteen of them (8 experimental, 8 control).

Heat Therapy Intervention. Heat therapy occurred over an eight to ten week period, with a total of 30 x one hour sessions scheduled three to four per week in all subjects enrolled in the experimental group (control subjects were not exposed to heat). Passive hot water immersion was selected as the method of heat stress because it is capable of increasing core temperature at a rate similar to moderate-intensity exercise (Kenny *et al.*, 1996), while also allowing high skin temperature and sweating rate, all requisite components for adaptation to heat (Fox *et al.*, 1963; Buono *et al.*, 2009).

Subjects reported to the laboratory to undergo passive heating, which entailed 60 min of water immersion in a bath set to 40.5°C. Previous research in our laboratory suggests this temperature is optimal to raise core temperature ≥ 38.5°C within 20-30 minutes. Once core temperature rose to 38.5°C, subjects sat upright (immersed to the waist) for the remainder of the one hour session in order to maintain core temperature between 38.5-39.0°C. If temperature dropped below 38.5, subjects were asked to

submerge again. After 60 minutes of exposure, subjects were asked to sit next to the tub until core temperature fell below 38.5˚C (10-15 minutes) for safety monitoring, and for monitoring the total exposure duration where core temperature was above 38.5°C. This threshold was selected based on human heat acclimation literature using isothermic models (Fox *et al.*, 1963), and is additionally important as a threshold for induction of heat shock proteins (Taylor, 2014).

On each heat therapy day, subjects provided a urine sample for measurement of specific gravity (USG) to confirm euhydration (USG ≤ 1.02) prior to heat exposure. Subjects were also weighed pre- and post-heat exposure (nude, towel-dried, behind a privacy screen) and given water to drink ad libitum during heat exposure and post-heat exposure if necessary to match fluid losses. Heart rate was monitored throughout heating using commercially-available heart rate monitors (Polar Electro, New York, NY), while rectal temperature (Tre) was monitored throughout passive heating by rectal thermistor (YSI, Yellow Springs, OH).

Oral Glucose Tolerance Test (OGTT). Selection of a 2-hr, 75g OGTT was based on its wide clinical utility, well-established thresholds for impaired fasting glucose and impaired glucose tolerance, and its inclusion as part of recommended screening for all obese women diagnosed with PCOS (Azziz *et al.*, 2006). An OGTT was performed in all subjects at the beginning (Pre, 0 HT sessions or time control), mid-point (Mid, 14-16 HT sessions or equivalent 4-5wk time control), and end (Post, 36-72h after 30th heat session or equivalent 8-10 week time control). Subjects arrived at the laboratory following an overnight fast (≥12hr) and having refrained from caffeine and alcohol for 12 hours, vitamin supplements and medications for 24 hours, and heavy exercise or heat exposure

for >24 hours. In addition, diet was recorded using a 24-hour food recall to ensure similar macronutrient composition between tests for each subject.

Upon arrival, body mass index (BMI=Weight in kg/[Height in m]^2), waist and hip circumference, and 3-site skinfold thickness (tricep, suprailiac, thigh) were assessed using established techniques. Following anthropometric assessment, subjects were seated in a comfortable chair for a minimum of 15 minutes prior to catheter placement. A venous catheter was inserted into a vein in the antecubital space or hand and baseline samples were drawn into syringes, then placed into appropriate tubes. Samples were placed in EDTA tubes for analysis of glucose (YSI 2300 Stat Plus, Yellow Springs, OH) and serum separator tubes (SST) for analysis of insulin. The EDTA tubes were immediately placed on ice and centrifuged, while the SSTs were allowed to clot at room temperature for 30 minutes prior to centrifugation. In addition, fasting samples were also drawn for measurement of various cytokines and adipokines associated with impaired metabolic health. All samples were centrifuged at 1500 x *g* for 10 minutes at 4°C, and plasma or serum was aliquoted into cryovials and placed in -80°C freezer.

After baseline (fasted) sampling, a 75-g glucose drink was ingested in a 3-5 minute period, and blood samples were taken at 15, 30, 45, 60, 90, and 120 minutes for analysis of blood glucose and insulin. Glucose was analyzed using the glucose oxidase method (YSI 2300 Stat Plus, Yellow Springs, OH), and insulin samples were frozen at -80°C for batch analysis by Oregon Clinical and Translational Research Institute (OCTRI).

The resulting glucose and insulin curves provide an indication of glucose tolerance and insulin sensitivity through measurement of the area under the curve for

both glucose and insulin. In addition, commonly used fasting markers of insulin resistance were calculated as assessments of insulin resistance (Piché *et al.*, 2007). These included the homeostatic model assessment for insulin resistance (HOMA-IR=[glucose mg/dL*insulin mU/L]/405) and quantitative insulin sensitivity check index (QUICKI=1/[Log(glucose) +Log(insulin)]), both calculated on values obtained after a 12-hr overnight fast.

