FACTORS INFLUENCING ENDOTHELIAL FUNCTION

THE INFLUENCE OF ESTROGEN AND SPRINT INTERVAL EXERCISE ON BRACHIAL ARTERY ENDOTHELIAL FUNCTION IN HEALTHY ADULTS

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LAY ABSTRACT

The endothelium is the inner lining of an artery that separates it from the flowing blood. A healthy endothelium responds to increases in blood flow by producing substances that enable an artery to widen. The projects in this thesis examined whether the responsiveness, and overall function, of the endothelium in healthy young adults is enhanced by the sex hormone estrogen or by “all-out” cycling sprints, an exercise protocol that has gained appeal for its time-efficiency. We demonstrated that estrogen does not enhance endothelial function in women, compared to men, at any phase of a menstrual or birth control pill cycle. A single session or 12-weeks of the intense but brief interval exercise also does not enhance endothelial function. This work suggests it may be easier to include women in future research assessing this measure and that this particular interval exercise protocol may not enhance endothelial function in healthy adults.

ABSTRACT

Endothelium-dependent vasodilation is an important marker of vascular function. Brachial artery flow-mediated dilation (FMD) is a noninvasive assessment of peripheral artery endothelial function that is associated with coronary artery endothelial function and is an index of cardiovascular health. This thesis sought to investigate factors that may influence the brachial artery FMD response in humans, particularly the sex hormone estrogen and low-volume sprint interval training (SIT). We first demonstrated the intra-individual consistency of the FMD response pattern in healthy young adults and introduced visual data screening as a tool for improving data accuracy. Having established best practices for FMD data analysis, we investigated the brachial artery FMD response in adults with different estrogen profiles: men, premenopausal women with a natural menstrual cycle (NAT), and premenopausal women using combined oral contraceptive pills (OCP). Our findings suggest that estrogen does not augment FMD during high-estrogen phases of a NAT or OCP cycle compared to low-estrogen phases or to men. We also investigated the acute and chronic brachial artery FMD response to a 3x20-s low-volume SIT model. Following a single SIT session, FMD was unchanged in men or women. These findings demonstrate that estrogen does not influence endothelium-dependent dilation at rest or following intense intermittent exercise, but also suggest that low-volume SIT may be an insufficient stimulus for eliciting changes in endothelial function. This stimulus magnitude postulation was further supported by a 12-wk exercise training study, whereby vascular changes were evident following moderate-intensity continuous training but not SIT. Taken together, this work suggests that controlling for menstrual cycle phase and/or OCP use in premenopausal women may not be necessary, making it more feasible to include women as research participants, and highlights the need for future characterization of the minimum low-volume interval stimulus that evokes improvements in endothelial function in healthy young adults.

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"Trust in the Lord with all your heart and lean not on your own understanding; in all your ways acknowledge him, and he will make your paths straight"

-Proverbs 3:5-6

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LIST OF ABBREVIATIONS

AUC	Area under the curve
BF	Blood flow
BMI	Body mass index
Ca^{2+}	Calcium
CaM	Calmodulin
cGMP	Cyclic guanosine monophosphate
CO	Cardiac output
CTL	Control
CV	Coefficient of variance
CVD	Cardiovascular disease
DBP	Diastolic blood pressure
DICOM	Digital Imaging and Communications in Medicine
E2	17β-estradiol
E2/Prog	Estradiol to progesterone ratio
ECG	Electrocardiogram
EE	Ethinyl estradiol
eNOS	Endothelial nitric oxide synthase
ER	Estrogen receptor
FFM	Fat free mass
FMD	Flow-mediated dilation
GC	Guanylate cyclase
GTP	Guanosine triphosphate
HIIT	High intensity interval training
HR	Heart rate
ICC	Intraclass correlation coefficient
IPAQ	International physical activity questionnaire
MAP	Mean arterial pressure
MBV	Mean blood velocity
MET	Metabolic equivalent
MICT	Moderate-intensity continuous training
NAT	Natural menstrual cycle
NO	Nitric oxide
NTG	Nitroglycerin
OCP	Oral contraceptive pill
PPO	Peak power output
PWV	Pulse wave velocity
RH	Reactive hyperemia
ROI	Region of interest
SBP	Systolic blood pressure

SIT	Sprint interval training
SR	Shear rate
VO_2 peak	Peak oxygen uptake

DECLARATION OF ACADEMIC ACHIEVEMENT

This thesis, presented in "sandwich" format, includes a general introduction, four independent manuscripts for which the candidate is first author, and an overall discussion. At the time of the thesis preparation, Chapter 2 was in preparation for submission, Chapters 3 and 4 were in review, and Chapter 5 was published in a peer-reviewed journal. The contribution of the candidate and all coauthors are outlined below for each manuscript:

CHAPTER 2

Shenouda N, MacDonald MJ. Noninvasive assessment of endothelial function: Best practices for flow-mediated dilation data analysis. In preparation for submission to *American Journal of Physiology – Heart and Circulatory Physiology*.

Contributions

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Critical revision of the manuscript: NS, MJM

CHAPTER 3

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CHAPTER 4

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CHAPTER 5

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Analyzed and interpreted the data: NS, MJM

Drafted the manuscript: NS

Critical revision of the manuscript: NS, JBG, MJG, MJM

CHAPTER 1: INTRODUCTION

1.1 PREAMBLE

The cardiovascular system is comprised of a rich network of blood vessels. Arteries, in particular, regulate the transportation of freshly oxygenated blood from the heart to the rest of the body. The function and structure of an artery varies with location in the vascular tree (101); however, a unifying factor is that every artery has a thin, single-cell membrane that separates the flowing blood from the rest of the arterial wall – *the endothelium*. The endothelium is a highly active interface that enables an artery to respond to changes in its internal milieu (101). Central to this thesis is the endothelium's ability to induce arterial vasodilation, and assessment of this ability is considered an index of endothelial function (70). Estrogen is a sex hormone that has the potential to augment endothelial function through its interaction with estrogen receptors (ERs) located on the endothelium (57). Although estrogen is produced in both men and women, its concentrations are significantly elevated during certain phases of a natural menstrual cycle in premenopausal women to levels that well-exceed those observed in men (63). Therefore, it is important to determine whether estrogen may be associated with cyclic changes and/or sex differences in endothelial function. With millions of women worldwide using oral contraceptive pills (OCPs) (114), it is equally important that comparisons of estrogen's potential effects on endothelial function between the sexes are not limited to naturally cycling women but also include women using OCPs. Exercise is another factor that may, acutely and/or chronically, augment endothelial function by increasing the frictional force, or shear

stress, that blood flow exerts on the arterial wall (43). Traditional endurance exercise has been shown to improve endothelial function (10), but a large proportion of adults fail to achieve the minimum activity levels recommended by public health guidelines (98). Low-volume sprint interval training offers a time-efficient alternative to moderate intensity endurance exercise and has been shown to improve cardiometabolic health (38), but investigations of its effects on endothelial function have been limited. This thesis, therefore, sought to investigate the potential influence of estrogen and low-volume sprint interval training on endothelial function in healthy young adults.

1.2 ENDOTHELIAL FUNCTION

The endothelium is an interactive interface between the arterial wall and flowing blood that is capable of many regulatory functions, most notably arterial vasodilation. Endothelium-dependent dilation is therefore a key index of endothelial function and overall vascular health. The following sections review the anatomy of the arterial wall, the mechanisms regulating endothelium-dependent dilation, and the use of high-frequency ultrasonography for the noninvasive assessment of peripheral artery endothelial function.

1.2.1 Arterial Anatomy

Arteries are comprised of three layers: the tunica adventitia (outer layer), the tunica media (middle layer), and the tunica intima (innermost layer) (Figure 1).

The tunica adventitia is composed mainly of collagen fibres and provides structural integrity to the arterial wall. The tunica media is composed predominantly of smooth muscle arranged in circular layers and intermixed with elastin fibres. The ratio of collagen to elastin determines an artery's passive mechanical properties, while the ratio of smooth muscle to connective tissue determines an artery's active mechanical properties, both of which vary along the arterial tree (101, 102). Large central arteries are elastic in nature, allowing for passive stretch and recoil with each cardiac contraction so as to dampen the pressure generated by left ventricular ejection. In contrast, peripheral conduit arteries and the resistance arterioles of the microcirculation are more muscular in nature, allowing for active regulation of arterial diameter and ultimately blood flow through effects on this smooth muscle layer (101). Specifically, vascular smooth muscle contraction results in arterial constriction and reduced blood flow, whereas vascular smooth muscle relaxation results in arterial dilation and increased blood flow. Lastly, the tunica intima separates the smooth muscle from the arterial lumen, and is comprised of a single layer of flattened endothelial cells anchored to a basement membrane (101). Once believed to be a passive lining of the arterial wall, the endothelium is now regarded as a highly dynamic interface that regulates vascular homeostasis (33).

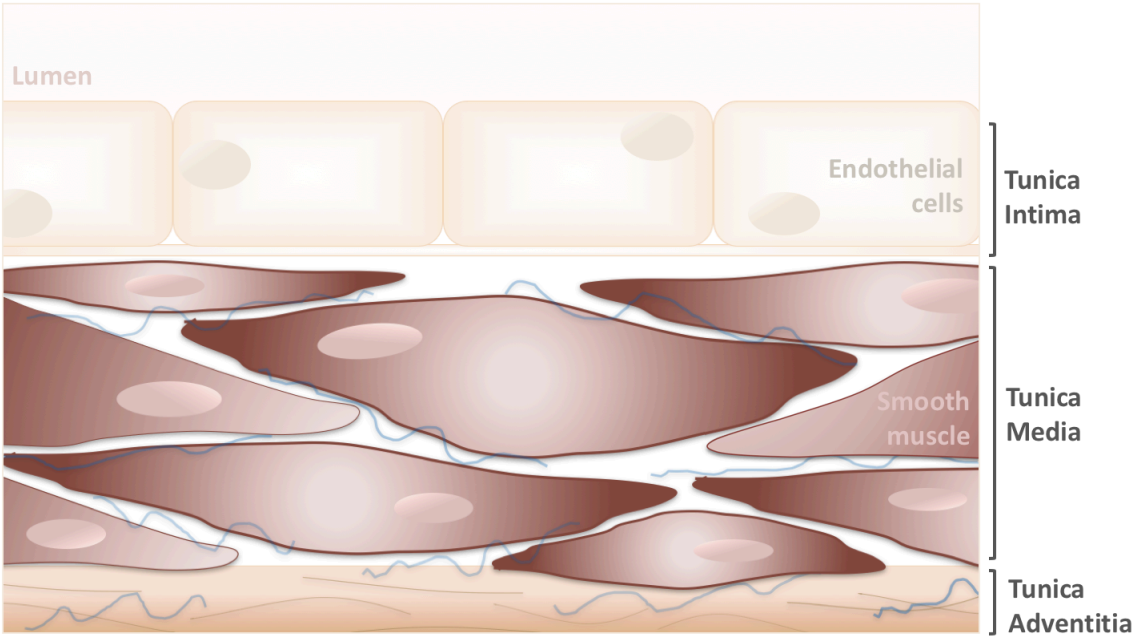

Figure 1. Arterial wall layers.

1.2.2 Regulatory Function of the Endothelium

The dominant function of the endothelium varies along the arterial tree. At the level of the capillaries, which are comprised only of a thin intima layer, the endothelium serves mainly as a selectively permeable barrier that regulates gas, metabolite, and nutrient exchange between the blood and surrounding tissue (101). However, of particular interest to this thesis is the function of the endothelium in peripheral conduit arteries. In these muscular arteries, the endothelium responds to chemical and physical stimuli by synthesizing and releasing vasoactive factors, which can have dilatory or constrictive effects on the underlying smooth muscle (33). Along with establishing an artery's basal tone and influencing its dilatory properties, endothelial vasoactive factors can also protect against or promote the

development and progression of atherosclerosis. Vasodilators like nitric oxide (NO), prostaglandin I_2, and endothelium-derived hyperpolarizing factor are considered to be antiatherogenic as they have been linked to attenuation of processes involved in plaque formation (i.e. inflammation, the adhesion of platelets and leukocytes to the endothelium, and the migration and proliferation of vascular smooth muscle cells into the tunica intima) (97). In contrast, endothelial vasoconstrictors like endothelin-1 and thromboxane are considered proatherogenic as they are linked to the promotion of the aforementioned processes (97). A healthy endothelium is characterized by the maintenance of endothelial vasodilatory function and antiatherogenic properties, whereas an inability to maintain these vasoprotective functions is considered to be indicative of endothelial impairment or dysfunction (21).

1.2.3 Endothelium-Dependent Dilation

An important milestone in vascular physiology was the chance discovery by Furchgott and Zawadzki in 1980 that an intact endothelium was needed to induce arterial dilation (32). This discovery initiated the notion of the existence of a potent endothelium-derived vasodilator, termed endothelium-derived relaxing factor, which was later identified as NO (59, 83). It is now understood that NO is synthesized from the amino acid L-arginine through the actions of the enzyme endothelial nitric oxide synthase (eNOS) (82). In its inactive state, eNOS is anchored to small invaginations in the plasma membrane of the endothelium,

known as caveolae, by the protein caveolin-1 (30, 97). The activation of eNOS is complex and can involve calcium (Ca^{2+}) dependent or independent mechanisms (24), neither of which have been fully elucidated. In the first mechanism, eNOS is activated when intracellular Ca^{2+} binds to the signaling protein calmodulin (CaM) and the Ca^{2+}-CaM complex binds to eNOS and displaces caveolin-1 (21). An influx of intracellular Ca^{2+}, and therefore this Ca^{2+} dependent mechanism, can be initiated by the activation of cell membrane receptors or by the frictional force (shear stress) that flowing blood exerts on the endothelium. Alternatively, shear stress can activate eNOS independently of Ca^{2+} through serine phosphorylation (26, 69); however, it has also been suggested that this mechanism simply increases eNOS sensitivity to lower levels of Ca^{2+} (72). In any case, eNOS activation results in the production and subsequent diffusion of NO into the vascular smooth muscle (Figure 2). In the smooth muscle, NO activates guanylate cyclase (GC), which in turn converts guanosine triphosphate (GTP) into cyclic guanosine monophosphate (cGMP). By reducing cytosolic Ca^{2+} concentrations and desensitizing actin-myosin contractility to Ca^{2+}, cGMP induces vascular smooth muscle relaxation and thus arterial dilation (79, 123). As previously alluded to, NO is not the only endothelial derived vasodilator as prostaglandins play a compensatory role when NO is chronically inhibited (9, 116) and endothelium-derived hyperpolarizing factor can elicit relaxation and dilation by opening potassium channels in the smooth muscle (115). Additionally, other unknown mechanisms have been proposed to regulate the vasodilatory response when a shear stimulus is sustained (86). Nevertheless,

NO is considered the primary and initial regulator of endothelium-dependent dilation, and therefore, NO-mediated vasodilation has become a widely used surrogate of endothelial function (70).

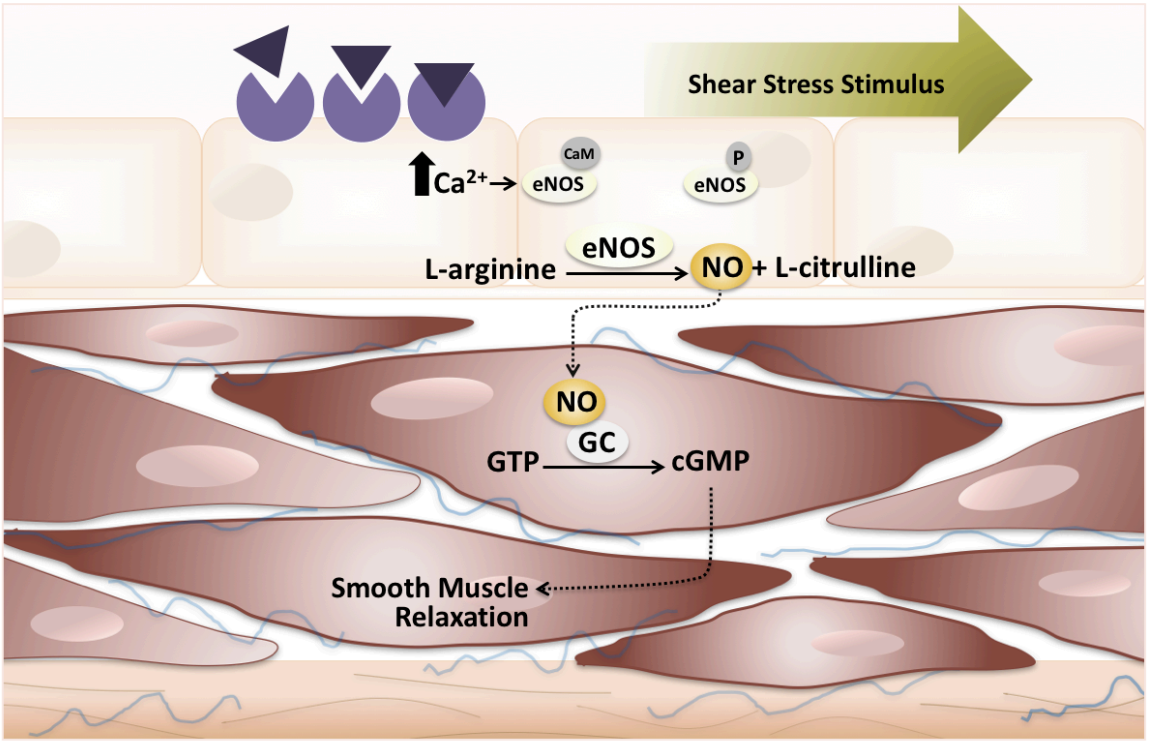

Figure 2. Endothelium-dependent dilation. The binding of ligands to plasma membrane receptors and/or the frictional shear stimulus of flowing blood activate eNOS through calcium-dependent (binding of calmodulin, $eNOS^{CaM}$) or calcium-independent (phosphorylation on serine site, $eNOS^{p}$) mechanisms. Activated eNOS synthesizes NO, a potent vasodilator that induces vascular smooth muscle relaxation and arterial dilation by initiating a G protein signaling cascade (GC, guanylate cyclase; GTP, guanosine triphosphate; cGMP, cyclic guanosine monophosphate).

1.2.4 Flow-Mediated Dilation Test

Popularized in 1992 by Celermajer and colleagues (14), the flow-mediated dilation (FMD) test is now widely used for assessing endothelium-dependent function noninvasively in humans (107). FMD describes the vasodilatory response of an artery to increased blood flow and shear stress. According to standard guidelines for FMD test conduction, using a pneumatic cuff inflated to suprasystolic pressure, an artery is occluded for 5 min in order to produce an ischemic condition. Upon rapid cuff deflation, a brief state of reactive hyperemia (RH) ensues, and the sudden increases in blood flow and shear stress stimulate NO production. Arterial diameter is monitored upstream using brightness mode ultrasonography and is typically quantified using edge-detection software (107). The peak dilatory diameter is then expressed as the absolute (mm) and/or relative (%) change from the baseline diameter. The FMD test infers endothelium-dependent function from the observed dilatory response, with larger responses indicating greater endothelial function, and lower responses indicating endothelial impairment. Sublingual administration of nitroglycerin (NTG), an NO donor, can be used as a control test to examine the downstream, endothelium-independent, response (107). FMD is commonly assessed in the brachial artery as brachial endothelial function has previously been related to coronary endothelial function (2). FMD is also an independent predictor of cardiovascular disease (CVD) risk (39, 124), and a 1% increase in FMD has been associated with a 9-13% decrease in CVD risk (44, 60). In young healthy adults, a brachial FMD response > 6% is considered

normal (117), although clinical cut points have not been established. Furthermore, while the FMD test is seemingly simple to conduct, obtaining reliable data requires highly trained personnel and careful data acquisition and analysis. A measurement is considered reliable if it is associated with a relatively small degree of error and high degree of repeatability (consistency under identical conditions) and/or reproducibility (consistency under different conditions) (5). Several guidelines and tutorials (18, 50, 92, 107) have helped to standardize subject preparation and data acquisition and their adherence has been shown to improve FMD reliability (46). Nevertheless, the variability in repeated brachial artery FMD measurements has ranged from 10-84% in healthy young adults (19, 34, 58, 67, 75, 95, 96, 105) and guideline adherence has only been able to explain ~36% of the discrepancy between studies (46). FMD data analysis likely accounts for some of the unexplained differences in FMD reliability, as it has not been adequately addressed in current guidelines and remains a highly variable process that is dependent on individual laboratory software and internal practices. As such, the FMD test has not been adopted for use in clinical CVD risk assessment but remains a widely used research tool (45).