Adipose Tissue Biopsies. Subcutaneous white adipose tissue samples were obtained after a 4-hour fast from the peri-umbilical area at the beginning (Pre, 0 HT sessions or time control) and end (Post, 36-72h after 30th heat session or equivalent 8-10 week time control) of the study in HT and CON subjects. This tissue depot was selected due to the high proportion of abdominal obesity in PCOS, and the ease of sampling & relative comfort of participants. The sampling area was cleaned with antiseptic (Chloraprep) and a local anesthesia was induced by intracutaenous and subcutaneous injection of 5ml of 1% lidocaine near the site of incision in the side of the umbilicus. Following local anesthesia, a 3mm wide skin incision was made with a sterile scalpel at the edge of the umbilicus, and 15 cm lontrog, 16 gauge infiltration cannula (Millenium Surgical, Narbeth, PA) was advanced through the incision. This cannula was connected to a 60-ml syringe containing a mixture of 50 ml of 0.9% sterile saline and 7.5mL of 1% lidocaine. This fluid was injected in the sub-cutaneous fat in a fan-like fashion from the incision site, covering a total area of ~5x5cm. Next, an 11 gauge aspiration cannula (Millenium Surgical, Narbeth, PA) connected to a 20mL sterile syringe was advanced in the same fan-like fashion while gentle suction was applied. A total sample of 10-12mL of fluid and fat was taken, resulting in an adipose tissue sample of 4-7mL. This sample was

collected and washed three times in sterile saline before a portion of whole adipose tissue was snap-frozen in liquid nitrogen for later analysis of HSPs and inflammatory markers. The remaining sample was placed in phosphate-buffered saline (PBS) for adipocyte isolation.

Primary adipocytes were isolated by digesting tissue with collagenase (1mg/mL in KRH buffer) at 37°C. When tissue chunks were no longer visible, the sample was passed through a 250um Pierce tissue strainer (Thermofisher Scientific, Rockford, IL) and washed with an equal volume of wash buffer (KRH buffer with 10uL adenosine and 2% BSA) before centrifugation at 500 x *g* for 5 minutes. The wash buffer and centrifugation process was repeated, then 3 x 100uL cell samples were each placed in wash buffer for a two hour serum-starve incubation at 37°C.

After incubation, one sample was left untreated and 20 µL insulin (Humulin-R; Lilly Pharmaceuticals) was added to the second and third samples in physiological (1.2ng/mL) and supra-physiological (12ng/mL) concentrations. These samples were placed in a 37°C incubator for five minutes before adipocytes were extracted and snap-frozen in liquid nitrogen for later analysis of insulin signaling by measuring phosphorylated AKT (p-AKT). Isolated adipocytes were thawed and combined with a 1:1 volume of protease inhibitor cocktail for tissue homogenization. Homogenized samples were rotated at 4°C for one hour, then spun at 1200 x *g* for 15 minutes prior to protein subnatant extraction and quantification. Analysis of p-AKT was completed using Wes (ProteinSimple, San Jose, CA) as previously described (Harris, 2015).

Whole adipose tissue samples were thawed and combined with a cocktail of 10X RIPA (AbCam, Cambridge, MA) and protease inhibitors in a 1:1 sample:cocktail mixture

before homogenization. Homogenized samples were spun at 1500 x *g* for 10 minutes, and protein extract was isolated from beneath the lipid layer of each sample. Samples were analyzed for Hsp27 (Santa Cruz Biotecnology, Santa Cruz, CA), Hsp70 (AbCam, Cambridge, MA), Hsp90 (AbCam, Cambridge, MA) and inflammatory markers JNK, p-JNK, and IKKβ (Santa Cruz Biotechnology, Santa Cruz, CA) using Western Blotting, with α-Vinculin (AbCam, Cambridge, MA) used as a loading control.

*Statistics***.** All data are presented as mean ± SEM. Results were analyzed using mixed-model ANOVA in GraphPad Prism 6, with repeated measures within HT or CON groups for each subject over time, and non-repeated measures comparison between groups of subjects. If a significant main effect was observed, Holm-Sidak post-hoc analysis was utilized to examine within- or between-group effects over time.

Results

All HT subjects completed the heat therapy intervention, and exhibited classic signs of heat adaptation including a reduced basal body temperature, lower heart rate, and increased sweating rate upon exposure to heat (Chapter IV Table 3). One CON subject withdrew after all Pre testing was complete, and was therefore not included in analyses. CON and HT subjects did not exhibit any changes in BMI, waist circumfrence, waist to hip ratio, or skinfold thickness over the course of 8-10 weeks (Table 8). Despite this, HOMA-IR decreased and QUICKI increased from Pre to Post, both indicating increased insulin sensitivity.

Table 8. A summary of anthropometric and blood variables related to metabolic health in heat therapy (HT) and control (CON) subjects. * indicates significantly ($p<0.05$) different from Pre.

	Group	Pre	Mid	Post
BMI	**HT**	41.8 ± 1.4	41.9 ± 1.4	41.8 ± 1.5
(kg/m^2)	**CON**	39.9 ± 1.9	39.8 ± 1.8	39.5 ± 1.8
Waist circumfrence	**HT**	111 ± 2	111 ± 2	110 ± 3
(cm)	**CON**	109 ± 4	109 ± 4	109 ± 4
Waist:Hip ratio	**HT**	0.85 ± 0.01	0.85 ± 0.01	0.84 ± 0.01
	CON	0.86 ± 0.02	0.86 ± 0.02	0.85 ± 0.02
Sum of skinfolds	**HT**	137 ± 6	138 ± 8	135 ± 7
(mm)	**CON**	132 ± 8	134 ± 7	138 ± 8
HOMA-IR	**HT**	5.9 ± 0.9	5.5 ± 1.2	4.9 ± 0.9*
	CON	6.4 ± 0.5	6.3 ± 0.5	6.2 ± 0.5
QUICKI	**HT**	0.30 ± 0.01	0.31 ± 0.01	0.31 ± 0.01*
	CON	0.29 ± 0.01	0.29 ± 0.01	0.29 ± 0.01

OGTT. All subjects exhibited abnormalities in the OGTT at baseline including impaired fasting glucose (n=2 HT, 4 CON; with n=1 HT and 1 CON in pre-diabetic range), elevated 2-hr glucose (n=3 HT, 1 CON), or both (n=4 HT, 4 CON). No glucose parameters significantly changed in CON or HT subjects at the mid-point of the study; however; at the end of the intervention HT subjects displayed reduced fasting glucose, and lower blood glucose values beginning at 45 minutes (Figure 22). These reductions in glucose resulted in a decreased glucose area under the curve (AUC, Figure 22 insets) in HT subjects at Post-testing. Fasting insulin did not change over HT, but insulin during the OGTT was significantly reduced at the 45, 60, 90, and 120min timepoints in Post, resulting in a decreased insulin AUC in HT subjects. Inulin AUC was also significantly

reduced at the Mid-point in HT subjects. All glucose and insulin data for HT and CON subjects are displayed in Figure 22.

Insulin Signaling. Subjects in HT and CON displayed a stepwise increase in p-AKT with increasing insulin dose in Pre samples (Figure 23). In Post samples, this stepwise increase was still evident in CON, but in HT subjects, sub-max and max doses were not different, indicating that the sub-max dose was adequate to maximize response after HT. Individual data for the sub-max dose are displayed in Figure 24. This change in p-AKT in response to insulin following HT represented a ~500% increase Pre to Post.