1.3 POTENTIAL INFLUENCE OF ESTRADIOL ON ENDOTHELIAL FUNCTION

The influence of estrogen on endothelial function has been of particular interest in vascular physiology. It has long been known that premenopausal women have a reduced incidence of CVD compared to age-matched men and age-matched postmenopausal women (61). The cardioprotection afforded to women during the reproductive years has largely been attributed to the sex hormone estrogen, particularly the biologically available variant 17β-estradiol (or simply estradiol). Estradiol is the predominant type of estrogen produced by the ovaries during the premenopausal years, while estrone and estriol are more prevalent after menopause and during pregnancy, respectively (71). Estradiol is also the most potent estrogen, having 12 times the estrogenic potency of estrone and 80 times that of estriol (47). Although primarily considered a female sex hormone, men produce estradiol in the testes and non-gonadal tissues, albeit at much lower levels than women (119). Estradiol concentrations and time course patterns, to which we refer to as an estradiol profile, differ between men, premenopausal women with natural menstrual cycles (NAT), and premenopausal women using combined monophasic OCPs. Moreover, although not central to this thesis, progesterone profiles are also distinct between men, and NAT and OCP women. Therefore, comparing these groups of adults enables us to examine the potential influence of differing estradiol profiles on endothelial function while also investigating the effects of sex, OCP use, and potential interactions of progesterone.

1.3.1 Estradiol Profiles in Premenopausal Women

As mentioned previously, men produce small amounts of estradiol, with the majority being converted from testosterone in non-gonadal tissue (119). In contrast to the consistently low concentrations observed in men, premenopausal women experience large fluctuations in estradiol across a NAT cycle or receive fixed doses of synthetic ethinyl estradiol (EE) for the majority of an OCP cycle (Figure 3).

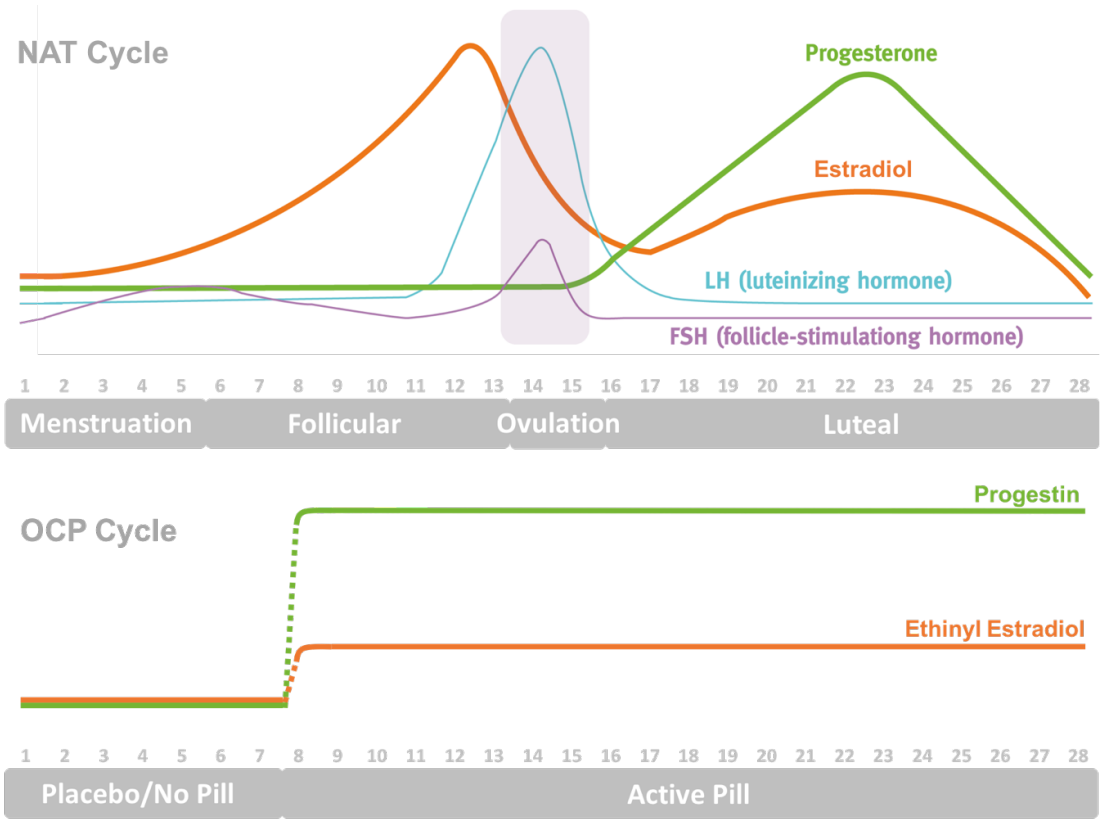

Figure 3. Sex hormone profiles across a natural menstrual cycle (NAT) and combined monophasic oral contraceptive pill cycle (OCP) in women. [NAT cycle adapted from http://www.newhealthadvisor.com/Menstrual-Cycle-Hormones.html]

Natural Menstrual Cycle

In naturally cycling premenopausal women, ovarian production of estradiol varies across the different menstrual cycle phases. By convention, the onset of menstruation marks the first day of a cycle, which is comprised of a follicular phase, ovulation, and a luteal phase. Menstruation often lasts 3 to 6 days and is regarded as the *early* follicular phase (48). However, for ease of clarity, this thesis distinguishes between menstruation and follicular/mid-follicular, with the latter terms indicating the time between menstruation and ovulation. A typical menstrual cycle lasts 28 days but ranges from 26 to 34 days, varying by 1 to 5 days from one cycle to the next (17, 48). This variability in cycle length, both between and within women, results from variability in the length of the follicular phase (first 14 days), as the length of the luteal phase (last 14 days) is highly consistent (8).

During menstruation, estradiol's production rate is low, approximately 80 μg/day (104), resulting in serum concentrations that are comparable to levels observed in men (menstruation, ~110 pmol/L; men, ~115 pmol/L) (53, 63). Throughout the follicular phase, granulosa cells surrounding the growing follicles produce estradiol at a rate of 440-940 μg/day (8, 47, 104), causing serum estradiol to reach pre-ovulation concentrations of 550-1285 pmol/L (63). Peak estradiol concentrations typically occur 1 to 3 days prior to the mid-cycle surge in luteinizing hormone that triggers ovulation (94, 106). Lastly, during the luteal phase, the corpus luteum forms from the granulosa cells and secretes some estradiol (270 μg/day) but mostly progesterone (25,000 μg/day) (8, 47, 104). The luteal phase is

therefore characterized by a large surge in progesterone, and a second modest increase in estradiol (367-771 pmol/L) (63), with both hormones peaking approximately 1 week after ovulation (8). If an ovum is not fertilized, the corpus luteum degenerates and the production of estradiol and progesterone ceases, prompting the shedding of the uterine lining and initiating the subsequent cycle (47). Throughout the menstrual cycle, the majority of estradiol and progesterone circulating in the blood are bound to carrier proteins, but approximately 2% are unbound and free to enter target cells (8).

Combined Monophasic Oral Contraceptive Pill Cycle

A combined monophasic OCP contains fixed doses of EE and a progestin, with progestin being the main active ingredient (40). To mimic a 28 day menstrual cycle, a typical OCP cycle is comprised of a 7 day placebo/no pill phase that triggers withdrawal bleeding and a 21 day active pill phase (40). Fixed doses of EE and progestin in the active pill phase inhibit the release of regulatory gonadotropin hormones, effectively preventing follicular maturation and ovulation (91). By interrupting the regular menstrual cycle, OCPs also suppress ovarian production of endogenous estradiol and progesterone (28). Nevertheless, EE and progestin exert the same biological effects as their endogenous counterparts, and EE interacts with ERs in much the same way as estradiol (104). Owing to a high first-pass metabolism in the gastrointestinal tract and liver, approximately 45% of an EE dose enters circulation (80). Serum EE concentrations following the ingestion of

combined monophasic OCPs containing 30-35 μg EE have been shown to range between 277 and 354 pmol/L (12, 25), comparable to estradiol concentrations observed in the mid-follicular phase of a menstrual cycle (106). Serum EE levels may also increase by 30-60% by the end of a cycle; however, considerable intra- and inter-individual variability in EE bioavailability exists (25, 80, 103). Regardless, the estradiol profile in women taking combined monophasic OCPs differs from both men and naturally cycling women, as EE and progestin are simultaneously elevated for the majority of a cycle in those taking OCPs.

Over the years, modifications have been made to EE doses and the progestin component, resulting in the establishment of four different generations of OCPs. First generation pills are rarely still in use as they contained high doses of EE (50 μg) that caused major adverse side effects (40). Subsequent second, third, and fourth generation pills all contain lower doses of EE (20-35 μg) but differ in the composition and concentration of progestins as they were progressively designed to elicit fewer androgenic effects (40). Similar to endogenous progesterone, progestins are capable of interacting with steroid hormone receptors other than just progesterone receptors (100). Both second and third generation progestins are testosterone derivatives that have an affinity for binding to androgen receptors. Second generation progestins (i.e. levonorgestrel) are the most androgenic with a 70% binding affinity to androgen receptors, while third generation progestins (i.e. desogestrel) have fewer androgenic properties and a reduced binding affinity of 40% (64, 99). Further modifications with progressive OCP generations resulted in

the development of fourth generation progestins (i.e. drospirenone) that are progesterone derivatives and have anti-androgenic properties (99). Therefore, OCPs provide different hormonal profiles which can be employed in examinations of the influence of estradiol, and its potential interactions with progesterone, on brachial artery FMD, as well as the influence of different progestin generations.

1.3.2 Estradiol-Mediated Dilation

Investigations in animal models and cultured endothelial cells have shown that estradiol increases NO production (54, 55). There are several mechanisms by which estradiol can interact with ERs on the endothelium and vascular smooth muscle to increase NO-bioavailability and possibly augment brachial artery FMD responses (77). ERs (ERα and ERβ) are nuclear hormone receptors that, once activated by the binding of estradiol, can elicit genomic or nongenomic signaling mechanisms (57). Most ERs are situated in the nucleus and cytosol but approximately 5-10% are localized on the plasma membrane (65). Through mechanisms involving the translocation of ERs to the nucleus and the upregulation of gene transcription, estradiol can increase eNOS gene expression, thereby increasing NO bioavailability (77, 85). The mechanism involving gene transcription is said to be *genomic* and takes hours or days from exposure to effect. Estradiol can also elicit rapid *nongenomic* vasodilation within 5 to 20 min of its exposure (76) by initiating an influx of Ca^{2+} and activating protein kinases (57). Several ER isoforms have been identified and shorter variants of ERα appear to exclusively

mediate estradiol's rapid activation of eNOS (66, 120). A structurally distinct G protein–coupled estrogen receptor was also discovered to mediate estradiol's rapid dilatory effects (84, 90, 109). It should be noted that progesterone can inhibit estradiol from binding to ERs (16), and inhibit L-arginine from being transported to eNOS (6), potentially antagonizing estradiol's dilatory effects.

1.3.3 Potential Influence of Cycle Phase, Oral Contraceptive Pill Use, and Sex on FMD

In 1995, Hashimoto *et al.* (53) reported that, within a NAT menstrual cycle in pre-menopausal women, brachial artery FMD increased by 7% from the menstrual to follicular phase and remained elevated in the luteal phase despite the surge in progesterone, a phenomenon that has only been replicated by Harris *et al.* (51). In both studies, sex differences (men versus women) in FMD were not apparent during menstruation but emerged when women were tested in the follicular and luteal phases, with women demonstrating larger responses than men (51, 53). In contrast, others have observed smaller increases in brachial FMD of 1-3% during the follicular phase that reversed in the luteal phase (1, 29, 122). Similar increases in brachial FMD of 1-2% have been observed in an OCP cycle from the placebo/no pill phase to the active pill phase, particularly with third or fourth generation, but not second generation, pills (73, 74, 110). Direct comparisons of the brachial FMD response between, and across, a NAT and OCP cycle are lacking, as are comparisons to men. Previous comparisons between NAT and OCP women have been between the menstrual and active pill phases or did not control

for cycle phase (56, 68). Additionally, Hashimoto *et al.* (53) and Harris *et al.* (51) were the only previous studies to have compared men and women; however, sex differences in the resting arterial diameter (smaller brachial artery diameters in women at baseline) were not appropriately accounted for and may have confounded their findings (3). Therefore, while the influence of estradiol on brachial artery FMD has previously been examined, no studies have comprehensively compared its effects between men and premenopausal women across both a natural and OCP cycle.

1.4 POTENTIAL INFLUENCE OF SPRINT INTERVAL TRAINING ON ENDOTHELIAL FUNCTION

With its ability to improve cardiovascular health by exerting direct effects on the vasculature, the influence of exercise on endothelial function is a prominent area of vascular research (43). For health promotion, public health guidelines recommend a minimum of 150 min of aerobic activity per week at moderate-to-vigorous intensity (113). In contrast to traditional endurance training, otherwise known as moderate-intensity continuous training (MICT), interval training involves alternating periods of intense exercise separated by periods of low-intensity recovery (35). When matched for energy expenditure, interval training is an effective alternative to MICT, eliciting comparable, if not superior, changes in endothelial function (88). However, with ‘lack of time’ being a commonly cited barrier for engaging in physical activity (89), there has been growing interest in

interval protocols that involve a lower exercise volume and reduced time commitment.

1.4.1 Low-Volume Sprint Interval Training

Sprint interval training (SIT) is an interval model characterized by “all out” or “supramaximal” effort, with target intensities corresponding to workloads that are greater than what is required to elicit peak oxygen uptake (VO_2peak) (121). In comparison to MICT, which corresponds to ~40-60% of peak power output (PPO), SIT corresponds to ~175-200% of PPO when intervals are averaged (13, 35). Interval protocols involving submaximal intensities are instead differentiated by the term high-intensity interval training (HIIT) (121). Interval training can further be defined as *low-volume* if the summed duration of intense exercise (excluding periods of recovery, warm-up, or cool-down) does not exceed 10 min (35). While not all HIIT protocols are low-volume, as they can range from a 7x1-min protocol to a 5x5-min protocol (31, 93), SIT protocols are low-volume as a result of their intense and brief nature. The classic SIT protocol consists of four to six bouts of 30-s “all out” cycling sprints (Wingate tests) separated by 4.5 min of recovery (35). Despite Wingate-based SIT involving only 2-3 min of intense exercise, the protocol is physically demanding. Additionally, with a total time commitment of ~20-30 min, its purported time efficiency has been questioned (36). With a need for truly time efficient protocols that are more feasible for the general population, it has been recommended that SIT research focus on protocols with fewer and shorter sprints

(118). As such, this thesis reserves the term 'low-volume SIT' to distinguish protocols for which the total time commitment is ≤ 10 min per session. To date, one of the most time-efficient SIT protocols involves just three bouts of 20-s "all out" cycling sprints separated by 2 min of recovery (38). Including a 2 min warm-up and 3 min cool-down, the entire protocol is 10 min. Figure 4 is a schematic highlighting the shorter duration and supramaximal intensity of the 3x20-s SIT protocol relative to traditional MICT. This low-volume SIT protocol has been shown to increase peak aerobic capacity and some metabolic outcomes (37, 38, 78); however, its effects on the vasculature have not been investigated. When assessing the endothelial function response to exercise, it is important to consider the acute response after

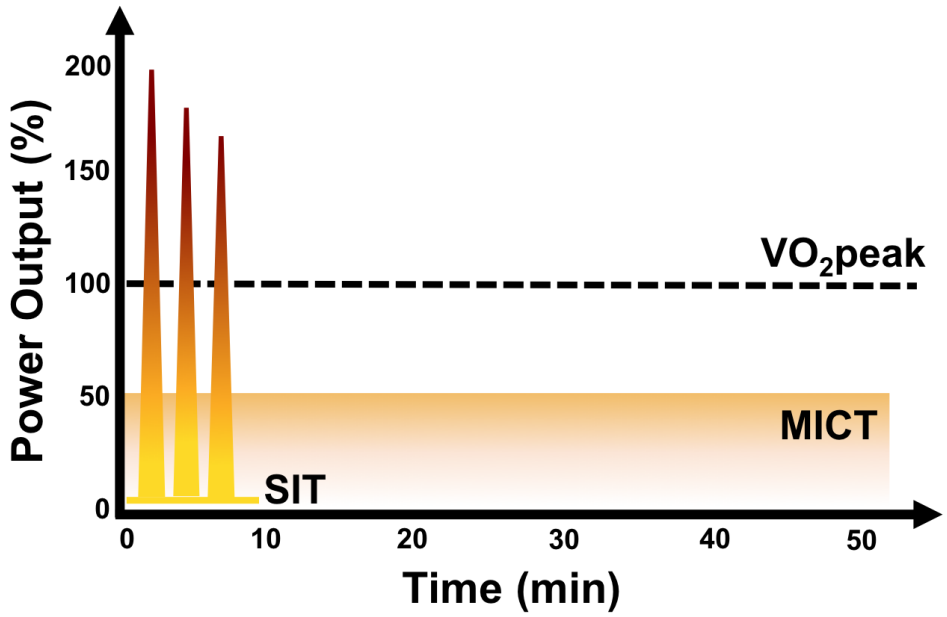

Figure 4. Sprint interval training (SIT) versus traditional moderate-intensity continuous training (MICT) [Adapted from Gibala *et al.* 2014 Sports Med]

a single exercise session as well as the chronic response following a period of exercise training. While investigations of the training response are important for determining the long-term benefits associated with regular exercise, the acute post-exercise response may help elucidate or predict an individual's training response (23). Therefore, by examining both the acute and chronic endothelial function responses to low-volume SIT we can determine whether its physiological benefits extend to the vasculature of healthy young adults.

1.4.2 Potential Acute and Chronic FMD Responses to SIT

To date, the endothelial function response to low-volume exercise has been dominated by HIIT investigations, as studies on the effects of SIT are sparse. Although changes in endothelial function acutely following low-volume HIIT may not necessarily reflect changes following low-volume SIT, brachial artery FMD has been shown to increase, by approximately 1-3%, 1 h after 12x1-min (4), 10x1-min (20), 8x1-min (11) and 7x1-min (31) HIIT protocols. While these increases in brachial artery FMD may seem small, a 1% increase in FMD has been associated with a 9-13% decrease in CVD risk (44, 60), thus changes of 1-1.5% have been deemed clinically and physiologically relevant (60, 112). To date, the lowest exercise volume for which the acute brachial artery FMD response was investigated was a SIT protocol involving 8x20-s cycling sprints (15). In this study, brachial artery FMD was shown to increase, by approximately 8%, immediately after cycling sprints performed at 170% PPO, but not at 100% or 130% PPO (15).

These findings suggest that exercise intensity matters in terms of eliciting acute changes to brachial artery FMD. The authors speculated that the highest exercise intensity likely stimulated higher brachial artery blood flow and shear stress, the main mechanisms by which exercise is purported to exert direct benefits on the endothelium (43). Although blood flow and shear stress have been shown to increase in the inactive brachial artery during continuous lower-limb cycling (42, 108), the arterial shear stress profile has yet to be characterized in either the active or inactive limbs during a HIIT and/or SIT session. Nevertheless, acute studies have demonstrated that interval exercise performed at high/supramaximal intensity, like SIT, can improve the post-exercise FMD response.

In examining the chronic endothelial function response, training studies have demonstrated differential effects of high/supramaximal intensity exercise that is performed in a continuous *versus* intermittent manner. Repeated exposure to high-intensity continuous exercise (i.e. 12 weeks of 30-60 min cycling or running at 70-80% of VO_2peak) has been shown to impair, or not change, the chronic endothelial function response (7, 41). In contrast, supramaximal exercise performed in intervals (i.e. 6 weeks of Wingate-based SIT) has been shown to improve endothelial function to the same degree as MICT, although this study assessed the active popliteal artery and not the inactive brachial artery (87). Even so, the intermittent nature of HIIT or SIT appears to be integral for inducing beneficial responses in the endothelium. This was elegantly demonstrated by Harris *et al.* (49) who compared Wingate-based SIT to a work-matched sprint

continuous training protocol involving a sustained maximal effort sprint. Although a group by time interaction did not reach statistical significance (p=0.08), after 4 weeks of training, brachial artery FMD increased in 67% of participants who completed SIT but decreased in 67% of participants who completed sprint continuous training (49). The study by Harris *et al.* (49) highlights the importance of shorter, repeated bouts of intense exercise, and aligns with *in vitro* research that has demonstrated increases in NO production with rapid and repeated impulses, but not gradual increases, in shear stress (27, 62). Collectively, these findings provide evidence suggesting that despite its low-volume, the 3x20-s SIT protocol has the potential to augment the acute and/or chronic brachial FMD response.