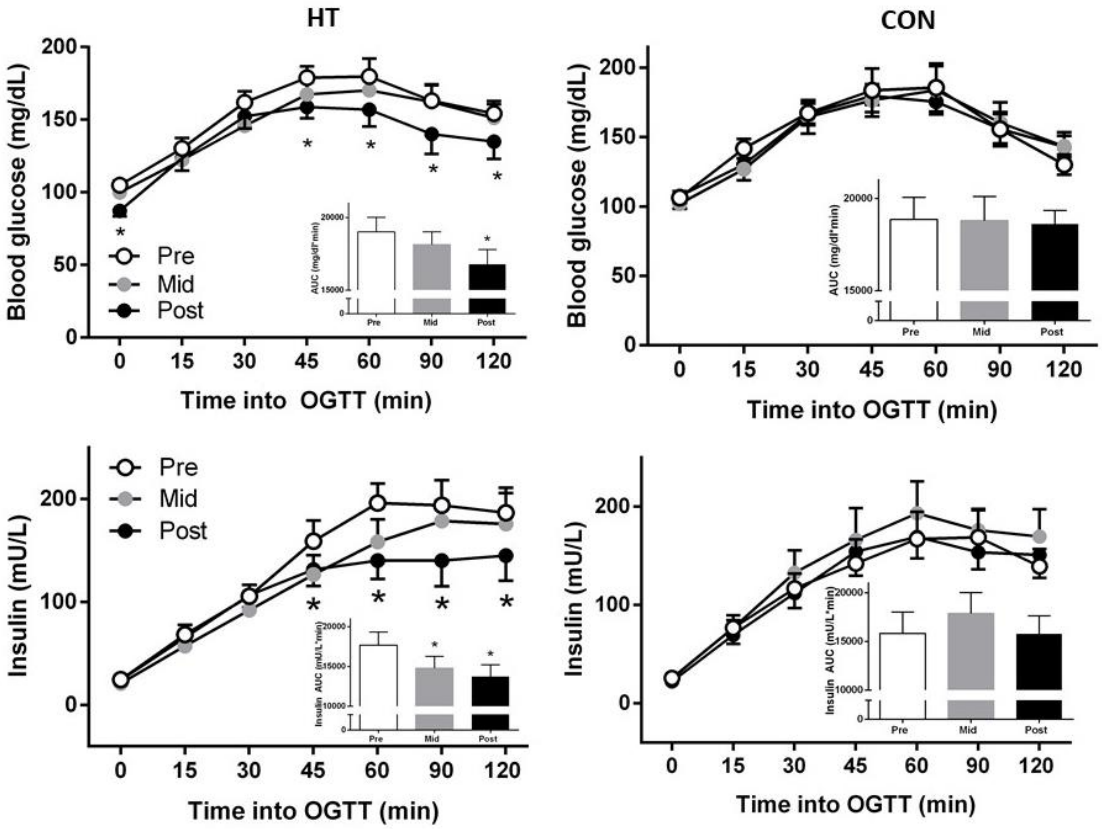


Figure 22. Glucose and insulin curves at Pre, Mid, and Post for HT (left panels) and CON (right panels). Glucose and insulin AUC are displayed as insets in each graph. * denotes significantly different from Pre

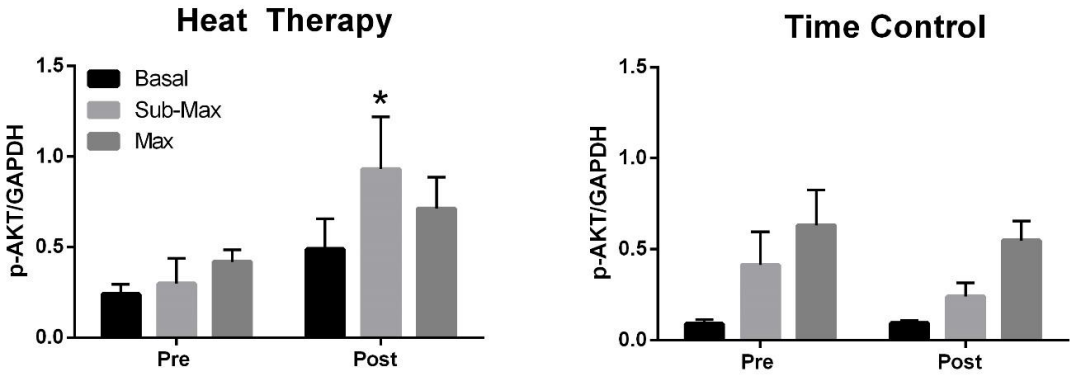


Figure 23. Insulin signaling (p-AKT) relative to loading control (GAPDH) at basal (no insulin), sub-max (1.2nM), and max (12nM) insulin doses in HT and CON. *Denotes significantly different from Pre.

In order to examine potential cellular signals that may be driving this increase in insulin signaling, common inflammatory markers IKKβ, JNK and p-JNK were examined in whole adipose tissue. As seen in Figure 25, a ~40% decrease in IKKβ (p=0.024) was observed following HT, with no change in CON subjects (p=0.49). While most HT subjects tended to decrease from Pre to Post, changes in JNK (~28% decrease; p=0.09 and p-JNK (~27% decrease; p=0.11) were not statistically significant following HT, and the ratio of pJNK/JNK (p=0.70) did not change. No changes were observed in CON subjects for JNK (p=0.32), p-JNK (0.42), or p-JNK/JNK (p=0.29).

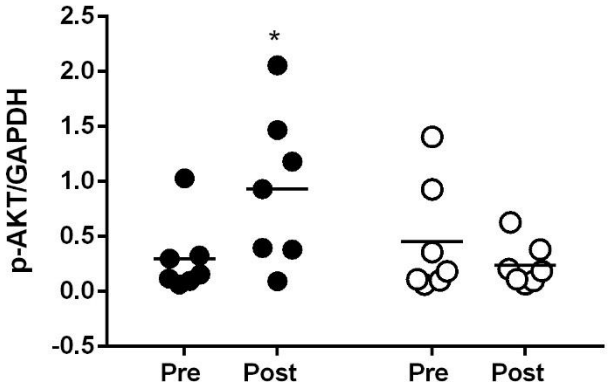


Figure 24. Individual responses of insulin signaling (p-AKT) relative to loading control (GAPDH) to 1.2nM insulin dose in HT (dark circles) and CON (open circles). *denotes significantly ($p<0.05$) different from Pre.

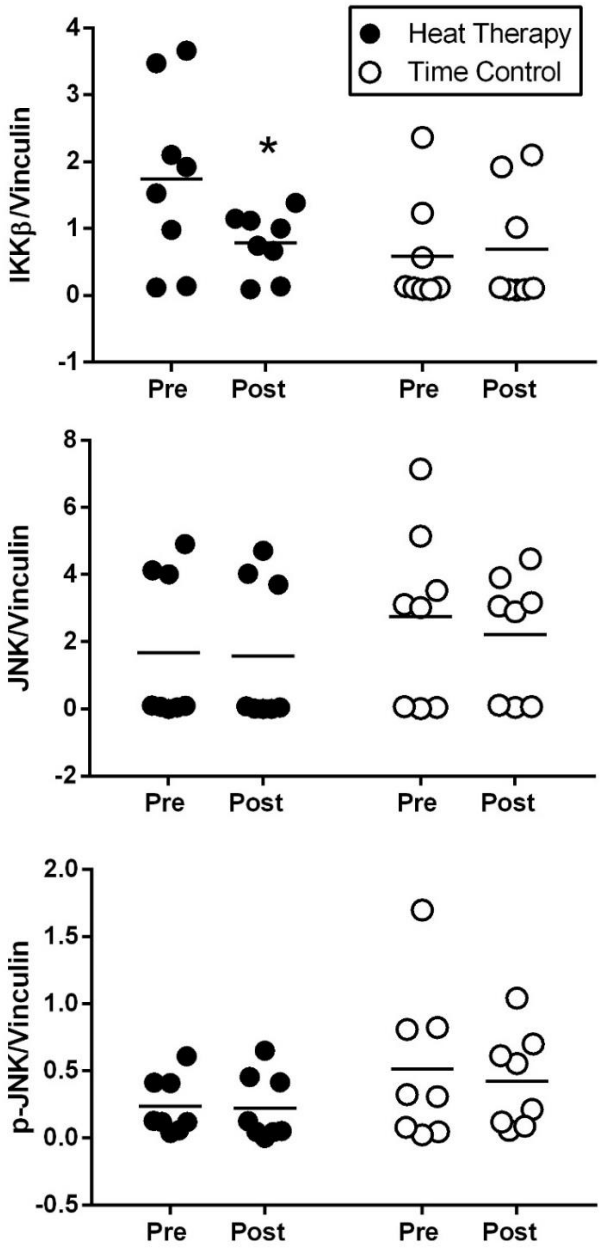


Figure 25. Individual IKKβ, JNK, and p-JNK abundance in HT and CON subjects, expressed relative to loading control (Vinculin). *Denotes significant difference from Pre.

Hsp27 abundance increased in HT subjects ($p=0.008$), with a mean increase of ~70% and no change in CON ($p=0.72$). However, no changes in Hsp70 ($p=0.79$) or Hsp90 abundance ($p=0.50$) were observed following HT.

Discussion

This study examined the impact of a 30-session, 8-10 week heat therapy intervention on markers of metabolic health in obese women with PCOS. Our primary findings indicate that repeated heat exposure is a potent stimulus to improve glucose tolerance, reduce markers of inflammation, and improve insulin sensitivity in a population with marked insulin resistance. Importantly, these improvements occurred in the absence of any

changes in BMI or body composition in our subjects. This lack of change in anthropometric measures suggests that heat therapy-mediated improvements in metabolic function are body mass-independent, and could therefore complement lifestyle modifications that lead to weight loss in obese women with PCOS.

The improvements in blood glucose during an OGTT in HT subjects are on par with those observed in hot yoga interventions in obese men and women (Hunter *et al.*, 2013*a*), and greater than those observed in exercise training interventions in PCOS (Costa *et al.*, 2018). Additionally, the decrease in fasting and 2-hr