1.4.3 Proposed Time Course for Acute and Chronic FMD Responses

Although not specific to interval training, the acute FMD response has been proposed to decrease immediately after exercise, increase between 1 and 24 h post-exercise, and return to pre-exercise levels by 24 to 48 h (Figure 5) (22). However, it is important to note that several factors relating to exercise and participant characteristics can alter this response pattern. In particular, the immediate post-exercise response may be confounded by oxidative stress, an increased arterial diameter, and/or altered sympathetic activity (81). Estrogen has also been identified as another potential mechanism relating to the post-exercise FMD response (22). Harvey *et al.* (52) previously showed that estrogen replacement therapy and exercise both improved FMD in postmenopausal women

but that their effects were not additive. To date, the potential interaction between exercise and estrogen in premenopausal women has received minimal attention and warrants further investigation. Additionally, it is currently unclear what specific acute FMD response pattern is important for initiating improvements in the chronic FMD response to exercise training. Nevertheless, with continued exercise training, endothelial function has been shown to increase after 2-4 weeks of training. In healthy adults, as training continues, the artery enlarges thereby 'normalizing' the recurring elevations in the shear stimulus and returning endothelial function to pre-training levels (Figure 5) (111). Therefore, in order to gain a more comprehensive understanding of the effects of low-volume SIT on the vasculature, it is important to examine both the acute and chronic brachial artery FMD responses.

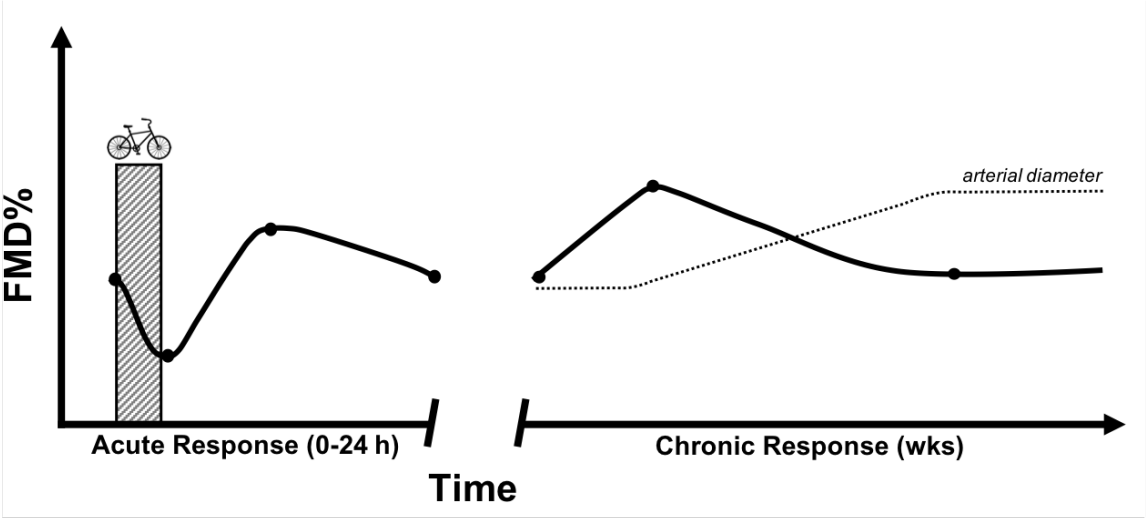

Figure 5. Proposed time course for acute and chronic changes in brachial artery FMD. [Adapted from Dawson *et al.* 2013 JAP and Green 2009 Exerc Sport Sci Rev]

1.5 STUDY OBJECTIVES AND HYPOTHESES

The overarching purpose of this thesis is to investigate factors that influence brachial artery endothelial function in healthy young adults. Of particular interest are the independent and combined effects of the sex hormone estradiol and the effects of low-volume sprint interval exercise as both an acute and chronic (training) stimulus.

In Chapter 2, we address a current gap in FMD guidelines by establishing best practices for FMD data analysis. We highlight the importance of maintaining consistency during image acquisition and analysis and demonstrate that the RH dilatory response pattern is highly consistent within an individual. Given this observation of intra-individual FMD response pattern consistency, we introduce visual data screening as a useful tool for ensuring that differences between serial FMD responses within an individual are not spurious, but reflective of physiological changes in endothelial function.

In Chapter 3, we investigate the role of estradiol on endothelial function. Specifically, we sought to comprehensively compare resting brachial artery endothelial function between men, premenopausal women with natural menstrual cycles, and premenopausal women taking combined monophasic OCPs, while investigating potential changes across a NAT or OCP cycle. We hypothesized that brachial artery endothelial function would be similar between men and women when estradiol levels are comparable but elevated in NAT and OCP women during a high-estradiol natural cycle or active pill phase, respectively.

In Chapter 4, we examine the acute endothelial function response to a single session of low-volume SIT in healthy men and premenopausal women. We also sought to explore the potential of estradiol to augment the post-exercise endothelial function response by studying women during a high-estradiol menstrual cycle phase. We hypothesized that endothelial function would be similar between men and women at baseline and would increase at 1 h post-exercise in both sexes. We also speculated that women would demonstrate larger acute increases in FMD compared to men reflective of estradiol further augmenting the post-exercise endothelial function response.

In Chapter 5, we examine the chronic endothelial function response following 12 weeks of SIT or MICT in the inactive upper-limb and active lower-limb of sedentary but otherwise healthy men. We hypothesized that SIT and MICT would elicit comparable increases in brachial and popliteal endothelial function after 6 weeks of training but that structural arterial changes would return endothelial function to pre-training levels by 12 weeks of training.

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CHAPTER 2

Noninvasive assessment of endothelial function:

Best practices for flow-mediated dilation data analysis

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ABSTRACT

The endothelium is a single layer of cells that lines the inner walls of an artery. Among its many functions, the endothelium regulates arterial dilation in response to increases in blood flow. As endothelial impairment precedes overt cardiovascular disease (CVD), endothelial function has become a widely investigated CVD risk marker. Specifically, the flow-mediated dilation (FMD) test is a noninvasive assessment of endothelial function. However, technical challenges relating to the standardization of the FMD test is one of the reasons it has remained a research tool rather than a clinical tool. While guideline and technical papers have helped to standardize FMD methodology, they have focussed mainly on data collection and there remains a gap in knowledge and standardization of practice in terms of data analysis. In this paper, we discuss strategies for the assessment of image quality as it pertains to continuous edge-detection analysis, provide recommendations for obtaining accurate arterial diameter measurements, and highlight statistical considerations for adjusting for resting arterial diameter. Additionally, we demonstrate that the shape of the reactive hyperemia arterial diameter response, although variable between individuals, is consistent within an individual. Utilizing this intra-individual consistency, we introduce visual data screening as a valuable tool for detecting uncharacteristic discrepancies that could indicate potential edge-detection errors with serial FMD measurements. We conclude by providing test-retest repeatability data from a cohort of healthy young men and women when these recommendations are followed.

KEY WORDS

Endothelial function, edge-detection, reproducibility, guidelines

NEW & NOTEWORTHY

The flow-mediated dilation (FMD) test is widely used for assessing endothelial function noninvasively. We consolidate best practices for FMD data analysis, demonstrate intra-individual consistency in the shape of the reactive hyperemia arterial diameter response, and introduce visual data screening as a useful tool for detecting potential arterial wall edge-detection errors. Additionally, we provide representative test-retest repeatability data for a cohort of healthy young men and women when these analysis techniques are applied.

INTRODUCTION

The endothelium is a single layer of endothelial cells that lines the arterial wall and serves as a homeostatic interface between the artery and flowing blood. Endothelial function refers to the endothelium's ability to respond to hemodynamic changes and regulate vascular tone through the production and release of vasoactive factors (14). Developed in 1992, the flow-mediated dilation (FMD) test provides a tool for the non-invasive assessment of endothelial function. The FMD test uses a combination of brightness mode and Doppler ultrasound to monitor the arterial dilatory response to increased blood flow following a period of distal limb ischemia (9, 36). Edge-detection software is commonly used to measure arterial

diameter at rest and throughout reactive hyperemia (RH). FMD is then typically expressed as a percent change between the resting and peak RH diameters, with a larger dilatory response indicative of greater endothelial function.

As originally hypothesized, and later confirmed, FMD is endothelium-dependent and mainly nitric oxide (NO) mediated (21). NO is an important endothelium-derived vasodilator as it helps to maintain the anti-inflammatory and anti-thrombogenic properties of a healthy endothelium (24). A reduction in NO bioavailability disrupts endothelial function, as observed with cardiovascular disease risk factors (13). Consequently, the impaired endothelium takes on pro-inflammatory and pro-thrombogenic properties, facilitating atherosclerotic progression and the development of cardiovascular disease (38). Indeed, peripheral endothelial function, assessed by brachial artery FMD, is associated with coronary endothelial function and is an independent predictor of cardiovascular disease risk (2, 40). As such, the FMD test has become widely used both in clinical-based and basic science cardiovascular research.

While the concept underlying the FMD test is rather simple, to increase blood flow through an artery and monitor the change in diameter, technical challenges relating to the standardization of its measurement is one of the reasons it has not been adopted for use in cardiovascular disease risk assessment (16). The technical execution of the FMD test requires a high degree of vigilance so as to ensure accurate findings and avoid erroneous conclusions. For a comprehensive outline of subject preparation and methodological considerations,

we direct the reader to the FMD guideline paper by Thijssen *et al.* (36), the tutorial by Harris *et al.* (18), and the video article by Rodriguez-Miguelez *et al.* (32). However, while these papers focus mainly on the standardization of data acquisition, the goal of the present paper is to discuss best practices pertinent to data analysis. In line with current recommendations (36), we focus on continuous edge-detection analysis.

I. IMAGE QUALITY

Since data analysis is highly dependent on the quality of the collected images, we begin our discussion by outlining the key characteristics of a high-quality image. In particular, we highlight two features that considerably affect edge-detection analysis: 1) the clarity of visualization of the arterial wall, and 2) the consistency of the artery's position in the ultrasound field of view, particularly during RH. While the specifics of edge-detection analysis are software-dependent, in general, images with clear, and distinct, near and far wall boundaries are preferable. A blurred near wall is often observed when an artery is superficial in the image. In such cases, the boundaries can be enhanced by using the muscle as a standoff (i.e. positioning the biceps directly in between the ultrasound probe and artery), as the homogeneous tissue focuses the ultrasound beam and reduces scatter. Secondly, an artery that maintains a consistent position in the ultrasound field of view presents fewer challenges to accurate boundary detection in comparison to one that shifts position due to probe or participant movement.

Sudden shifts typically result from abrupt movements by the operator or participant, while gradual shifts often result from a slipping probe. In the latter case, operators can use external probe holders to help stabilize the probe's position on the participant's arm and need only to make slight adjustments to the probe if necessary. In any case, excessive shifting of an artery during RH requires that the region of interest (ROI) tracking box be expanded to accommodate movement of the artery. This expansion of the ROI, in turn, increases the likelihood of the edge-detection software incorrectly tracking other echogenic borders. Ultrasonography is a technically challenging skill; thus, we strongly recommend that ultrasound operators master it prior to starting a study. A minimum of 20 h of scanning practice has been suggested for gaining proficiency (20). We recommend that operators also practice analyzing images, prior to data collection, as familiarity with edge-detection software helps in gauging image quality during scans.

II. ELECTROCARDIOGRAM (ECG) GATING

During systole, arterial diameter increases to buffer the increased pressure and volume generated by left ventricular contraction. To account for the cyclic diameter change within a heart cycle, the 2002 FMD guidelines (10) recommended measuring arterial diameter at a consistent phase of the heart cycle. Specifically, end diastole is a stable cardiac phase that is easily identifiable by the ECG R-wave. ECG gating is a feature available in some ultrasound software that allows for automatic R-wave-triggered image acquisition. Alternatively, commercially

available editing software (Sante DICOM Editor, Version 3.1.20; Santesoft, Athens, Greece) can be used to manually extract end-diastolic frames from an acquired image and merge them into a single Digital Imaging and Communications in Medicine (DICOM) file. More recently, however, the 2011 FMD guidelines (36) expressed the emerging opinion that ECG gating may not be necessary after Kizhakekuttu *et al.* (23) demonstrated that averaging diameters over a heart cycle yields similar FMDs as using end-diastolic diameters, and with established labortories reporting similar internal findings (8, 29). Averaging diameters over a heart cycle is appealing when ECG gating is not available, or requires expensive software upgrades, as post-acquisition editing increases analysis time. Nevertheless, averaging diameters over a heart cycle yields more image frames for edge-detection analysis and operator review. Therefore, operators should consider that the apparent time saved by forgoing manual extraction of end-diastolic frames may instead be spent reviewing, and potentially correcting, additional image frames. More importantly, averaging over a heart cycle can be problematic when images of suboptimal quality are analyzed using completely automated edge-detection software. Since operators using fully automated systems cannot correct improperly detected frames, they are forced to delete them and/or exclude them from the analysis. In such cases, entire heart cycles should be removed so as to avoid averaging over partial heart cycles, which can misrepresent the arterial diameter. For the aforementioned reasons, we continue to support the analysis of end-diastolic frames. Nevertheless, whether laboratories

choose to use end-diastolic diameters or continuously average across heart cycles, it is recommended they remain consistent within a study and clearly report the selected method.

III. ARTERIAL DIAMETER ANALYSIS

An artery's dilatory response is expressed as the absolute and relative change between resting and peak RH diameters as per *Eqs.* 1 and 2, respectively.

$$[1]\ \text{FMD}\ (\text{mm}) = \text{peak RH diameter} - \text{resting diameter}$$

$$[2]\ \text{FMD}\ (\%) = \frac{\text{absolute FMD}}{\text{resting diameter}}\ \text{x}\ 100$$

Accurate resting and peak RH diameters are therefore essential for obtaining a true FMD response. In this section, we note several factors that can influence arterial diameter measurements and, wherever possible, present recommendations for increased accuracy.

Arterial Segment Consistency: The clarity and consistency of the echogenic boundaries of an arterial wall can vary along the length of an image, and although the difference is at times subtle, it may be enough to impact the estimated arterial diameter and the calculated FMD response. It is therefore imperative that the arterial segment that is imaged and analyzed at rest and during RH be consistent, both within a single test and between repeated tests. To ensure consistent placement of the ultrasound probe, a non-toxic washable marker can be used to mark the proximal and distal edges of the probe on the biceps. Alternatively, and

more appropriate for tests performed on different days, an anthropometric measuring tape can be used to record the distance from the antecubital fossa to the distal edge of the probe. Additionally, we recommend that ultrasound operators refer to images from a participant's previous test(s) and identify structural features that can serve as landmarks. These anatomical landmarks can be used to guide the orientation of the ultrasound probe during data acquisition, as well as the placement of the ROI during data analysis (Figure 1).

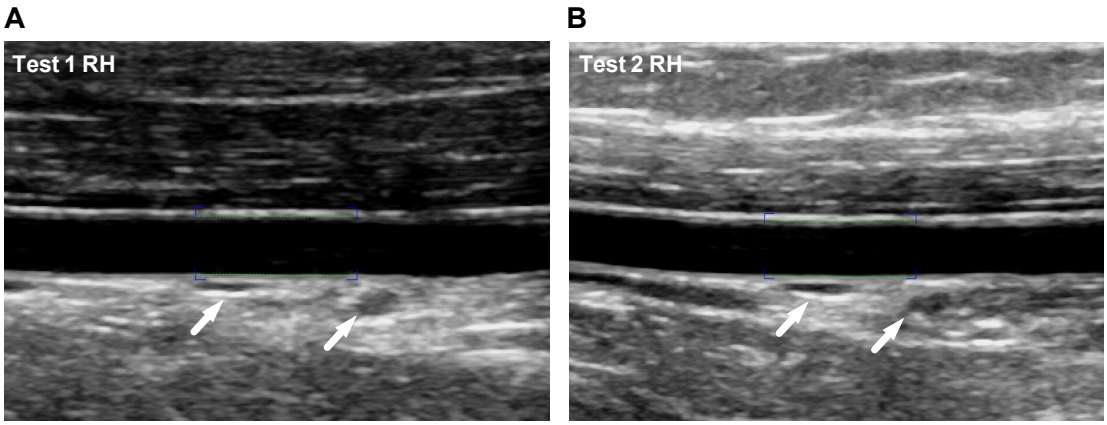

Figure 1. The use of anatomical landmarks (white arrows) to maintain consistency in brachial artery image acquisition and analysis in the same individual for two tests performed on different days.

Software Calibration: Edge-detection software computes arterial diameter as the distance between the echogenic boundaries it detects on the near and far wall of an artery. Therefore, it is important to ensure that the software is appropriately calibrated to the image resolution (i.e., pixels/mm) prior to boundary detection. Edge-detection software may either prompt the operator to select a calibration ROI

or may automatically import the information from the DICOM header. In either case, the calibration should be checked and corrected if necessary.

Boundary Selection: A true arterial diameter reflects the lumen diameter, or the distance between the near and far wall lumen-intima boundaries. However, it is not uncommon for the visibility of the intima layer of the arterial wall to be inconsistent. Since analysis consistency is far more important than a true lumen diameter, we instead recommend analysing the stable media-adventitia boundaries. Operators should also review previously analyzed images from the same participant, as inadvertently detecting the lumen-intima boundaries for one image/test and the media-adventitia boundaries for another image/test, would result in inaccurate FMD responses/comparisons. Special attention should be paid to images in which the intima fades in and out or is not easily distinguishable from the adventitia, as the detected boundaries may also be inconsistent. As edge-detection errors can at times still produce a seemingly typical RH tracing, operators should review each frame of an image clip, particularly where the peak occurs. In cases of improper tracking, operators can redirect the boundary if using semi-automated software or may need to delete the corresponding diameter if using fully automated software.

Identification of Peak RH Diameter: To determine the peak RH diameter, rolling or smoothing averages are applied to the RH tracing. In comparing the effects of 3, 5, and 10 s smoothing averages, Harris *et al.* (18) observed that longer averages

result in a lower peak RH diameter and lower FMD response. With longer averages (i.e., 10 s) abating the true peak and shorter averages (i.e., 3 s or less) confounded by too much 'noise', we support the recommendation by Harris and colleagues that rolling averages of approximately 5 s be used to identify the peak RH diameter. For laboratories analyzing end-diastolic frames, a 5-frame rolling average can be used in place of the 5 s rolling average.