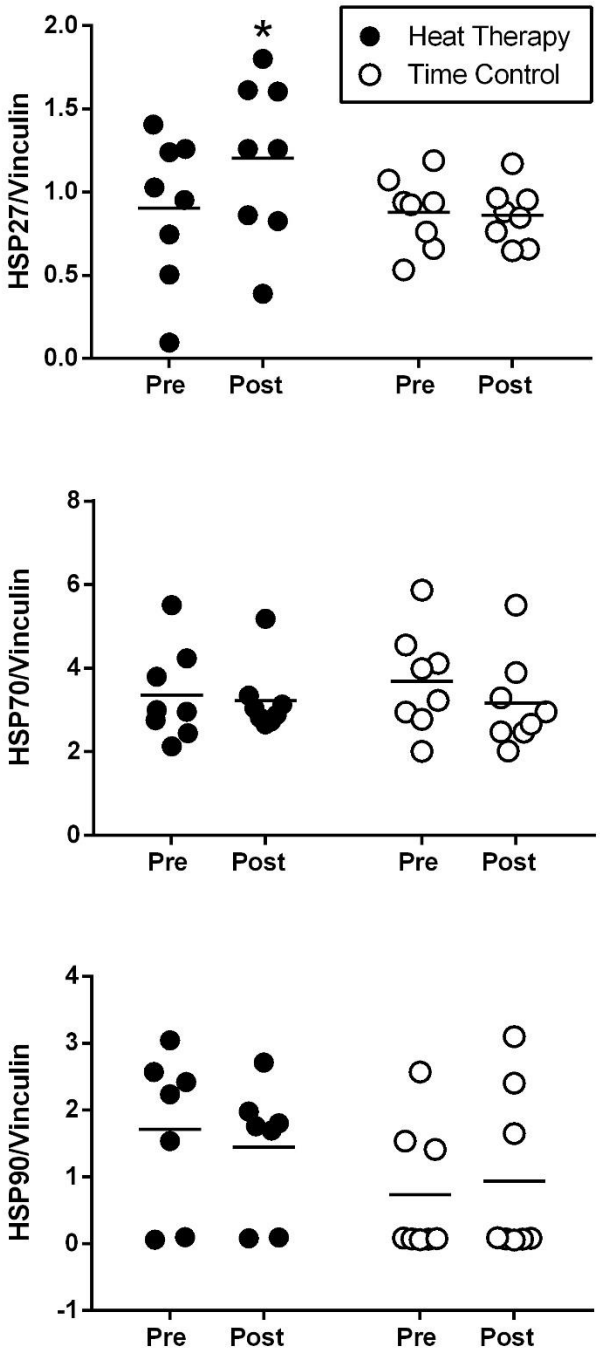


Figure 26. Individual Hsp27, 70 and 90 abundance in HT and CON subjects, expressed relative to loading control (Vinculin). *Denotes significant difference from Pre.

glucose observed in HT individuals changed the risk category for six of nine HT subjects (from pre-diabetic to impaired, or from impaired to optimal classification). While we realize an OGTT is not specifically a measure of insulin sensitivity, results correlate well with gold standard measures of insulin sensitivity (Euglycemic-Hyperinsulinemic clamp technique) (DeFronzo *et al.*, 1979), with the strongest correlations in individuals with some degree of insulin resistance (Stumvoll *et al.*, 2000; Piché *et al.*, 2007). Additionally, we observed a significant decrease in HOMA-IR and insulin AUC, and a significant increase in QUICKI, which in concert indicate increased whole-body insulin sensitivity. HOMA-IR and QUICKI values for the women in this study met established thresholds for insulin resistance (>2.0 and ≤0.30, respectively), and the changes in these values were clinically meaningful but not large enough to alter risk classification.

The systemic improvement in markers of insulin sensitivity and insulin-stimulated glucose uptake during the OGTT were supported by changes in insulin signaling in subcutaneous fat samples. While visceral fat appears to have a stronger relationship with metabolic dysfunction (Wajchenberg *et al.*, 2002) and insulin resistance (Preis *et al.*, 2010) in obesity, obese women with PCOS exhibit dysfunction in both visceral and subcutaneous depots in the abdominal region (Echiburú *et al.*, 2018). The improvement in insulin signaling observed in subcutaneous white adipose tissue along with a reduction in IKKβ and increase in Hsp27 creates a possible mechanism for heat-mediated improvements in whole-body glucose disposal. It appears that heat stress, potentially through changes in Hsp27 abundance, reduced adipose tissue inflammation such that insulin signaling was improved within fat. Based on the increased glucose uptake despite a decreased insulin response during the OGTT, heat therapy also likely improved insulin

sensitivity in the liver and skeletal muscle. While we did not examine HSPs, inflammatory proteins, or insulin signaling in skeletal muscle, muscle is the largest glucose sink and primarily responsible for glucose uptake in an OGTT. Therefore, the reduced blood glucose after 45 minutes would suggest improvements in skeletal muscle glucose uptake and insulin signaling, as seen in animal heat stress models (Gupte *et al.*, 2009).