IV. VISUAL DATA SCREENING

Our laboratory has observed that the shape of the RH response, though variable between individuals is often quite consistent within an individual (Figure 2). This consistency makes visual screening a useful tool for identifying data points that could indicate analysis errors. We propose a protocol for visual data screening that involves plotting individual participant resting and RH diameters from all FMD tests onto a single graph. Figure 3 demonstrates consistent resting arterial diameters and RH responses at baseline (Figure 3A), and a maintained RH pattern that is shifted upward as the artery increases in size following an exercise-training intervention (Figure 3B). In the corresponding right panels, the visit 3 resting diameter (black circle) has been altered to demonstrate an uncharacteristic discrepancy that would flag an operator to review the detected contours and/or reanalyze the image. As our laboratory analyzes the media-adventitia boundaries, such a discrepancy could result from the edge-detection software incorrectly tracking the lumen-intima boundaries, or from the operator being inconsistent in

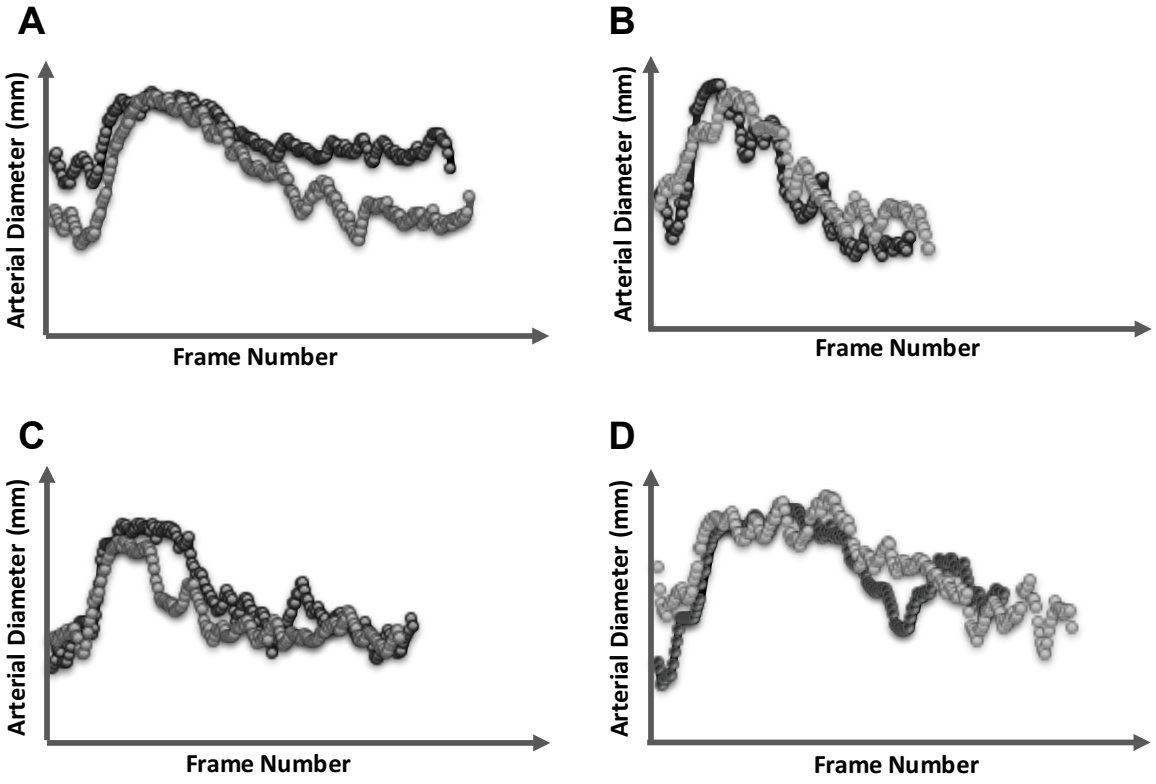

Figure 2. Inter-individual variability but intra-individual consistency of the FMD RH response. RH arterial diameter tracings differ between individuals (panels A to D) but are consistent within an individual.

his or her placement of the ROI. It is important to note that not all discrepancies are the result of analysis errors, as some may in fact be physiological in nature. The goal of visual screening is not to manipulate data, but rather to help identify potential analysis errors that may otherwise go undetected if the operator were to simply review the numerical data. In particular, novice operators may overlook seemingly small errors, as they may be unaware that small differences in arterial diameter can have a large impact on the calculated FMD response. For example,

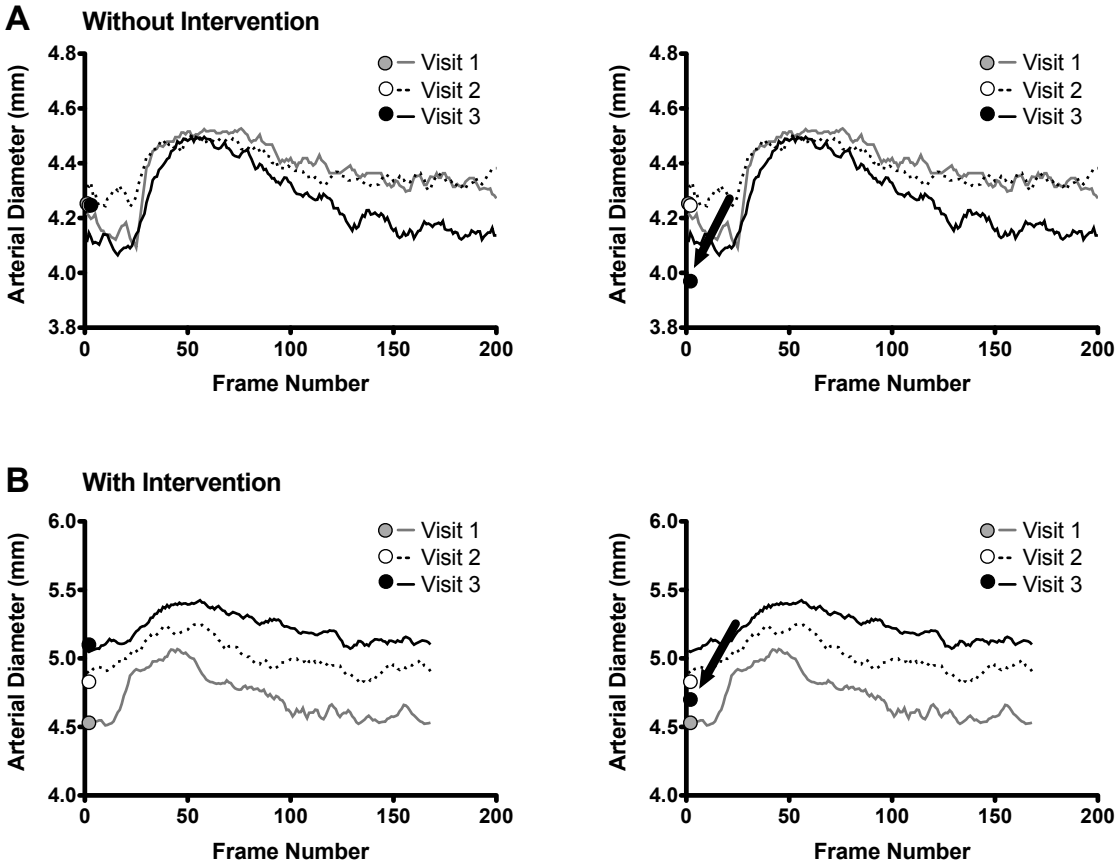

Figure 3. Visual data screening. Resting diameters (circles) and RH arterial diameters (line tracings) at three separate visits from an investigation of FMD repeatability (A) and from an exercise-training intervention (B). Original data is presented in the left panels, and hypothetical errors in visit 3 resting diameter are presented in the right panel (black circles highlighted by arrows).

the 0.3-0.4 mm discrepancies between the original and altered resting diameters presented in Figure 3 more than double the relative FMD response (i.e. from ~6 to 13%). While the provided examples relate to errors in resting diameter, visual screening can also be used to flag potential errors in the RH tracing (i.e. artificial peak, irregular shape, higher/lower position relative to other tests and/or resting diameters etc.). To minimize the impact of operator bias on data analysis, we

recommend that data be visually screened following all resting and RH edge-detection analyses but prior to calculating the FMD response. Additionally, images can be labelled such that operators are blinded to the participant ID, test/visit number, and intervention group. However, in order for visual screening to remain effective, the operator should have knowledge of which resting and RH images correspond to one other, and for repeated tests, which FMD tests belong to the same participant.

V. STATISTICAL CONSIDERATIONS

Since the publication of the landmark paper by Celermajer *et al.* (9), FMD has been typically expressed as a percent change between resting and peak RH diameters (FMD%). Celermajer and colleagues presumably used a percent change to account for differences in arterial size. However, it was evident early on that a negative relationship exists between resting diameter and FMD%, such that smaller arteries demonstrate larger responses compared to bigger arteries, even in healthy subjects (9). The influence of resting diameter on FMD% was acknowledged in the early FMD guidelines (10), but no suitable alternatives were offered. The larger FMD% observed in smaller arteries has been partially attributed to larger levels of shear stress (30), or the frictional force of blood against the endothelium. Normalization of FMD% to the shear stimulus has been proposed (31) but is not endorsed by current FMD guidelines (36).

In recent years, Atkinson and Batterham have focused attention on the

issues associated with using a ratio statistic such as percent change to adjust for arterial size, and have instead advocated for the use of allometric scaling (4, 5, 7). Ratios are effective at controlling for differences in resting arterial diameter only if the relationship between the numerator and denominator is a straight line through the origin (12). A violation of this fundamental assumption results in inaccurate scaling, whereby endothelial function is overestimated for relatively smaller arteries and underestimated for relatively larger arteries. Therefore, non-significant group differences in resting diameter cannot alone substantiate the use of FMD%. The assumption of unity must be verified with linear regression analysis on the natural log-transformed data, with logged peak diameter, $ln(Dpeak)$, as the dependent variable and logged resting diameter, $ln(Drest)$, as the independent predictor. An unstandardized β coefficient deviating from 1 and 95% confidence intervals with an upper limit less than 1 indicate a violation of the ratio assumption (5).

To appropriately adjust for differences in resting diameter, Atkinson and Batterham recommend an analysis of covariance (ANCOVA) on the natural log-transformed data, with $[ln(Dpeak) - ln(Drest)]$ as the dependent variable and $ln(Drest)$ as the covariate. Although the shear stimulus can be included in the model as an additional covariate (6), advancements in this area suggest it may not be necessary (3). The ANCOVA estimated means are then back-transformed to obtain scaled FMD% for group averages, while the regression slope is used to obtain a scaled FMD ratio for individual participants (6, 7). It is currently unclear whether scaled FMD for individual participants can be expressed as a percent for

conventional interpretation. Furthermore, while Atkinson and Batterham have appended allometric scaling instructions for a cross-sectional, between group comparison (6), similar step-by-step instructions are needed for longitudinal repeated measures and mixed model comparisons. As allometric scaling is still in its infancy and not yet widely adopted, we recommend that absolute and relative FMD continue to be reported alongside scaled FMD%. Additionally, it may be beneficial to report the slope and 95% confidence intervals of the regression analysis.

VI. TEST-RETEST REPEATABILITY

Utilizing the recommendations and procedures presented in this paper, we provide test-retest repeatability data from a cohort of healthy young men (n=20, 21 ± 1 yr), and women with natural menstrual cycles (n=15, 22 ± 3 yr). Group-averaged resting diameters and RH diameter traces for men and women are shown in Figure 4. Men underwent three brachial artery FMD tests scheduled one week apart. Women underwent five FMD tests scheduled on different days of a single menstrual cycle (average cycle length: 32 ± 4 days): two consecutive visits in the menstrual phase (days 2-5), two consecutive visits in the follicular phase (days 8-13), and one visit in the luteal phase (5-11 days post-ovulation). All participants were free of any known cardiovascular disease and were not using long-term drug prescriptions (i.e. statins, vasoactive medication, anti-inflammatory medication). Intraclass correlation coefficients (ICC; two-way random for within

phase repeatability and two-way mixed for cycle repeatability; single measures reported) and coefficients of variance (CV; $[(SD/mean)\ x\ 100]$) are presented in Table 1.

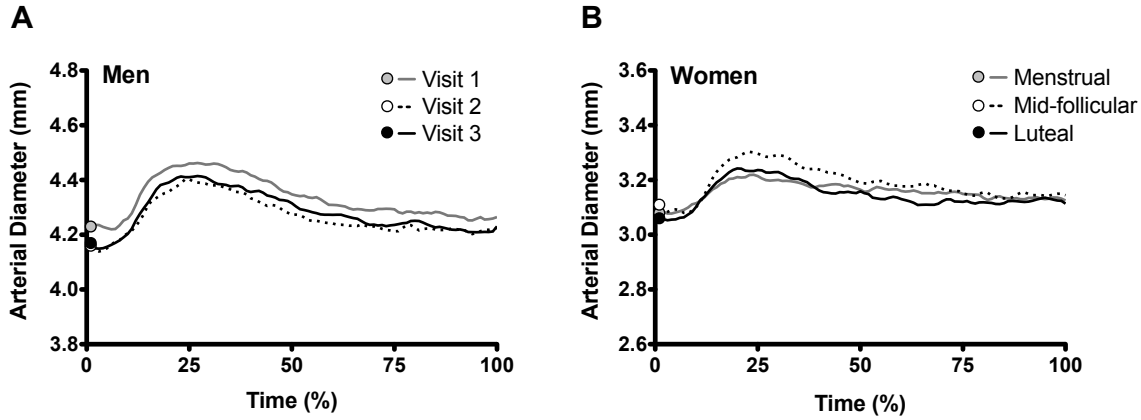

Figure 4. Group-averaged resting diameters (circles) and RH traces (lines) in men (A) and women (B) at three separate visits. RH data were linearly interpolated to 100 discrete points and expressed as a percentage of the 3-min RH period.

We observed high test-retest repeatability for resting arterial diameter (ICC, 0.91-0.98; CV, 1-2%), with little difference between sexes. Others have observed resting diameter ICCs of 0.81-0.99 (15, 19, 22, 25, 28) and CVs of 2-13% (11, 15, 20, 28, 34). While our ICCs are comparable to what has been previously reported in the literature, our lower CVs suggest less variability in our resting diameter data. With regards to relative FMD, test-retest repeatability was highest in men (ICC, 0.93; CV, 10%), slightly lower within women's menstrual (ICC, 0.80; CV, 20%) and mid-follicular phases (ICC, 0.76; CV, 20%), and lowest across the menstrual cycle (ICC, 0.60; CV, 25%). FMD repeatability in the literature has been variable, with studies in healthy young adults reporting acceptable (ICC, 0.60-0.92; CV, 10-28%)

(15, 22, 25, 28, 35, 37) and poor repeatability (ICC, 0.1; CV, 42-84%) (20, 26, 27, 33, 34). To our knowledge, no other studies have reported sex-specific repeatability data or compared FMD repeatability within women's menstrual cycle phases and across a single menstrual cycle.

Table 1. FMD Repeatability in Men and Women

	Men	Women		
		Menstrual	Mid-Follicular	Cycle
ICC (r)				
Resting Diameter	0.91	0.98	0.97	0.94
Peak RH Diameter	0.89	0.94	0.95	0.92
Absolute FMD	0.92	0.71	0.62	0.42
Relative FMD	0.93	0.80	0.76	0.60
CV (%)				
Resting Diameter	2	1	1	2
Peak RH Diameter	2	2	2	3
Absolute FMD	10	20	21	24
Relative FMD	10	20	20	25

ICC, intraclass correlation coefficient; CV, coefficient of variance; RH, reactive hyperemia; Men, 3 visits one week apart; Menstrual, 2-5 days after onset of menstruation; Mid-follicular, 8-13 days after onset of menstruation; Cycle, across menstrual (Visit 1), mid-follicular (Visit 4), and luteal (Visit 5) phases.

With some studies suggesting that estradiol influences the FMD response (1, 39), it could be speculated that the slightly reduced FMD repeatability we observed in women is the result of natural fluctuations in estradiol concentrations across the menstrual cycle. However, estradiol is stable during the menstrual phase, and comparable to levels observed in men. Therefore, we would have

expected menstrual phase FMD repeatability to have been more similar to men than to women's mid-follicular phase when estradiol concentrations are on the rise. While the potential influence of sex hormones on FMD repeatability remains to be elucidated, our data suggest that FMD repeatability is higher within women's menstrual and mid-follicular phases, but still acceptable across a single menstrual cycle. In summary, applying the recommendations outlined in this paper resulted in high and acceptable arterial diameter and FMD test-retest repeatability for healthy young men and women. With limited studies reporting FMD repeatability (17), and with a large discrepancy in what has been previously reported in the literature, we encourage more researchers to incorporate FMD repeatability data in their publications.

CONCLUDING REMARKS

FMD is a widely used test for assessing endothelial function noninvasively. FMD is easily calculated from measurements of resting and peak RH diameters, which can be obtained using continuous edge-detection software. However, poor quality images and inconsistencies in edge-detection can greatly affect the FMD analysis. In this paper, we provided strategies for improving arterial diameter accuracy and FMD reproducibility. We also demonstrated intra-individual consistency in the RH tracings and introduced visual data screening as a useful tool for detecting potential analysis errors. Lastly, we demonstrated high test-retest repeatability and low variability in healthy young adults when these

recommendations were applied.

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CHAPTER 3

Brachial artery endothelial function is stable across a menstrual and oral contraceptive pill cycle, but lower in premenopausal women than age-matched men

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ABSTRACT

Sex hormone concentrations differ between men, premenopausal women with natural menstrual cycles (NAT), and premenopausal women using oral contraceptive pills (OCP), as well as across menstrual or OCP phases. This study sought to investigate how differences in sex hormones, particularly estradiol, between men and women and across cycle phases, may influence brachial artery endothelial function. Fifty-three healthy adults (22±3 yr; 20 men, 15 NAT, 18 second, third or fourth generation OCP) underwent assessments of sex hormones and endothelial (flow-mediated dilation test, FMD) and smooth muscle (nitroglycerin test, NTG) function. Men were tested three times at one-week intervals, and women were tested three times throughout a single menstrual or OCP cycle (NAT: menstrual, mid-follicular, luteal; OCP: placebo/no pill, 'early' and 'late' active pill). Endogenous estradiol concentration was comparable between men and women in their NAT menstrual or OCP placebo phase ($p=0.36$) but increased throughout a NAT cycle ($p<0.001$). Allometrically scaled FMD did not change across a NAT or OCP cycle but was lower in both groups of women than men ($p=0.005$), whereas scaled NTG was lower only in NAT women ($p=0.001$). Changes in estradiol across a NAT cycle were not associated with changes in relative FMD ($r^2=0.01$, $p=0.62$) or NTG ($r^2=0.09$, $p=0.13$). Duration of OCP use was negatively associated with the average relative FMD for second generation OCP users only ($r=-0.65$, $p=0.04$). Our findings suggest that brachial endothelial function

is unaffected by cyclic hormonal changes in premenopausal women but may be negatively impacted by longer term use of second generation OCPs.

KEYWORDS

Flow-mediated dilation, estrogen, sex differences, pill generation, endothelium-independent function

NEW & NOTEWORTHY

We demonstrate that brachial artery flow-mediated dilation (FMD) does not change across a menstrual or oral contraceptive pill (OCP) cycle in premenopausal women but is lower in women than men. Although unaffected by within-cycle changes in estradiol, brachial FMD is negatively correlated with duration of OCP use for second-generation pills.

INTRODUCTION

Brachial artery endothelial function is an indicator of arterial health and an independent predictor of cardiovascular disease (CVD) risk (38). Compared to age-matched men, premenopausal women have a lower incidence of CVD that then rises steadily after menopause (17). The cardioprotection observed in premenopausal women has been attributed to the sex hormone estrogen, particularly 17β-estradiol (or simply estradiol). In contrast to the low and steady estradiol concentrations observed in men, premenopausal women with natural

menstrual cycles (NAT) or those using combined monophasic oral contraceptive pills (OCPs) experience cyclic changes in endogenous or synthetic estradiol, respectively. Differences in estradiol levels and patterns between men and women, and across a NAT or OCP cycle, may elicit differential responses in brachial artery endothelial function.

Brachial artery endothelial function can be assessed noninvasively using the flow-mediated dilation (FMD) test (31). Brachial artery FMD has been observed to increase in the high-estradiol follicular phase of a NAT cycle (1, 7, 12, 13, 37), but most studies have not actually assessed the relationship between FMD and estradiol. Progesterone has also been suggested to antagonize the beneficial effects of estradiol (3, 5), but while some studies reported that increases in FMD during the follicular phase reversed in the high-progesterone luteal phase (1, 7, 37), other studies reported that increases in FMD were sustained in the luteal phase (12, 13). Given these findings, the ratio of estradiol to progesterone may be useful for elucidating the relationship between sex hormone concentrations and changes in FMD across a NAT cycle but this ratio has not been reported in previous studies.

To date, only two studies have compared brachial artery FMD between men and premenopausal women across different cycle phases. While both studies found women to have a larger FMD response than men during high-estradiol phases (12, 13), neither study accounted for women having smaller arterial diameters, likely confounding their findings (2, 19). Moreover, neither study

included women using OCPs despite an estimated 67 million women using OCPs worldwide (34). Although some studies have compared endothelial function between OCP and NAT women, their findings are inconclusive with reports of OCP women having larger (20), smaller (14, 21), and similar (10, 16, 35) responses. These differences may stem from studies not controlling for cycle phase (14), comparing between the menstrual and active pill phases (21), or assessing resistance vessel function (16, 20, 35), which may not be reflective of conduit vessel function (11).

Additionally, previous comparisons between OCP and NAT women have not always controlled for, or investigated, the effects of different pill generations or duration of OCP use. OCP generations differ in progestin type, which has been shown to strongly predict endothelial function (8). Short-term *versus* long-term use of OCPs may also influence the endothelial function response. Although brachial artery endothelial function is unchanged following 6-months of OCP use (10, 35), other studies suggest it may be inversely associated with prolonged use of OCPs (8, 14). With previous studies observing a confounding effect of age (8) or a negative but nonsignificant relationship (14), additional investigations on the influence of the duration of OCP use on endothelial function, for different pill generations, are warranted.

Therefore, to gain a more comprehensive understanding of the effects of estradiol, menstrual cycle and pill cycle phases, this study sought to compare brachial artery endothelium-dependent and independent function between age-

matched, healthy young men, naturally cycling premenopausal women (menstrual, mid-follicular, luteal phases), and premenopausal women using combined monophasic OCPs (placebo/no pill, and early and late active pill phases). We hypothesized that brachial artery endothelial function would be comparable between men, the menstrual phase of naturally cycling women, and the placebo/no pill phase of women using OCPs. We further hypothesized that endothelial function would be elevated in the mid-follicular and active pill phases of a menstrual and pill cycle, respectively, compared to all other phases and compared to men. As a secondary objective, this study investigated the influence of OCP generation and duration of OCP use on endothelial and smooth muscle function.

METHODS

Participants

Fifty-six healthy, recreationally active adults between the ages of 18 and 32 yr were recruited through poster advertisement in and around McMaster University. The arterial stiffness profiles of the same participants are reported in a parallel manuscript (Priest *et al.*, in review). Participants included healthy young men (n=20), premenopausal women with natural menstrual cycles (NAT, n=18), and premenopausal women using monophasic oral contraceptive pills (OCP, n=18). NAT women were excluded if they experienced anovulatory and/or irregular menstrual cycles defined through monitoring of the timing of ovulation and menstruation, had cycles exceeding 40 days in length, were pregnant, or used

hormonal contraceptives within the 12 months prior to recruitment. OCP women were excluded if they were using any methods of hormonal contraception other than a combined, cyclic, low-dose monophasic OCP (i.e. pill, patch, ring, intrauterine device) or if they were using a first-generation pill, progestin-only pill, continuous pill, or combined multiphasic pill. OCP women were using a second-generation (n=10), third-generation (n=3), or fourth-generation (n=5) pill for an average of 41 months (range: 5 to 144 months). Additional exclusion criteria for all participants included any known cardiovascular disease or use of long-term drug prescriptions including statins and vasoactive medication. This study was approved by the Hamilton Integrated Research Ethics Board (#0507) and conformed to the Declaration of Helsinki. Written informed consent was obtained from participants before participation.

Study Design

All study visits were conducted in the Vascular Dynamics Laboratory at McMaster University. Participants attended an initial visit where they were screened for eligibility, provided written informed consent, and were familiarized with laboratory techniques. Men underwent three morning testing sessions scheduled the same day and time, one week apart (e.g., three consecutive Mondays at 9 AM). NAT women completed a minimum of one monitoring cycle, during which they tracked the first and last day of menstruation and the day of a positive ovulation test result (BFP Mid-Stream Ovulation Test; Fairhaven Health).

During their subsequent testing cycle, NAT women continued to monitor for ovulation and underwent three morning testing sessions scheduled during the menstrual, mid-follicular, and luteal phases of a single menstrual cycle. OCP women also underwent three morning testing sessions scheduled during the placebo/no pill phase (withdrawal bleeding), early in the active pill phase, and later in the active pill phase. Figure 1 depicts the study design. Menstrual and pill cycle characteristics, along with the timing of women's testing sessions, are detailed in

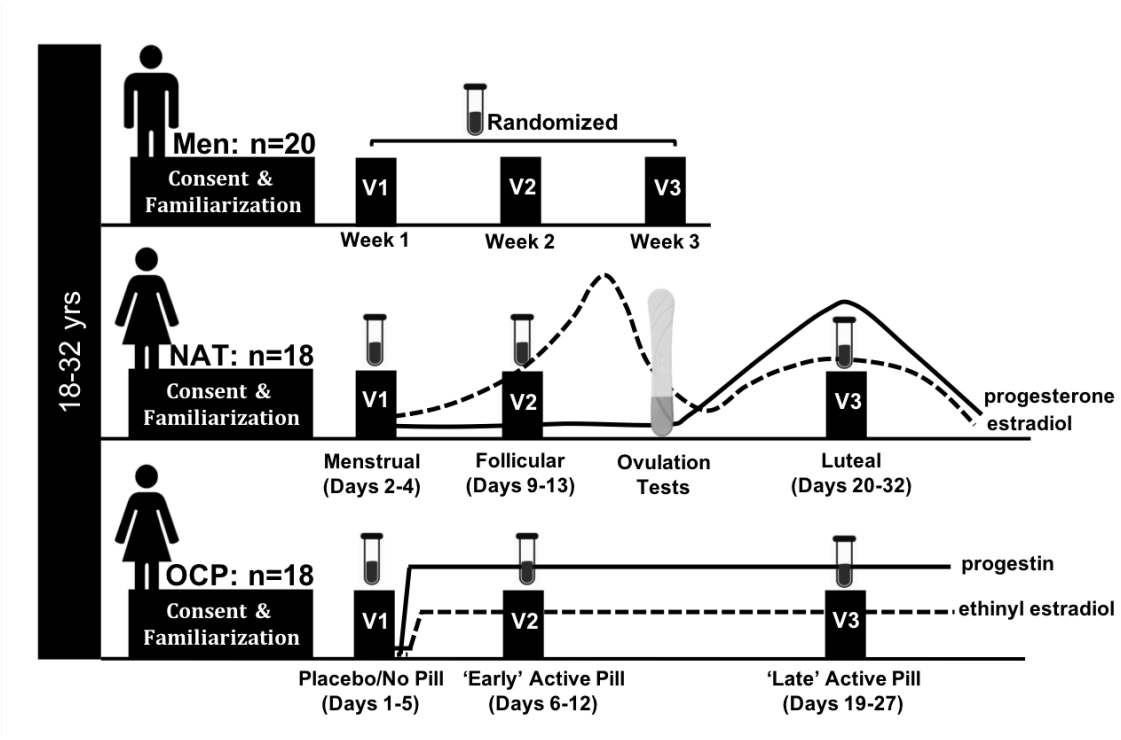

Figure 1. Study Design. Men underwent weekly testing sessions with a fasted blood sample randomized at one of the three visits. Women with natural menstrual cycles (NAT, cycle length: 22-37 days) underwent three testing sessions scheduled during the menstrual, mid-follicular, and luteal phases of a single cycle. Women taking monophasic oral contraceptive pills (OCP, cycle length: 28 days) underwent three testing sessions scheduled during the placebo/no pill phase (withdrawal bleeding), and early and late active pill phase to correspond with the timing of NAT women's visits.

the section below. Prior to all testing sessions, all participants were asked to fast overnight for 10 h (only water permitted) and refrain from moderate-to-vigorous intensity activity for 24 h. Anthropometric measures of height (m) and weight (kg) were obtained at the first visit and used to determine body mass index (BMI, kg/m^2). Blood samples for sex hormone analyses were obtained at the start of a visit, and vascular assessments were made after a 10-min supine rest in a quiet, temperature-controlled room. Resting hemodynamics were monitored continuously with a single-lead ECG (PowerLab model ML 132; ADInstruments Inc., Colorado Springs, CO, USA) and beat-to-beat finger blood pressure (Finometer MIDI, Finapres Medical Systems; Amsterdam, The Netherlands).

Menstrual and Pill Cycle Characteristics

NAT women had an average menstrual cycle length of 32 days (range: 22-37), with menstruation lasting 6 days (range: 4-7) and ovulation occurring 19 days (range: 13-23) after the onset of menstruation. On average, testing for NAT women occurred on day 3 (range: 2-4) for the menstrual phase, day 11 (range: 9-13) for the mid-follicular phase (6±1 days after end of menstruation and 7±3 days prior to positive ovulation), and day 26 (range: 20-32) for the luteal phase (7±2 days post-ovulation and 6±2 days prior to end of cycle). OCP women had a standard 28-day pill cycle, with 7 days of a placebo/ no pill phase and 21 days of an active pill phase. On average, testing for OCP women occurred on day 3 (range: 1-5) for the placebo/no pill phase relative to the onset of withdrawal bleeding (5±2 days into

the placebo/no pill phase), day 10 (range: 6-12) for the early active pill phase (5±1 days into the active pill phase), and day 23 (range: 19-27) for the late active pill phase (18±2 days into the active pill phase).

Sex Hormones

Standard venipuncture techniques were used to collect two 4 mL blood samples (BD Vacutainer Plus, Red BD Hemogard Closure, Franklin Lakes, NJ) at one randomized testing session for men and at each testing session for NAT and OCP women. Samples were set aside for 45-60 min to allow for clotting prior to being spun at 4000 rpm at 4°C for 10 min (Sorvall Legend XTR Centrifuge, Thermo Scientific, Waltham, MA). Serum was aliquoted into three polypropylene tubes (Falcon, Corning Science, Corning, NY) and frozen at -20°C. Frozen samples were sent to the Hamilton Regional Medicine Program Core Laboratory for analysis of endogenous concentrations of serum 17β-estradiol (E2; Architect Estradiol Chemiluminescent Microparticle Immunoassay; Abbott Diagnostics, Abbott Park, IL; sensitivity, <92 pmol/L), progesterone (Architect Progesterone Chemiluminescent Microparticle Immunoassay; Abbott Diagnostics, Abbott Park, IL; sensitivity, <0.3 nmol/L) and testosterone (Immulite 2000 chemiluminescent enzyme immunoassay; Siemens Healthcare Diagnostics, Tarrytown, NY; sensitivity, <0.7 nmol/L). Estradiol and progesterone concentrations were used to calculate the estradiol to progesterone (E2/Prog) ratio. Estradiol concentrations for samples obtained during the active pill phase were below the detection limit of the

17β-estradiol assay (<92 pmol/l for both), which has no cross-reactivity with the synthetic ethinyl estradiol (EE) found in OCPs, confirming that OCP use suppressed endogenous sex hormones (28).

Flow-Mediated Dilation Test

Endothelium-dependent function of the brachial artery was measured using the non-invasive flow-mediated dilation (FMD) test (4). In accordance with current guidelines (31), a blood pressure cuff positioned on the right forearm was rapidly inflated to 200 mmHg for 5 min in order to occlude blood flow to the distal vascular bed. Using a 12 MHz linear array probe and ultrasound with simultaneous electrocardiogram (ECG) (Vivid Q, GE Medical Systems, Horten, Norway), longitudinal images of the right brachial artery were obtained proximal to the antecubital fossa. All images were collected in duplex mode to obtain both brightness-mode images and pulse wave velocity profiles, with an insonation angle of 68° to maximize image quality (26). A 30-s resting image was acquired prior to cuff inflation and a continuous reactive hyperemia (RH) image was acquired from 5 s prior to cuff deflation up to 3 min afterwards. Images were stored in Digital Imaging and Communications in Medicine (DICOM) format. End-diastolic frames were extracted from each heart cycle and compiled into a new standard DICOM file (Sante DICOM Editor, Version 3.1.20; Santesoft, Athens, Greece), which was analyzed for arterial diameters using semi-automated edge-tracking software (Artery Measurement System (AMS) II, Version 1.141; Gothenburg, Sweden) (36).

Resting diameter was determined by averaging the end-diastolic diameters obtained from the 30-s resting image. Peak RH diameter was identified as the largest 5-cycle rolling average of the end-diastolic RH diameters. The resting and peak RH diameters were then used to calculate the absolute and relative changes in FMD, as per *Eqs. 1* and *2*:

$$[1]\ \text{FMD}\ (\text{mm}) = \ \text{peak RH diameter} - \text{resting diameter}$$

$$[2]\ \text{FMD}\ (\%) = \frac{\text{absolute FMD}}{\text{resting diameter}}\ \text{x}\ 100$$

Mean blood velocity (MBV) was obtained from offline analysis of the pulse wave velocity profile (LabChart 7, ADInstruments Inc., Colorado Springs, CO, USA). MBV (cm/s) and arterial diameters (cm; 5-cycle rolling averages) were used to calculate blood flow (BF) and shear rate (SR), as per *Eqs. 3 and 4*. We report the peak post-deflation BF (peak RH BF), the MBV averaged to the peak RH diameter (MBV to peak), the SR area under the curve to the peak RH diameter (SR AUC to peak), and the time to peak RH diameter (time to peak).

$$[3]\ \text{BF}\ (\text{ml/min}) = \ (\pi \text{r}^2 \text{x MBV})\ \text{x}\ 60\ ,\ \ \text{where}\ r = (\text{arterial diameter}/2)$$

$$[4]\ \text{SR}\ (\text{s}^{-1}) = \frac{\text{MBV x 8}}{\text{arterial diameter}}$$

Nitroglycerin Test

Endothelium-independent dilation, an index of smooth muscle cell function, was assessed using an exogenous nitric-oxide donor after a 10-min rest. A 30-s

resting image of the brachial artery was acquired, after which participants received a 0.4 mg sublingual dose of nitroglycerin (NTG). Following NTG administration, 30-s images were obtained every minute for 10 min. For consistency, the same ultrasound settings were retained from the FMD test. Resting diameter and peak NTG diameter were used to calculate the absolute and relative changes in NTG, as per *Eqs. 5* and *6*:

$$[5]\ \text{NTG (mm)} = \text{peak NTG diameter} - \text{resting diameter}$$

$$[6]\ \text{NTG (\%)} = \frac{\text{absolute NTG}}{\text{resting diameter}}\ \text{x}\ 100$$

Statistical Analyses

All data were assessed for normality using Shapiro-Wilk tests. Sex hormone data was not normally distributed and analyzed using nonparametric tests. Differences in sex hormone concentrations across a single NAT cycle were analyzed using Friedman tests with significant effects followed with Wilcoxon signed-rank tests. Differences between men, OCP placebo/no pill phase, and each NAT cycle phase were analyzed using a Kruskal-Wallis test with significant effects followed with Mann Whitney U tests. A Bonferroni correction ($p<0.017$) was applied for multiple comparisons to reduce the likelihood of a Type 1 error. Comparisons of FMD and NTG between men's weekly visits, NAT menstrual cycle phases, and OCP cycle phases were examined using a 3 x 3 (group x visit) mixed model ANOVA, and significant interactions or main effects were assessed using Tukey's HSD post hoc test.

To account for sex differences in resting arterial diameter, allometrically scaled FMD and NTG were analyzed using linear mixed model analyses with the diameter change on the natural log scale as the dependent variable (i.e. $lnDpeak - lnDrest$), group and visit as fixed factors, and the log-transformed resting diameter ($lnDrest$) as the covariate. As recommended for allometric scaling, significant interactions or main effects were assessed with pairwise comparisons and Fisher's least significant difference (LSD) post hoc test (2).

To further investigate the potential influence of endogenous estradiol on endothelium-dependent and endothelium-independent function, NAT women were divided into tertiles based on the changes in estradiol from the menstrual to the follicular phase. Independent t-tests were used to compare the corresponding changes in relative FMD and NTG between women with the smallest (lowest tertile) and largest (highest tertile) changes in estradiol. Additionally, a robust regression (*vce(cluster)* option in Stata 14.2) was used to assess the relationship between changes in estradiol, from the menstrual to follicular and follicular to luteal phases, and the corresponding changes in relative FMD and NTG.

To explore the potential influence of OCP generation, a comparison of relative FMD and NTG between women using a second generation pill (n=10) and those using a third or fourth generation pill (n=8) was conducted using a 2 x 3 (pill generation x visit) mixed model ANOVA. To explore the potential influence of the duration of OCP use on endothelium-dependent or endothelium-independent function, Pearson bivariate correlations were used to investigate the relationship

between OCP duration with relative FMD and relative NTG, respectively. Partial correlations were also used to control for the potential influence of age and 95% confidence intervals (95% CI) are presented for all correlations. Statistical analyses were performed using SPSS Statistics (Version 20.0, Chicago, IL), GraphPad Prism (Version 4.0b; La Jolla, CA), and Stata (Version 14.2; College Station, TX). Significance was set at $p \leq 0.05$, normal data are expressed as mean ± standard deviation (SD), and non-normal data as median ± interquartile range (IQ).

RESULTS

Three NAT women were excluded from all data analyses as a result of having an anovulatory testing cycle (n=2), or an uncharacteristic surge in progesterone prior to ovulation (n=1). Participant characteristics for the remaining 53 participants are reported in Table 1.

Sex Hormones

Sex hormones concentrations are reported as median ± IQ for 50 participants (20 men, 15 NAT, 15 OCP) due to an inability to acquire blood samples from 3 OCP women at one or more visit. Estradiol concentrations were comparable between men (119 ± 40 pmol/l) and women during NAT menstrual phase (126 ± 75 pmol/l) and OCP placebo/no pill phase (97 ± 45 pmol/l; p=0.36). Within a NAT cycle, estradiol increased from the menstrual to the follicular phase (230 ± 207 pmol/l, p=0.003 vs. menstrual) and was highest in the luteal phase (649 ± 490

Table 1. Participant Characteristics

	Men (20)	NAT (15)	OCP (18)
Age, yr	21 ± 1	22 ± 3	22 ± 3
BMI, kg/m^2	24 ± 3	22 ± 2	23 ± 4
IPAQ, x10^2 MET-min/week	44 ± 24	32 ± 23	29 ± 22
Cycle length, days		32 ± 4	28 ± 0
OCP duration, months			41 ± 34
OCP generation, n			
Second			10
Third			3
Fourth			5

Data are means ± SD. BMI, body mass index; IPAQ, International Physical Activity Questionnaire; NAT, natural menstrual cycle; OCP, oral contraceptive pill. OCP generation: *Second*, 20 µg ethinyl estradiol (EE) + 100 µg levonorgestrel (n=9, Alesse®, Alysena®) or 30 µg EE + 150 µg levonorgestrel (n=1, Ciclo®); *Third*, 30 µg EE + 150 µg desogestrel (n=2, Mirvala®, Marvelon®) or 35 µg EE + 250 µg norgestimate (n=1, Cyclen®); *Fourth*, 30 µg EE + 3000 µg drospirenone (n=5, Yaz®, Yasmin®, Femelle®).

pmol/l, p=0.001 vs. menstrual, p=0.003 vs. follicular, Figure 2A). The elevated estradiol concentrations in the NAT follicular and luteal phases were also greater than concentrations observed in men and the OCP placebo/no pill phase (p≤0.001 for all). Following ovulation in a NAT cycle, progesterone concentrations increased in the luteal phase (menstrual 1.1 ± 0.5 nmol/l, follicular 1.0 ± 0.7 nmol/l, luteal 43.6 ± 38.3 nmol/l, p<0.001, Figure 2B), whereas progesterone remained steady across an OCP cycle (placebo/no pill 0.8 ± 0.6 nmol/l, early active pill 0.7 ± 1.0 nmol/l, late active pill 0.7 ± 1.0 nmol/l, p=0.41). As a result of the progesterone surge in the luteal phase, the E2/Prog ratio was highest in the follicular phase and lowest in the luteal phase (p<0.001, Figure 2C). The E2/Prog ratio was 0.14 ± 0.12 in the

menstrual phase, 0.19 ± 0.51 in the follicular phase (p=0.005 vs. menstrual), and 0.02 ± 0.02 in the luteal phase (p=0.001 vs. menstrual and follicular). As anticipated, testosterone concentrations were greater in men than NAT and OCP women (p<0.001 for all comparisons, Figure 2D).

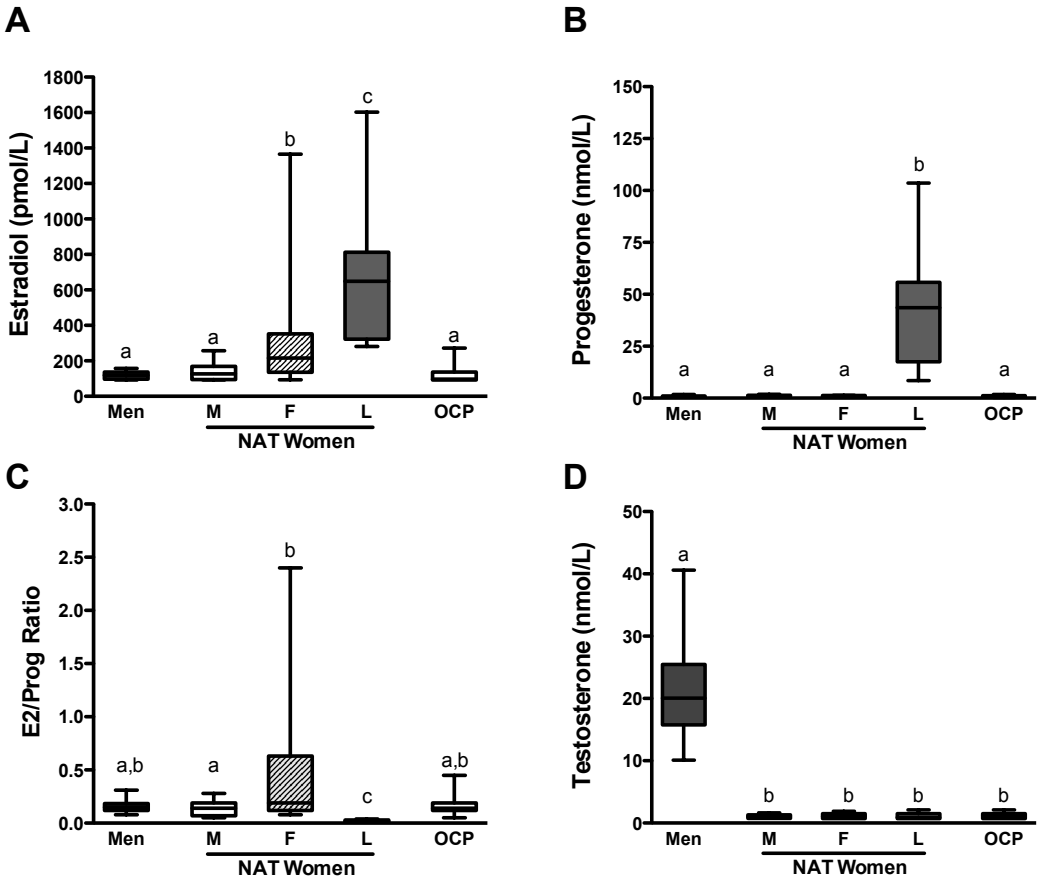

Figure 2. Serum concentrations of estradiol (A), progesterone (B), calculated estradiol/progesterone ratio (C), and testosterone (D) in men, at different phases of women's natural menstrual cycles (NAT; M: menstrual, F: follicular, L: luteal), and during the placebo/no pill phase of women's oral contraceptive pill cycles (OCP; withdrawal bleeding). Data are presented as box and whisker plots: the box extends from the 25th to 75th percentiles, the middle line represents the median, and the whiskers represent the range. Different letters denote different medians (Bonferroni correction, p<0.005).