There are multiple potential mechanisms for the observed changes in whole-body and adipose tissue-specific changes in glucose tolerance and insulin sensitivity. As stated above, the increase in Hsp27 and decrease in IKKβ observed in adipose tissue is likely in part responsible for enhanced insulin signaling in adipocytes. While adipose tissue plays a small role in whole-body glucose uptake, insulin's role in suppressing hormone-sensitive lipase can impact insulin sensitivity and glucose uptake in other tissues, as enhanced suppression of lipolysis in HT subjects would reduce fatty acid accumulation in liver and skeletal muscle. Since this lipid accumulation is involved in obesity-induced systemic inflammation and insulin resistance, future work examining changes in insulin sensitivity, glucose uptake, HSP, and inflammatory protein abundance in skeletal muscle biopsies can elucidate the impact of heat therapy on other tissues. In addition, heat acclimation in human (Brunt *et al.*, 2016*a*) and animal models (Maloyan *et al.*, 2005) increased angiogenic signals, which could impact blood supply to adipose tissue and therefore reduce the hypoxia and inflammation associated with hypertrophic adipocytes. The women in this study also experienced a decrease in sympathetic outflow, serum testosterone, and circulating inflammatory cytokines (see Chapter V), which can impact adipose tissue function by catecholamine-induced fatty acid release, promotion of

adipocyte hypertrophy, and promotion of intracellular inflammation, respectively. Finally, repeated heat shock in mice induced increases in serum adiponectin (Morera *et al.*, 2012), an adipokine highly associated with insulin sensitivity (Díez & Iglesias, 2003; Albers *et al.*, 2015*b*). As adiponectin is decreased in obese women with PCOS (Spritzer *et al.*, 2015), this may exacerbate insulin resistance (Villa & Pratley, 2011) and a possible increase in circulating adiponectin could contribute to the observed systemic improvements in this study.

In summary, repeated heat exposure improved whole-body glucose uptake, systemic insulin sensitivity, and insulin signaling in subcutaneous adipose tissue in obese women with PCOS. These improvements may be related to increased Hsp27 abundance and reduced IKKβ abundance in subcutaneous adipose tissue, but given the global and multifaceted stimulus of heat therapy, future work should examine the contribution of other potential underpinnings in these profound improvements including changes in visceral fat, liver, and skeletal muscle insulin signaling, potential adipose tissue angiogenesis, as well as circulating catecholamines and adipokines.

CHAPTER VII

SUMMARY & FUTURE DIRECTIONS

Heat has been used in various cultures for centuries with purported physiological and psychological benefit. However, until recently these potential physiological benefits were relatively unexplored in scientific literature. The pioneering work by Michal Horowitz using long-term (30 day) passive heat acclimation in animal models to examine systemic and cellular adaptations to heat exposure (Horowitz & Meiri, 1993; Maloyan *et al.*, 2005; Horowitz & Assadi, 2010) brought forth many new avenues to explore in humans, including the potential for heat acclimation to improve cardiovascular and metabolic health. However, long-term passive heat interventions in humans with impaired cardiometabolic health have been limited, with indications of improved cardiovascular (Kihara *et al.*, 2002) and metabolic (Hooper, 1992) health in heart failure and diabetes, respectively.

This study builds upon previous work examining heat therapy by assessing changes in cardiometabolic health in women with PCOS, a population that serves as 'a paradigm from prehypertension, prediabetes, and preobesity' (Luque-Ramírez & Escobar-Morreale, 2014). While PCOS is a complex and multifaceted syndrome with a spectrum of dysfunction, the cardiometabolic health impairments in obese young women with PCOS create an ideal model to examine the impact of heat therapy in an at-risk population before long-term damage occurs due to hypertension, diabetes, or other complications.

The primary findings in Chapter IV include reduced arterial wall thickness, improved arterial compliance, enhanced endothelial function, reduced C-reactive protein, and increased tolerance to IR injury, which together create a profile of profound cardiovascular risk reduction in obese women with PCOS. While the magnitude of changes in some variables (FMD, arterial stiffness) was smaller than observed in healthy, inactive humans, other health markers (blood pressure, wall thickness, inflammation) exhibited larger improvements than seen in healthy humans (Brunt *et al.*, 2016*c*). The large decrease in blood pressure in this pre-hypertensive population is particularly promising as an indication that heat therapy could be added to a lifestyle treatment in individuals with elevated blood pressure.

The large reduction in MSNA observed over the course of heat therapy (Chapter V) may be in part responsible for the decrease in blood pressure, but changes in MSNA have bearing beyond blood pressure regulation in obesity and PCOS. Elevated MSNA can contribute to adipose tissue dysfunction in obesity (Smith & Minson, 2012), and elevated sympathetic activity to the ovary may play a role in ovarian dysfunction and androgen production in PCOS (Lara *et al.*, 1993, 2005). The change in serum testosterone and the reports from multiple subjects that they resumed regular menstrual cycles are perhaps the most novel and exciting findings in this study, as they go beyond cardiometabolic risk reduction and potentially impact the pathogenesis of PCOS.