Endothelial Function

There was no interactive effect between group and visit for resting diameter, peak RH diameter, absolute FMD, relative FMD, or any RH hemodynamic parameter ($p>0.05$ for all, Table 2). Compared to NAT and OCP women, men had larger resting diameters (main effect of group $p<0.001$; men vs. NAT $p<0.001$, men vs. OCP $p<0.001$, NAT vs. OCP $p=0.19$), larger peak RH diameters (main effect of group $p<0.001$; men vs. NAT $p<0.001$, men vs. OCP $p<0.001$, NAT vs. OCP $p=0.12$), and larger peak RH BF (main effect of group $p<0.001$; men vs. NAT and OCP $p<0.001$ for each, NAT vs. OCP $p=1.00$). There were no group differences in absolute ($p=0.07$) or relative FMD ($p=0.60$); however, group differences were unmasked with allometrically scaled FMD (main effect of group $p=0.005$, Figure 3A). Specifically, men had a higher scaled FMD response (9.0 ± 2.6%) than NAT (5.5 ± 2.2%, $p=0.001$ vs. men) and OCP women (6.5 ± 2.0%, $p=0.007$ vs. men), with no differences observed between the two groups of women ($p=0.15$) or across cycle phases in women.

Smooth Muscle Function

There was no interactive effect of group by visit for resting or peak NTG diameters or absolute and relative NTG ($p>0.05$ for all, Table 2). Resting brachial artery diameter prior to the NTG test was largest in men, followed by OCP women, and smallest in NAT women (main effect of group $p<0.001$; men vs. NAT $p<0.001$, men vs. OCP $p<0.001$, NAT vs. OCP $p=0.03$), as was the peak dilatory diameter

attained following NTG administration (main effect of group $p<0.001$; $p\leq0.001$ for all individual comparisons). After adjusting for group differences in resting diameter, allometrically scaled NTG revealed that smooth muscle function was lower in NAT women (main effect of group $p=0.001$; NAT 19.2 ± 4.1%) compared to men (24.5 ± 4.4%, $p=0.01$ vs. NAT) and OCP women (24.9 ± 2.0%, $p<0.001$), with no differences between men and OCP women ($p=0.80$; Figure 3B).

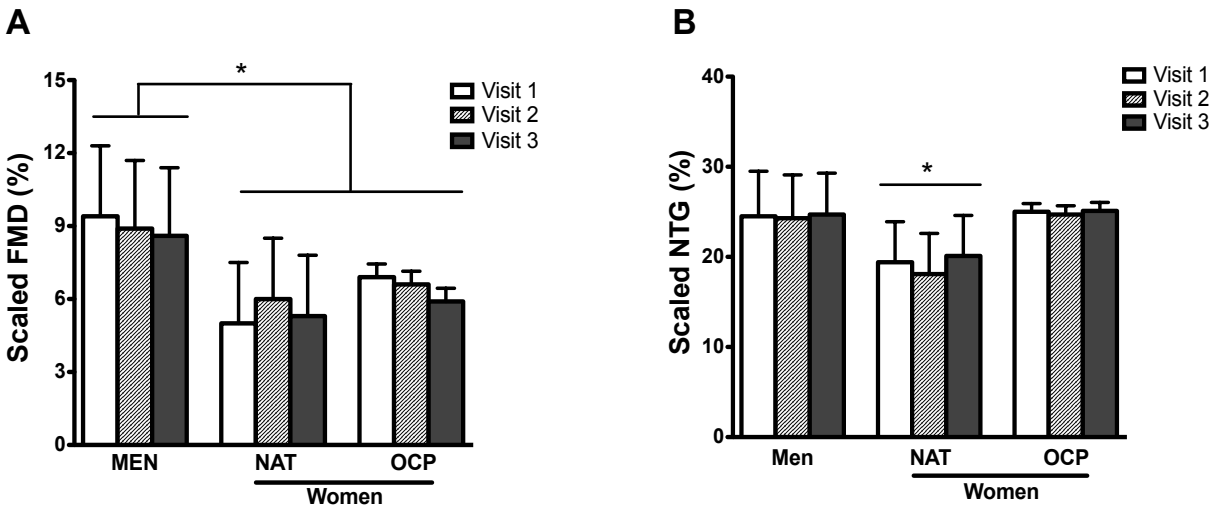

Figure 3. Allometrically scaled brachial artery endothelial (FMD, flow-mediated dilation test) (A) and smooth muscle function (NTG, nitroglycerin test) (B) in men, women with natural menstrual cycles (NAT), and women using monophasic oral contraceptive pills (OCP). Visits 1-3 were scheduled weekly for men, and across different phases of a single NAT (menstrual, follicular, luteal) or OCP cycle (placebo/ no pill, and early and later in active pill phase) for women. Data are mean ± SD. No group x visit interactions. Main effect of sex for scaled FMD% ($p=0.005$, Men > NAT and OCP) and for scaled NTG% ($p=0.001$, NAT < Men and OCP).

Table 2. Brachial Artery FMD and NTG Characteristics

	Men			NAT Women			OCP Women			
	Visit 1	Visit 2	Visit 3	Menstrual	Follicular	Luteal	Placebo	Early Active	Late Active	P
FMD Test										
Resting diameter, mm	4.23 ± 0.35	4.16 ± 0.39	4.17 ± 0.36	3.08 ± 0.36	3.11 ± 0.35	3.06 ± 0.37	3.31 ± 0.24	3.25 ± 0.29	3.30 ± 0.32	0.27
Peak RH diameter, mm	4.52 ± 0.34	4.44 ± 0.39	4.44 ± 0.39	3.28 ± 0.31	3.34 ± 0.31	3.28 ± 0.37	3.57 ± 0.26	3.50 ± 0.28	3.52 ± 0.31	0.10
Absolute FMD, mm	0.29 ± 0.11	0.28 ± 0.11	0.27 ± 0.10	0.20 ± 0.09	0.23 ± 0.08	0.22 ± 0.06	0.26 ± 0.08	0.25 ± 0.06	0.22 ± 0.06	0.14
Relative FMD, %	6.9 ± 2.8	6.7 ± 2.9	6.6 ± 2.4	6.9 ± 3.5	7.8 ± 3.2	7.3 ± 2.3	7.8 ± 2.4	7.7 ± 2.2	6.8 ± 2.0	0.30
Peak RH BF, mL/min	494 ± 123	497 ± 156	496 ± 134	248 ± 77	262 ± 84	242 ± 104	257 ± 89	239 ± 41	248 ± 75	0.85
MBV to peak, cm/s	34 ± 7	33 ± 5	34 ± 8	28 ± 13	33 ± 12	31 ± 14	30 ± 18	30 ± 8	28 ± 9	0.65
SR AUC to peak, $x10^3$	34 ± 12	32 ± 13	32 ± 14	34 ± 11	38 ± 17	32 ± 10	29 ± 16	28 ± 14	27 ± 12	0.32
Time to peak, s	50 ± 17	49 ± 17	49 ± 16	56 ± 32	46 ± 19	43 ± 22	40 ± 13	35 ± 17	42 ± 28	0.58
NTG Test										
Resting diameter, mm	4.17 ± 0.33	4.08 ± 0.33	4.04 ± 0.37	3.06 ± 0.35	3.09 ± 0.33	3.06 ± 0.37	3.42 ± 0.25	3.31 ± 0.29	3.35 ± 0.28	0.15
Peak diameter, mm	4.98 ± 0.37	4.89 ± 0.37	4.87 ± 0.36	3.79 ± 0.44	3.77 ± 0.38	3.80 ± 0.40	4.30 ± 0.30	4.19 ± 0.31	4.24 ± 0.36	0.35
Absolute NTG, mm	0.80 ± 0.20	0.81 ± 0.20	0.82 ± 0.20	0.73 ± 0.15	0.68 ± 0.15	0.74 ± 0.14	0.89 ± 0.13	0.88 ± 0.13	0.89 ± 0.16	0.80
Relative NTG, %	19.4 ± 5.0	20.0 ± 5.3	20.7 ± 6.0	23.9 ± 4.4	22.2 ± 5.2	24.6 ± 5.5	26.0 ± 3.9	26.8 ± 4.8	26.8 ± 4.7	0.50

FMD, flow-mediated dilation; RH, reactive hyperemia; BF, peak post-deflation blood flow; MBV, mean blood velocity up to peak RH diameter; SR AUC, shear rate area under curve to peak RH diameter; Time to peak, time to peak RH diameter; NTG, nitroglycerin. Data are means ± SD and p values are 3 x 3 (group x visit) interactions. Main effect of visit: NTG resting diameter ($p=0.03$, V1 > V3) and peak diameters ($p=0.03$, V1 > V2). Main effect of sex: FMD resting diameter, peak RH diameter, peak RH BF ($p<0.001$, Men > NAT and OCP); NTG resting and peak diameters ($p<0.001$, Men > OCP > NAT); absolute NTG ($p=0.005$, OCP > NAT) and relative NTG ($p<0.001$, NAT and OCP > Men).

Influence of Endogenous Estradiol

When NAT women were stratified into tertiles based on changes in estradiol from the menstrual to the follicular phase, women in the lowest tertile for changes in estradiol experienced decreases or slight increases in estradiol (median ± IQ, 2.0 ± 47.0 pmol/l, n=5), whereas women in the highest tertile experienced large surges in estradiol (488 ± 809 pmol/l, n=5). However, no companion differences were observed in the corresponding change in relative FMD between the two tertiles (lowest tertile 1.4 ± 2.5% increase in FMD, highest tertile 1.3 ± 3.3% decrease in FMD, p=0.19; Figure 4), while changes in relative NTG mirrored those of estradiol (lowest tertile 3.2 ± 3.7% decrease in NTG, highest tertile 1.4 ± 1.8% increase in NTG, p=0.02). Nevertheless, robust regression revealed a non-significant relationship between changes in estradiol across menstrual cycle phases and the corresponding changes in relative FMD (r^2=0.01, p=0.62) or relative NTG (r^2=0.09, p=0.13). Repeating all analyses using the E2/Prog ratio in place of estradiol for the independent variable resulted in similar outcomes (data not shown).

Influence of OCP Generation and Duration of Use

When women using a second generation pill were compared to women using a third or fourth generation pill, no group differences were observed in resting brachial artery diameter at any visit (p>0.05 for all comparisons), and pill generation

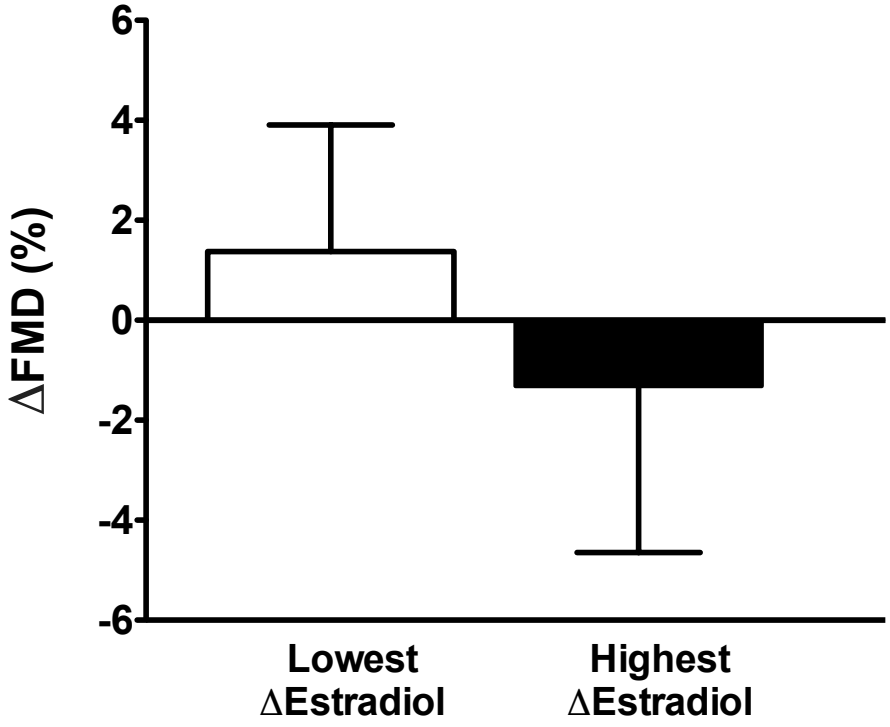

Figure 4. Changes in relative FMD from the menstrual to the follicular phase in NAT women with smallest increases in estradiol (□; ΔEstradiol=2 ± 47 pmol/l, ΔFMD%=1.4 ± 2.5% increase; n=5) compared to those with largest surges in estradiol (■; ΔEstradiol=488 ± 809 pmol/l, ΔFMD%=1.3 ± 3.3% decrease; n=5). ΔFMD% are mean ± SD; no group differences were observed (p=0.19).

did not influence relative FMD (pill generation p=0.16, visit p=0.24, interaction p=0.61) or relative NTG (pill generation p=0.53, visit p=0.59, interaction p=0.34). Duration of OCP use, for all generations combined, was negatively correlated with the cycle average relative FMD (r=-0.54, 95% CI=-0.81 to -0.10, p=0.002) but was not associated with the cycle average relative NTG (r=-0.44, 95% CI=-0.75 to 0.04, p=0.07). Furthermore, when OCP women were grouped based on pill generation, the negative association between duration of OCP use and brachial FMD persisted

for women using a second generation pill (r=-0.65, 95% CI=-0.91 to -0.04, p=0.04; mean duration: 29 months, range: 5 to 72 months) but not for women using a third or fourth generation pill (r=-0.55, 95% CI=-0.90 to 0.26, p=0.16; mean duration: 57 months, range: 5 to 144 months). Partial correlations between duration of OCP use and brachial FMD, controlling for age, also yielded similar results (second generation, r=-0.72, 95% CI=-0.93 to -0.17, p=0.03; third or fourth generation, r=-0.59, 95% CI=-0.91 to 0.20, p=0.17).

DISCUSSION

This study is the first to directly compare both endothelium-dependent and independent dilation between men and premenopausal women with natural menstrual cycles or those using combined monophasic OCPs. Allometrically scaled brachial FMD, indicative of endothelium-dependent dilation, remained stable across both a menstrual and OCP cycle and was comparable between the two groups of women but lower than in age-matched men. Allometrically scaled brachial NTG, indicative of endothelium-independent dilation, was lower in naturally cycling women than OCP women and men. These findings suggest that brachial artery endothelial function may be well regulated in healthy young women.

Natural Menstrual Cycle

Across a NAT cycle, estradiol concentrations increased in the mid-follicular and luteal phases but did not appear to influence brachial artery FMD in either

phase. As a result of interindividual variability in menstrual cycle length and the timing of our mid-follicular assessments relative to a positive ovulation, changes in estradiol from the menstrual to the mid-follicular phase ranged from a decrease of 28 pmol/l to an increase of 1211 pmol/l, with women tested closer to ovulation demonstrating greater increases in estradiol. Nevertheless, a comparison between a subset of women with the smallest *versus* largest increases in estradiol concentrations also revealed no differences in the corresponding change in FMD.

Our findings are in contrast to frequently cited papers that reported increases in brachial FMD during the follicular phase (1, 7, 12, 13, 18, 37), yet they are in agreement with other, more recent, papers that also observed no changes in FMD across a menstrual cycle (6, 15, 22, 27, 29). It is unclear why there is a stark discrepancy in the literature, as previous studies that reported changes in FMD had a similar relative increase in estradiol from the menstrual to the follicular phase (mean increase: 332 pmol/l, range: 125 to 510 pmol/l) as studies that reported no changes in FMD (mean increase: 521 pmol/l, range: 257 to 900 pmol/l).

The lack of relationship between changes in estradiol and changes in relative FMD in the current study were further supported by the nonsignificant robust regression. Likewise, we did not observe any relationships between brachial artery FMD and progesterone or the estradiol/progesterone ratio. Others have also reported nonsignificant correlations between estradiol and FMD (6, 7, 15), with one study observing increases in FMD near ovulation but finding progesterone to be the only significant correlate of FMD ($r=-0.26$, $p=0.03$) (7). Therefore, while some

studies may have observed parallel increases in estradiol and brachial artery FMD from the menstrual to the mid/late-follicular phases, the two variables appear to be unrelated. We speculate that endothelium-dependent dilation in premenopausal women with natural menstrual cycles may instead be related to ER expression and/or eNOS activation (9). Alternatively, estradiol-mediated genomic and nongenomic mechanisms may be regulated in such a way as to enable endothelial function to remain stable across a menstrual cycle despite fluctuations in estradiol.

As for endothelium-independent function, Hashimoto *et al.* (13) are the only group to have reported augmented smooth muscle function in the follicular and luteal phases. Our findings and those of others (7, 15, 18, 29, 37) suggest that smooth muscle function is unaltered by changes in estrogen or progesterone across a natural menstrual cycle. In the current study, however, endothelium-independent function was lower in NAT women compared to men and OCP women. It is possible the reduced smooth muscle function in NAT women resulted from a lower cardiorespiratory fitness compared to men and OCP women (25); however, without an assessment of peak oxygen uptake, we are unable to confirm this speculation.

Combined Monophasic OCP Cycle

Across an OCP cycle, brachial artery endothelial function remained unchanged across the placebo/no pill and active pill phase, regardless of pill generation. Our findings are in contrast to previous reports that increases in

brachial artery FMD during the active pill phase are dependent on pill generation (32). Second generation pills, containing progestins such as levonorgestrel, are the most androgenic and have been shown to reduce brachial artery FMD by ~2% during the active pill phase or have no effect on FMD (32, 33). In contrast, third and fourth generation pills containing progestins such as desogestrel and drospirenone, respectively, have fewer or no androgenic effects and have been shown to increase brachial artery FMD by ~1.5 to 2% in the active pill phase (23, 24, 32).

In the current study, relative and scaled FMD were 1% lower in the late active pill phase than the placebo/no pill phase, but this was not a significant difference when OCP women were compared to NAT women and men (i.e. mixed model ANOVA) or when they were assessed separately (i.e. one-way repeated measures ANOVA). While our findings may have been limited by a small sample size, we also did not observe an effect of pill generation and/or pill cycle phase when we compared relative FMD just between OCP women using a second generation *versus* a third or fourth generation pill. Interestingly, despite not seeing differences in brachial artery FMD across an OCP cycle or between OCP generations, we did observe a negative relationship between duration of OCP use and the average relative FMD. Furthermore, when OCP women were stratified based on pill generation, the negative relationship persisted only for women using a second generation but not a third or fourth generation pill.

To date, the effect of prolonged OCP use on endothelial function has received little attention (8, 14). Friedman *et al.* (8) previously observed a moderately negative relationship between duration of OCP use and FMD (r=-0.44, p=0.04), but found age to be a confounding factor. Heidarzadeh *et al.* (14) also reported a negative, albeit nonsignificant, relationship between duration of OCP use and FMD in women using second generation OCPs (14). In the current study, the association between duration of use and FMD for second generation pills was significant even after accounting for age. Moreover, the duration of OCP use in our cohort of women using second generation pills was 5-72 months, whereas it was ~30-80 months in the study by Heidarzadeh *et al.* (14). Thus, our inclusion of women with shorter durations of OCP use (i.e. 5-30 months) may have strengthened the negative relationship we observed. Our finding that prolonged use of a second generation OCP is associated with reduced endothelial function is clinically relevant as extended use of this type of OCP may augment age-related declines in endothelial function (30).

Group Differences

Comparing between men, NAT women and OCP women, brachial artery FMD was not different between the two groups of women but was lower in women than men. These sex differences in endothelial function were not apparent with absolute or relative FMD but became evident once group differences in baseline arterial diameter were accounted for with allometric scaling. Compared to men,

scaled FMD was 3.5% and 2.5% lower in NAT and OCP women, respectively. It should be noted that the FMD test assesses endothelial function (NO bioavailability) indirectly by quantifying arterial dilation, a process that is dependent on smooth muscle relaxation. Thus, with NAT women demonstrating a 5.3% lower NTG response than men, it remains unclear how much of the FMD response in NAT women is truly reflective of reduced NO-bioavailability and how much is a result of attenuated smooth muscle function.

The lower smooth muscle function in NAT women may have also masked group differences in brachial artery FMD between NAT and OCP women. It is possible the FMD response of NAT women was artificially attenuated and is actually more similar to men and higher than OCP women; however, adjusting for group differences in the NTG response did not alter our FMD findings (data not shown). The lack of difference between NAT and OCP women's FMD responses may have also been confounded by OCP women being grouped together regardless of pill generation. Compared to NAT women, previous studies have reported lower endothelial function in OCP women using a second generation pill (14, 21), but higher or similar endothelial function in OCP women using a third or fourth generation pill (10, 20, 35). Therefore, it would appear from previous studies that the greater degree of androgenicity in second generation OCPs may contribute to a lower endothelial function in women using these pills compared to women with natural menstrual cycles. Nevertheless, controlling for pill generation in our analyses also did not alter our FMD finding.

Limitations and Strengths

This study did not capture peak estradiol concentrations in naturally cycling women as women were tested in the mid-follicular phase, rather than prior to ovulation. Changes in serum estradiol from the menstrual to the mid-follicular phase were quite variable between participants but were not related to the corresponding changes in brachial FMD. It is possible the genomic effects of estradiol, such as increased expression of ER and/or eNOS, are more closely related to the FMD response and take full effect during a narrow window of time near ovulation; however, this postulation is beyond the scope of the current study and requires further investigation. As a secondary objective, this study assessed the influence of OCP pill generation and duration of OCP use, but our analyses of women using a second generation versus a third or fourth generation pill may have been limited by the relatively small sample sizes in these sub-groups. Moreover, while all participants reported being recreationally active, this study utilized the subjective International Physical Activity Questionnaire rather than an objective measure of fitness, such as a peak oxygen uptake test. Potential differences in fitness levels and/or in the nature of activities participants engage in (i.e. aerobic versus resistance) may have contributed to the group differences we observed in brachial artery FMD and NTG responses. Nevertheless, this study is the first to compare both endothelial and arterial smooth muscle function between age-matched men and premenopausal women with natural cycles or using combined monophasic OCPs across different cycle phases. Additionally, thorough monitoring

of menstrual cycle phases, confirmation of an ovulatory cycle, and inclusion of sex hormone analyses all helped to strengthen our study design.

Conclusions

We demonstrated that in young healthy women, brachial artery FMD remained stable across a NAT or OCP cycle, despite changes in sex hormones, and was not associated with serum estradiol concentrations in naturally cycling women. Comparing between men and women, brachial artery FMD was similar between naturally cycling women and those using combined monophasic OCPs but lower in both groups of women compared to age-matched men. Smooth muscle function was lower in naturally cycling women and may have confounded the group comparisons in endothelial function, but also highlights the importance of assessing both endothelium dependent and independent function. We also observed a negative relationship between longer durations of OCP use and brachial artery FMD in women using a second, but not a third or fourth, generation pill. Overall, our findings suggest that controlling for menstrual cycle phase, or use of a third or fourth generation OCP, may not be necessary for cross-sectional assessments of brachial artery endothelial function in premenopausal women, which would certainly increase the feasibility of their inclusion as research participants in this type of study design. Future studies should investigate how arteries regulate estradiol's genomic and nongenomic mechanisms, so as to

elucidate the relationship, or lack thereof, between estradiol and endothelial function in healthy premenopausal women.

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The authors have no conflicts of interest to disclose.

AUTHOR CONTRIBUTIONS

Study conceptualization and design: NS, SEP, MJM. Data collection: NS, SEP, VR. Data analysis: NS, VR. Data interpretation and manuscript preparation: NS, MJM. Edited manuscript: NS, SEP, VR, MJM.

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CHAPTER 4

Brachial artery endothelial function is unchanged after acute sprint interval exercise in sedentary men and women

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NEW FINDINGS

- What is the central question of this study?

  What is the acute brachial artery endothelial function response to sprint interval exercise and are there sex-based differences?

- What is the main finding and its importance?

  Brachial artery endothelial function did not change in either men or women following an acute session of SIT consisting of 3x20-s 'all-out' cycling sprints. Our findings suggest this low-volume protocol may not be sufficient to induce functional changes in the brachial artery of sedentary, but otherwise healthy adults.

ABSTRACT

Sprint interval training (SIT) is a potent metabolic stimulus, but studies examining its acute effects on brachial artery endothelial function are limited. The influence of estradiol on the acute arterial response to this type of exercise is also unknown. We investigated the brachial artery endothelial function response to a single session of SIT in sedentary healthy men (n=8; 22±4 yr) and premenopausal women tested in the mid-follicular phase of the menstrual cycle (n=8; 21±3 yr). Participants performed 3x20-s 'all-out' cycling sprints interspersed with 2 min of active recovery. Brachial artery flow-mediated dilation (FMD) and hemodynamic parameters were measured before and 1 and 24 h post-exercise. Despite attenuations in some hemodynamic parameters at 1 h post-exercise, there were no changes in absolute

(p=0.23), relative (p=0.23) or allometrically scaled FMD (p=0.38) following a single session of SIT. Resting and peak dilatory diameters did not change in men or women (p>0.05 for all) and there were no interactions between time and sex for any measure (p>0.05). Estradiol was not correlated with relative FMD at baseline (r=-0.22, p=0.42) or with the change in relative FMD from baseline to 1 h post-exercise (r=0.24, p=0.40). Overall, brachial artery FMD appears to be unchanged in men and women following an acute session of SIT, and the higher estradiol concentrations in women do not augment the baseline or post-exercise FMD response. The 3x20-s model of low-volume sprint interval exercise may not be sufficient to alter brachial artery endothelial function in healthy men and women.

KEYWORDS

Acute exercise, flow-mediated dilation, sex differences, estradiol

INTRODUCTION

Sprint interval training (SIT) is characterized by brief intermittent bursts of 'all-out' effort (37) and has gained traction as a time-efficient and metabolically potent training stimulus (13). However, classic SIT models are demanding, involving 4-6 repeated 30-s Wingate tests (13). As recently contended, there is a need to investigate SIT protocols that involve fewer and shorter sprints and that are truly time-efficient and more feasible for the general population (35). To date, the 3x20-s protocol is currently one of the most time-efficient SIT models that has

been demonstrated to generate increases in maximal aerobic capacity and some metabolic outcomes (14, 15). To our knowledge, vascular responses to this low-volume model have not been examined, thus our laboratory was interested in investigating its acute (current study) and chronic (27) effects on brachial artery endothelial function.

Brachial artery endothelial function is a marker of cardiovascular health (22) and can be assessed noninvasively using the flow-mediated dilation (FMD) test (30). Dawson *et al.* (9) have proposed a time course for the acute post-exercise FMD response, whereby it is attenuated immediately after exercise, increases between 1 and 24 h, and returns to pre-exercise levels by 24 to 48 h. Since the immediate post-exercise response may be confounded by changes in sympathetic activity, the shear stimulus, and/or arterial diameter (25), 1 h post-exercise is a common time for assessing the acute FMD response. At 1 h post-exercise, increases in brachial FMD of approximately 1-3% have been observed after a 12x1-min (2), 8x1-min (5), and 7x1-min (12) protocol. The lowest volume model for which the acute brachial FMD response has been investigated involved 8x20-s cycling sprints and observed increases in brachial FMD immediately after exercise, but a 1 h assessment was not included (7). Evidently, previous examinations of the acute FMD response following interval exercise are dominated by lengthy protocols involving high-intensity, but submaximal effort, and investigations of low-volume sprint interval paradigms are sparse.

Biological sex is another factor that may be influencing the acute arterial response to exercise. Differences in the brachial artery FMD response have been observed between men and women following a variety of acute (21, 38) and chronic (4, 26) exercise stimuli, but reports of which sex was more responsive are conflicting. Additionally, the majority of previous studies examined the post-exercise FMD response in older men and postmenopausal women (4, 26, 38) and their findings may not be generalizable to younger men and women. In premenopausal women, estradiol is elevated in the follicular phase of a natural menstrual cycle and has the potential to augment the brachial artery FMD response (6, 23). When tested during high-estradiol phases, women have been observed to have a 6-8% higher resting FMD response than men (19, 20); however, these observations were likely confounded by women having smaller arterial diameters (1). Recently, our laboratory observed that estradiol did not influence resting brachial artery FMD across cycle phases or between men and women (Shenouda *et al.*, in review). Whether estradiol augments the post-exercise endothelial function response in premenopausal women is currently unknown.

Understanding the endothelial function response to an acute SIT session, and investigating the potential for estradiol to elicit sex-based differences, may help in predicting long-term training responses (10) and provides a foundational framework for larger clinical trials. Therefore, the primary objective of this study was to examine brachial artery FMD at 1 and 24 h following a single session of SIT in healthy young men and women. We also sought to explore the potential

influence of sex on the acute arterial response to SIT and thereby inform the potential for this form of exercise to induce training associated changes in FMD in both men and women. In order to maximize any effects of estradiol, premenopausal women were tested in a high-estradiol phase of the menstrual cycle. Based on the proposed acute FMD response pattern (9), we hypothesized that, in both men and women, endothelial function would be elevated at 1 h post-exercise and would return to baseline levels at 24 h. We further hypothesized that if estradiol was influencing the post-exercise endothelial function response, then the relative increase from baseline to 1 h would be larger in women than men.

METHODS

Ethical Approval

This study was approved by the Hamilton Integrated Research Ethics Board (HIREB 14-299) and conformed to the Declaration of Helsinki, except for registration in a database. Written informed consent was obtained from all subjects prior to participation.

Participants

Eighteen sedentary, but otherwise healthy adults (9 men, 9 women) between 18-30 yr were recruited through poster advertisement in and around McMaster University. Participants were classified as sedentary if they did not meet the Canadian Physical Activity Guideline recommendations for adults (34) and self-

reported as not being habitually active (acquiring ≤ 1.5 h of physical activity per week). Men and women were matched for age and relative cardiorespiratory fitness per fat-free mass, and all were nonsmokers. Women were not using hormonal contraceptives and were tested in the mid-follicular phase of their menstrual cycle (day 9 ± 2 following onset of menstruation). Two participants were excluded from analyses due to atypical sex hormone concentrations: one male's estradiol level well exceeded the reference limit for men (485 pmol/L vs. <162 pmol/L), while one female's estradiol level was below the assay detection limit (<92 pmol/L). Descriptive characteristics for the remaining 16 participants (8 men, 8 women) are reported in Table 1. The acute skeletal muscle responses in some of the same individuals have been previously reported (28).

Table 1. Participant Characteristics

	Men (8)	Women (8)
Age, yr	22 ± 4	21 ± 3
Height, cm	177 ± 9	166 ± 7*
Weight, kg	84 ± 22	69 ± 24
BMI, kg/m^2	27 ± 6	24 ± 6
VO_2peak, ml/kg/min	36 ± 6	34 ± 8
VO_2peak, ml/kg FFM/min	45 ± 8	47 ± 9
Estradiol, pmol/L	124 ± 36	245 ± 88*
Progesterone, nmol/L	0.8 ± 0.2	0.8 ± 0.2
Testosterone, nmol/L	17.2 ± 6.6	1.1 ± 0.4*

BMI, body mass index; VO_2peak, peak oxygen uptake; FFM, fat-free mass. Sex hormone data reported for n=15 (8 men, 7 women) as one female participant had an adverse response to blood sampling. Data are mean ± SD; *p<0.05 vs. men.

Study Design

On separate visits prior to the exercise trial day, participants completed an incremental peak oxygen uptake (VO_2peak) test on an electronically braked cycle ergometer (Lode Excalibur Sport V2.0, Groningen, The Netherlands) as previously described (28), an assessment of fat-free mass (FFM) (BodPod®, COSMED Inc., Concord, CA, USA), and a familiarization with the sprint interval exercise (Velotron, RacerMate, Seattle, WA, USA). In preparation for the exercise trial day, participants were asked to refrain from exercise for 48 h and from alcohol for 24 h. All participants were tested in the morning after an overnight fast (no food or drink with the exception of water for 10 hours). Relevant to this publication, the exercise trial day involved blood sampling for sex hormone analyses, an acute session of SIT, and brachial artery FMD tests at baseline, and at 1 and 24 h post-exercise.

Blood Samples

Fasted serum samples were collected from an antecubital vein using standard venipuncture techniques (BD Vacutainer®, Becton, Dickinson and Company, Franklin Lakes, NJ, USA). Samples were processed in accordance with the manufacturer's instructions, aliquoted, and stored at -20°C until subsequent analyses. The Hamilton Regional Medicine Program Core Laboratory analyzed samples for estradiol (Abbott Architect, Abbott Laboratories, Chicago, IL, USA), progesterone (Abbott Architect, Abbott Laboratories, Chicago, IL, USA), and testosterone (Immulite 2000, Siemens, Erlangen, Germany).

Sprint Interval Exercise Protocol

Participants completed a single session of low-volume SIT, consisting of a 2-min warm-up, followed by 3x20-s 'all out' cycling sprints against 5.0% body weight (Velotron, RacerMate, Seattle, WA, USA). A resistance load of 5% body weight has been shown to be as equally effective as 7.5% body weight (15, 24), but is more manageable in sedentary populations as determined through pilot testing. Cycling sprints were interspersed with 2 min of active recovery at 25 W. Sprint and recovery loads were set using compatible computer software (Velotron Wingate Software Version 1.0, Seattle, WA, USA). Heart rate was measured continuously throughout the exercise session using telemetry (Polar A3, Lake Success, NY, USA).

Flow-Mediated Dilation

Brachial artery FMD was measured in accordance with current guidelines (30). Briefly, we inflated a blood pressure cuff positioned around the forearm to suprasystolic pressures for 5 min in order to occlude blood flow to the distal vascular beds. Using brightness-mode and Doppler ultrasound (Vivid Q, GE Medical Systems, Horten, Norway; Duplex mode), we simultaneously imaged the right brachial artery proximal to the antecubital fossa, while collecting mean blood velocity signals (MBV) for 30 s at rest and for 3 min after cuff deflation (reactive hyperemia, RH). Resting and RH end-diastolic arterial diameters were analyzed using semi-automated edge tracking software (Artery Measurement System (AMS)

II, Version 1.141; Gothenburg, Sweden) (36) and a 5-frame rolling average was used for calculating absolute and relative FMD, as per *Eqs.* 1 and 2, respectively. Test-retest repeatability in our laboratory is 1-2% for arterial diameter measurements, and 10-20% for absolute and relative FMD (Shenouda *et al.,* in preparation).

$$[1]\ \text{FMD}\ (\text{mm}) = \ \text{peak RH diameter} - \text{resting diameter}$$

$$[2]\ \text{FMD}\ (\%) = \frac{\text{absolute FMD}}{\text{resting diameter}}\ \text{x}\ 100$$

To account for differences in resting arterial diameter between men and women and/or in post-exercise diameter changes, FMD data were allometrically scaled in accordance with recommendations (1) and as previously described (27).

Hemodynamic Parameters

The MBV signals acquired at rest and during RH were analyzed using compatible software (EchoPAC PC, Version 110.0.2; GE Medical Systems, Horten, Norway). The MBV and arterial diameter data were used to calculate blood flow (BF) and shear rate (SR):

$$[3]\ \text{BF}\ (\text{mL/min}) = \ (\pi \text{r}^2 \text{x MBV})\ \text{x}\ 60\ ,\ \ \text{where}\ r = (\text{arterial diameter}/2)$$

$$[4]\ \text{SR}\ (\text{s}^{-1}) = \frac{\text{MBV x 8}}{\text{arterial diameter}}$$

We characterize resting hemodynamics by reporting resting brachial artery blood flow (Resting BF) and heart rate (HR), determined from Doppler ultrasound with built-in single lead ECG (Vivid Q, GE Medical Systems, Horten, Norway), as well

as mean arterial pressure (MAP) and cardiac output (CO), estimated by continuous hemodynamic monitoring (Nexfin, BMEYE, Amsterdam, The Netherlands). We characterize RH hemodynamics by reporting the peak postdeflation BF (peak RH BF), the BF averaged to the peak RH diameter (BF to peak), the MBV averaged to the peak RH diameter (MBV to peak), the SR area under the curve to the peak RH diameter (SR AUC to peak), and the time to the peak RH diameter (time to peak).

STATISTICAL ANALYSES

All data were assessed for normality using the Shapiro-Wilk test. Descriptive statistics and sex hormone data were analyzed using independent samples t-tests. A two-factor (time x sex) mixed-model ANOVA was used to examine the effects of SIT on arterial diameter, FMD, and hemodynamic parameters in men and women and to explore the potential influence of sex at rest and following exercise. Significant findings were followed up with pairwise comparisons and a Bonferroni correction was used to adjust for multiple comparisons. To account for differences in resting arterial diameter, allometrically scaled FMD was analyzed using linear mixed model analysis with the diameter change on the natural log scale as the dependent variable, [$ln(Dpeak) - ln(Drest)$], time (baseline, 1h post, 24 h post) and sex (men, women) as fixed factors, and the log-transformed resting diameter, $ln(Drest)$, as the covariate. Bivariate Pearson correlations were used to explore the relationships between sex hormone concentrations and FMD (baseline FMD and the change in FMD from baseline to 1 and 24 h). 95% confidence intervals

(95% CI) are reported for all correlation coefficients, for the change in relative FMD from baseline to 1 and 24 h post-exercise, and for the difference in these changes between men and women. Statistical analyses were performed using SPSS Statistics (Version 20.0, Chicago, IL) and GraphPad Prism (Version 4.0b; La Jolla, CA). Significance was set at $p \leq 0.05$ and data are expressed as mean ± standard deviation (SD) unless otherwise noted.

RESULTS

Sex Hormone Concentrations

Compared to men, women had higher estradiol ($p=0.003$), similar progesterone ($p=0.45$), and lower testosterone concentrations ($p<0.001$; Table 1).

Sprint Interval Exercise

Men and women exercised at similar percentages of their peak heart rate (91 ± 4% and 93 ± 4% respectively; $p=0.37$). Relative peak power output (PPO), averaged over all three sprints, was also similar between sexes (men: 8.6 ± 0.6 W/kg FFM; women: 9.1 ± 1.3 W/kg FFM; $p=0.35$), and corresponded to 217% (men) and 204% (women) of the PPO attained during their VO_2peak tests.

Brachial Artery Endothelial Function

We did not observe any post-exercise changes in resting diameter, peak RH diameter, or FMD in men or women ($p>0.05$ for all, Table 2). The mean change

in relative FMD from baseline to 1 h post-exercise was -0.6% (95% CI, -2.3 to 1.1%) for the pooled cohort, with a 0.4% difference in this change between men and women (95% CI, -3.0 to 3.7%). The mean change in relative FMD from baseline to 24 h post-exercise was +0.7% (95% CI, -0.7 to 2.0%) for the pooled cohort, with a 0.0% difference in this change between men and women (95% CI, -2.9 to 2.9%).

Compared to men, women tended to have smaller resting diameters (3.99 ± 0.17 mm vs. 3.51 ± 0.17 mm; main effect of sex, p=0.06) and they had smaller peak RH diameters (4.27 ± 0.17 mm vs. 3.72 ± 0.17 mm; p=0.03), but there were no effects of sex on absolute (p=0.21) or relative (p=0.42) FMD. After accounting for differences in arterial diameter with allometric scaling, scaled FMD appeared lower in women than men (5.1 ± 0.0% *vs.* 8.4 ± 0.0%, respectively) but this difference did not reach significance (p=0.08). There were no interactive effects of time and sex on resting diameter (p=0.33), peak RH diameter (p=0.25), absolute FMD (p=0.84), relative FMD (p=0.96), or allometrically scaled FMD (p=0.84) (Table 2). Figure 1 shows the group means and individual participant data for scaled FMD and resting diameters for men and women at baseline and 1 and 24 h after sprint interval exercise.