Insulin resistance also serves an important role in PCOS pathogenesis (Dunaif *et al.*, 1992, 2001; Dunaif, 2017) and can contribute to excess androgen production and elevated sympathetic outflow. Therefore, the change in adipocyte and whole-body insulin resistance over the course of heat therapy (Chapter VI) not only altered metabolic health,

but may have additionally contributed to decreased androgen production, decreased markers of inflammation, and restoration of eumenorrhea in some HT subjects. However, given that fasting insulin did not change over the course of heat therapy, it is unlikely that changes in insulin contributed to changes in baseline MSNA. In applying heat therapy to insulin-resistant populations beyond women with PCOS, the changes in glucose uptake, HOMA-IR, insulin AUC, and adipocyte insulin signaling observed in this study show great promise for pre-diabetic or type 2 diabetic individuals as an adjunctive therapy to reduce hyperglycemia as seen in Hooper & Hooper's early work (Hooper, 1992). This study builds on their work by implementing a longer heat intervention, observing glucose and insulin responses to an OGTT, and examining adipose tissue markers of insulin signaling and inflammation in order to examine potential mechanisms behind the observed decrease in blood glucose seen with hot tub use in type 2 diabetics.

The decreased markers of inflammation in subcutaneous adipose tissue (IKKβ) and in circulation (C-reactive protein, TNFα) are related to most cardiometabolic health outcomes, as meta-inflammation plays an important role in vascular health, sympathetic activity, and insulin resistance. It is possible that changes in HSP expression or abundance over time in various tissues are mediating these changes in inflammation; however; we only observed an increase in Hsp27 abundance after heat therapy in subcutaneous adipose tissue. The limited timepoints (Pre-Post) and tissue (subcutaneous adipose tissue) that we were able to observe did not allow for examination of a timecourse or tissue-specific effect. Considering that HSP abundance can be up-regulated in response to response to hypoxia or reactive oxygen species (Kregel, 2002), it is possible that an improvement in adipose tissue perfusion or oxidative stress over time

blunted a possible heat-mediated increase in HSP expression as seen in animal heat stress (Gupte *et al.*, 2009; Rogers *et al.*, 2015) or heat acclimation models (Horowitz & Assadi, 2010).

Implications and Future Directions. The overarching implication of these findings is that heat therapy is an extremely promising intervention that leads to robust improvements in cardiovascular and metabolic health in obese women with PCOS. While it appears that changes in HSP abundance, adipose tissue and systemic inflammation, and sympathetic activity are driving changes in this population, it remains difficult to tease apart the underlying mechanisms or relative influence of each component. We endeavored to examine mechanisms behind the observed improvements in cardiometabolic health with heat therapy; however; the reductions in inflammation, sympathetic nerve activity, and serum androgens are likely a small piece of the cellular and systemic adaptations that occur over heat therapy. For example, in animal work, changes in protein expression (Maloyan *et al.*, 2005; Horowitz & Assadi, 2010), hormone or neurotransmitter receptor density (Umschweif *et al.*, 2014) , epigenetic modification (Horowitz, 2007, 2014), and tissue-specific protein abundance (Gupte *et al.*, 2009; Rogers *et al.*, 2015) all occur with long-term heat exposure and likely play an important role in health outcomes. Future research examining these mechanisms in humans can further characterize the cellular and systemic changes that occur with heat therapy in health and disease.

The other large remaining gaps in current research are the time course of induction and decay of heat therapy benefits, the ‘dose’ (time x temperature) of heat needed to see these benefits, and the stimulus required to maintain benefits after an 8-10

week heat therapy intervention. We can clearly see from the current study and previous work using hot water immersion (Brunt *et al.*, 2016*c*) that most changes take the full 8-10 weeks (30-36 heat sessions) to manifest both in healthy individuals and in those with impaired cardiometabolic function. However, we do not know how long these changes persist after heat therapy, and whether it is possible to maintain them over time with regular heat doses after the initial induction. Based on Horowitz's work on epigenetic modification and cytoprotective memory in heat acclimation (Horowitz, 2014), it is possible that benefits could be maintained or even expanded with a less frequent heat stimulus after the initial intervention.

Heat therapy research in humans is still in its infancy, and future directions in diseased populations, optimal intervention timelines, and underlying mechanisms have important implications in human health through a spectrum of disease states. This study examining heat therapy in obese women with PCOS provides a glimpse of the myriad cardiovascular, autonomic, and metabolic benefits of heat therapy, and many potential avenues for further exploration.

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