Moreover, there were no associations between baseline relative FMD and serum concentrations of estradiol (r=-0.22, 95% CI=-0.65 to 0.31, p=0.42), progesterone (r=-0.31, 95% CI=-0.71 to 0.24, p=0.27), or testosterone (r=0.08, 95% CI=-0.45 to 0.57, p=0.78). Similarly, sex hormone concentrations were not associated with the change in relative FMD from baseline to 1 h post-exercise

(estradiol, r=0.24, 95% CI=-0.31 to 0.67, p=0.40; progesterone, r=-0.38, 95% CI=-0.75 to 0.17, p=0.16; testosterone, r=-0.02, 95% CI=-0.53 to 0.50, p=0.95) or with the change in relative FMD from baseline to 24 h post-exercise (estradiol, r=0.19, 95% CI=-0.34 to 0.63, p=0.49; progesterone, r=0.26, 95% CI=-0.27 to 0.67, p=0.34; testosterone, r=-0.17, 95% CI=-0.62 to 0.35, p=0.53).

Resting Hemodynamic Parameters

Resting brachial artery BF was attenuated at 1 h post-exercise compared to 24 h (main effect of time, $p<0.01$; 32 ± 49 ml/min lower vs. baseline, p=0.07; 40 ± 27 ml/min lower vs. 24 h, $p<0.001$). Heart rate was elevated at 1 h post-exercise compared to baseline and 24 h (main effect of time, $p<0.001$; 9 ± 8 beats/min higher vs. baseline, $p<0.001$; 11 ± 7 beats/min higher vs. 24 h, $p<0.001$), but there were no changes in MAP (p=0.17) or CO (p=0.13) (Table 2). There were no interactive effects of time and sex on any of the resting hemodynamic parameters (Table 2).

Reactive Hyperemia Hemodynamic Parameters

Peak RH BF was attenuated at 1 h post-exercise compared to 24 h (main effect of time, $p<0.01$; 38 ± 76 ml/min lower vs. baseline, p=0.22; 76 ± 89 ml/min lower vs. 24 h, p=0.008), with no differences between baseline and 24 h (p=0.17). The BF averaged to the peak RH diameter was also attenuated at 1 h post-exercise (main effect of time, p=0.001; 46 ± 59 ml/min lower vs. baseline, p=0.03; 63 ± 72 ml/min lower vs. 24 h, p=0.01), as was the MBV averaged to the peak RH diameter

(main effect of time $p<0.001$; 7 ± 7 cm/s slower vs. baseline, $p=0.003$; 9 ± 7 cm/s slower vs. 24 h, $p=0.001$). SR AUC to the peak RH diameter was unchanged following exercise ($p=0.60$), while time to peak dilation tended to be longer at 1 h post-exercise compared to baseline (main effect of time, $p=0.02$; 19 ± 4 s longer vs. baseline, $p=0.06$; 13 ± 1 s longer vs. 24 h, $p=0.25$). There were no interactive effects of time and sex on any of the RH hemodynamic parameters (Table 2).

DISCUSSION

The main findings from the present study are that brachial artery endothelial function was unchanged following a single session of SIT in healthy men and premenopausal women, and that estradiol did not augment the baseline or post-exercise FMD response in women compared to men.

The small mean changes in relative FMD from baseline to 1 and 24 h post-exercise and the wide 95% confidence intervals suggest that there is substantial uncertainty in the estimation of the true population effects. Furthermore, while it may appear that scaled FMD increased by 0.9% from baseline to 24 h post-exercise in men, examining the individual participant responses revealed that only two men were driving this change. Our findings are in contrast to previous reports that endothelial function increases between 1 and 24 h post-exercise before returning to baseline levels by 24-48 h (9) and to previously observed increases of 1-8% in endothelial function after higher-volume interval protocols (2, 5, 7, 12). Increases in brachial artery FMD of 1-1.5% have been deemed clinically and

Table 2. Brachial Artery FMD and Hemodynamic Characteristics

	Men			Women			P		
	Baseline	1 h Post	24 h Post	Baseline	1 h Post	24 h Post	TxS	Time	Sex
FMD									
Resting diameter, mm	3.94 ± 0.61	4.02 ± 0.56	4.00 ± 0.60	3.54 ± 0.39	3.57 ± 0.27	3.44 ± 0.36	0.33	0.32	0.06
Peak RH diameter, mm	4.22 ± 0.58	4.28 ± 0.48	4.32 ± 0.65	3.74 ± 0.37	3.76 ± 0.30	3.67 ± 0.41	0.25	0.76	0.03
Absolute FMD, mm	0.28 ± 0.12	0.26 ± 0.16	0.32 ± 0.20	0.21 ± 0.12	0.20 ± 0.07	0.23 ± 0.09	0.84	0.23	0.21
Relative FMD, %	7.6 ± 3.9	6.7 ± 5.0	8.3 ± 5.4	6.0 ± 3.6	5.5 ± 1.9	6.7 ± 2.6	0.96	0.23	0.42
Scaled FMD, %	8.3 ± 3.4	8.3 ± 3.5	9.2 ± 3.5	5.1 ± 3.5	4.9 ± 3.4	5.4 ± 3.6	0.84	0.38	0.08
Resting Hemodynamics									
Resting BF, ml/min	75 ± 42	49 ± 37	94 ± 41	61 ± 57	23 ±16	59 ± 18	0.66	<0.01	0.09
HR, beats/min	71 ± 5	83 ± 11	70 ± 7	68 ± 11	74 ± 12	65 ± 12	0.13	<0.001	0.24
MAP, mmHg	82 ± 8	83 ± 11	77 ± 6	82 ± 15	86 ± 11	80 ± 10	0.87	0.17	0.71
CO, L/min	7.3 ± 1.0	8.1 ± 1.4	7.8 ± 0.8	6.1 ± 1.5	6.3 ± 1.6	6.6 ± 1.1	0.66	0.13	0.02
RH Hemodynamics									
Peak RH BF, ml/min	382 ± 114	340 ± 84	454 ± 166	292 ± 118	258 ± 121	295 ± 100	0.11	<0.01	0.07
BF to peak, ml/min	234 ± 60	189 ± 46	275 ± 78	186 ± 70	139 ± 62	177 ± 58	0.18	0.001	0.03
MBV to peak, cm/s	32 ± 10	25 ± 10	35 ± 8	31 ± 9	22 ± 8	31 ± 7	0.57	<0.001	0.45
SR AUC to peak, $x10^3$	20 ± 11	26 ± 15	20 ± 14	22 ± 18	23 ± 15	23 ± 11	0.67	0.60	0.95
Time to peak, s	50 ± 13	71 ± 28	54 ± 22	45 ± 15	61 ± 31	53 ± 22	0.75	0.02	0.57

RH, reactive hyperemia; FMD, flow-mediated dilation; BF, blood flow; HR, heart rate; MAP, mean arterial pressure; CO, cardiac output; MBV, mean blood velocity; SR AUC, shear rate area under curve. Data are means ± SD. No significant time x sex (TxS) interactions. Main effect of time for resting BF (1 h < 24 h), HR (1 h > baseline and 24 h), peak RH BF (1 h < 24 h), BF to peak (1 h < baseline and 24 h), MBV to peak (1 h < baseline and 24 h), and time to peak (trend 1 h > baseline). Main effect of sex trending for resting diameter, and significant for peak RH diameter, CO, and BF to peak (men > women for all).

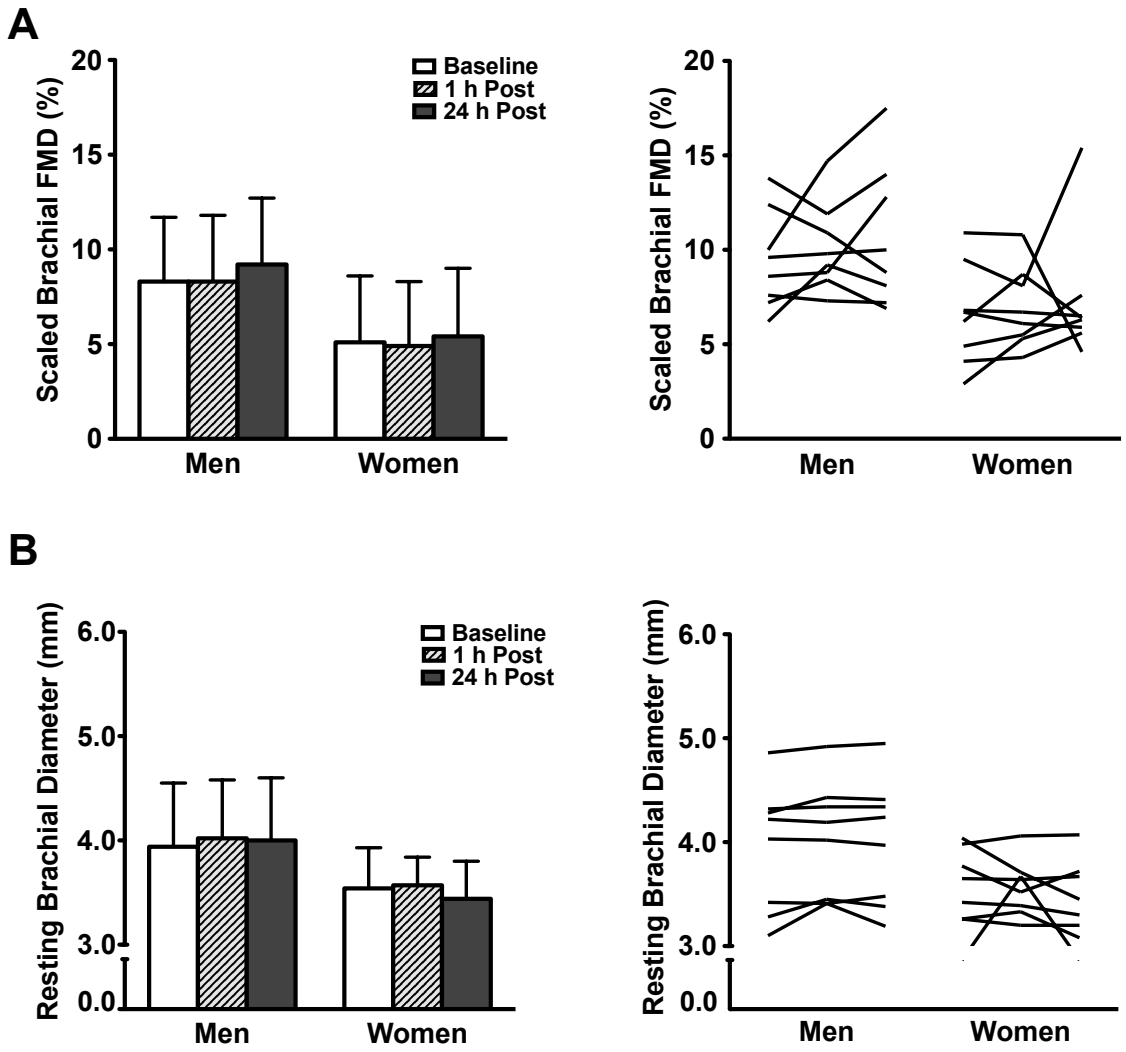

Figure 1. Allometrically scaled brachial FMD (A) and resting brachial diameter (B) at baseline, and 1 and 24 h following a single session of sprint interval exercise in men and women. Group means and standard deviations are presented in the left panel, while individual data are shown on the right. No interaction or main effects were observed. Main effect of sex trending for resting arterial diameter (p=0.06).

physiologically relevant (22, 33). It should be noted that Chuensiri *et al.* (7) observed increases in brachial FMD when an 8x20-s SIT protocol was performed at 170%, but not at 100% or 130%, of PPO, although it is unclear how long FMD remained elevated and whether it was confounded by concomitant changes in arterial diameter and/or hemodynamic parameters. Nevertheless, in the current study, both men and women exceeded 200% of their PPO during the cycling sprints, suggesting that exercise intensity is unlikely to be the reason FMD was unchanged. The absence of an acute FMD response is more likely attributed to our low-volume exercise stimulus and/or our healthy subject population and indicates that estradiol was unable to further influence this response.

A key physiological stimulus underlying endothelial function is shear stress, the frictional force of blood flow on the endothelium (17). Acute increases in anterograde or oscillatory (both anterograde and retrograde) shear during exercise appear to be essential for inducing improvements in endothelial function (3, 33), while unopposed retrograde shear is thought to be detrimental (32). Shear stress increases in the brachial artery during lower-limb exercise (29), and in particular, oscillatory shear has been shown to increase in the inactive brachial artery during rhythmic exercise like walking and cycling (31). To date, no studies have characterized the brachial artery shear response during high-intensity or sprint interval exercise.

Although acute increases in absolute and/or relative brachial artery FMD have been previously observed with interval exercise (2, 5, 7, 8, 12), these exercise

paradigms involved longer intervals at submaximal intensities (12x1-min at 70% PPO, 10x1-min at 80% PPO, 8x1-min at 90% PPO, 7x1-min at 85% PPO, 8x20-s at 170% PPO), whereas the current study utilized a low-volume model involving only 3x20-s supramaximal sprints. How this SIT model influences the brachial artery shear profile and what the minimum shear stimulus required to generate acute and lasting increases in endothelial function are both unknown. As our exercise protocol involved only a few brief bursts of intense work, it is possible the stimulus was insufficient to elicit acute functional changes in the endothelium of healthy adults. While the influence of baseline endothelial function on the acute FMD response to exercise is unclear, persons with cardiometabolic disorders typically demonstrate greater endothelial improvements with exercise training (16). Therefore, low-volume SIT may be a more potent stimulus in persons with impaired endothelial function. Although technically challenging to execute, characterizing the brachial shear stimulus during sprint interval exercise, and comparing it in healthy *versus* clinical populations, would vastly improve our understanding of the regulatory control of endothelial function.

An unanticipated finding in this study was the observation of a slight attenuation in some resting and RH hemodynamic parameters at 1 h post-exercise. While SR AUC remained unchanged and time to peak dilation tended to be longer at 1 h post-exercise, the attenuations in BF (resting BF, peak RH BF, and postdeflation BF averaged to the peak RH diameter) and MBV all seem to suggest that we may have captured the tail end of hemodynamic recovery. It is likely these

responses were greater immediately after exercise and lessened as participants recovered by 1 h post-exercise. Low-volume SIT is a metabolically potent stimulus (13, 15), thus we speculate that following exercise, increased vascular resistance served to redirect blood flow to the lower limbs for metabolite clearance, thereby generating reduced hemodynamic properties in the inactive upper limb where our assessments were conducted. Simultaneous examinations of the hemodynamic responses in the inactive upper limb and active lower limb during and/or after sprint interval exercise are needed to confirm this postulation.

We also did not observe any sex-based differences in the endothelial function response prior to or following an acute session of SIT. The binding of estradiol to estrogen receptors located on endothelial and smooth muscle cells has been shown to increase endothelial nitric oxide synthase expression and activation, thereby increasing nitric oxide bioavailability (6, 23). Previous studies that reported a higher FMD response in women compared to men did not adjust for women having smaller arterial diameters, thus FMD may have been overestimated in women and underestimated in men (19, 20). After accounting for sex differences in arterial diameter in the current study, we did not observe an enhancing effect of estradiol on endothelial function in women compared to men. Even with elevated estradiol concentrations, brachial artery FMD was not higher in women at baseline and was actually somewhat lower than men at all time points, albeit not significantly ($p=0.08$). Including estradiol as a covariate in our analyses did not alter our findings (data not shown), and serum estradiol concentrations

were not associated with the baseline relative FMD or with the change in relative FMD from baseline to 1 h or 24 h post-exercise.

Limitations

This study did not assess the effects of oxidative stress on the acute endothelial function response to sprint interval exercise. Oxidative stress can inactivate NO by converting it to peroxynitrite (11), potentially counteracting any exercise-induced increases in NO production, and has been shown to peak 20 min following a Wingate sprint and to subside by 40 min (18). With our earliest assessment at 1 h post-exercise, the influence of oxidative stress in the current study is likely minimal. Given the brief nature of our exercise stimulus, sprint interval exercise may have elicited changes in endothelial function and hemodynamic parameters that reversed soon after the completion of exercise. Our ability to detect post-exercise changes in endothelial function, interactive effects of time and sex, and/or associations between FMD and sex hormones was likely limited by our small sample size and needs to be confirmed by larger trials. Lastly, our study design did not include a within-subject, non-exercising, time-control condition, which would have substantially increased our statistical power. We do wish to note that any minor changes in absolute and relative FMD observed in this study were within our laboratory's established test-retest variability (coefficient of variance of 10% in men and 20% in women during the mid-follicular phase; in

preparation) and below the 1-1.5% change deemed clinically and physiologically relevant (22, 33).

Conclusions

We demonstrated that brachial artery endothelial function was not altered by an acute session of low-volume SIT in healthy young men or women, nor was it enhanced by higher estradiol concentrations in women. Owing to our small sample size, larger trials are needed to confirm these findings. We recommend that future studies also report hemodynamic parameters as they provide additional insight into the acute brachial artery response to exercise. Investigations in persons with cardiometabolic disorders may also elucidate the influence of baseline function on the acute FMD response to low-volume sprint interval exercise. Lastly, examinations of other interval models are needed to determine a low-volume stimulus that elicits an acute, and potentially chronic, arterial response in persons without baseline impairments in endothelial function.

ADDITIONAL INFORMATION

Competing Interests

None declared.

Author Contributions

Conception and design of the experiments: NS, LES, MJG and MJM. Collection,

analysis, or interpretation of the data: NS, LES and MJM. Drafting the article or revising it critically for important intellectual content: NS, LES, MJG and MJM. All authors approved the final version of the manuscript for publication. All authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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CHAPTER 5

Changes in brachial artery endothelial function and resting diameter with moderate-intensity continuous but not sprint interval training in sedentary men

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RESEARCH ARTICLE

Changes in brachial artery endothelial function and resting diameter with moderate-intensity continuous but not sprint interval training in sedentary men

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Shenouda N, Gillen JB, Gibala MJ, MacDonald MJ. Changes in brachial artery endothelial function and resting diameter with moderate-intensity continuous but not sprint interval training in sedentary men. *J Appl Physiol* 123: 773–780, 2017. First published May 24, 2017; doi:10.1152/japplphysiol.00058.2017.—Moderate-intensity continuous training (MICT) improves peripheral artery function in healthy adults, a phenomenon that reverses as continued training induces structural remodeling. Sprint interval training (SIT) elicits physiological adaptations similar to MICT, despite a lower exercise volume and time commitment; however, its effect on peripheral artery function and structure is largely unexplored. We compared peripheral artery responses to 12 wk of MICT and SIT in sedentary, healthy men (age = 27 ± 8 yr). Participants performed MICT (45 min of cycling at 70% peak heart rate; $n = 10$) or SIT (3 × 20-s "all out" cycling sprints with 2 min of recovery; $n = 9$), and responses were compared with a nontraining control group (CTL, $n = 6$). Allometrically scaled brachial flow-mediated dilation (FMD) increased 2.2% after 6 wk of MICT and returned to baseline levels by 12 wk, but did not change in SIT or CTL (group × time interaction, $P = 0.04$). Brachial artery diameter increased after 6 and 12 wk (main effect, $P = 0.03$), with the largest increases observed in MICT. Neither training protocol affected popliteal relative FMD and diameter, or central and lower limb arterial stiffness (carotid distensibility, central and leg pulse wave velocity) ($P > 0.05$ for all). Whereas earlier and more frequent measurements are needed to establish the potential presence and time course of arterial responses to low-volume SIT, our findings suggest that MICT was superior to the intense, but brief and intermittent SIT stimulus at inducing brachial artery responses in healthy men.

NEW & NOTEWORTHY We compared the effects of 12 wk of moderate-intensity continuous training (MICT) and sprint interval training (SIT) on peripheral artery endothelial function and diameter, and central and lower limb stiffness in sedentary, healthy men. Whereas neither training program affected the popliteal artery or stiffness indexes, we observed changes in brachial artery function and diameter with MICT but not SIT. Brachial artery responses to SIT may follow a different time course or may not occur at all.

arterial stiffness; pulse wave velocity; popliteal artery; exercise training

THE CARDIOPROTECTIVE BENEFITS of regular exercise are well established and include reduced risk for cardiovascular disease (CVD) (23) and improved arterial function and structure (15). Moderate-intensity continuous training (MICT), as reflected in physical activity guidelines (37), increases endothelial function (5, 36), reduces central arterial stiffness (17, 20), and elicits structural remodeling (36). Sprint interval training (SIT) and its companion, high-intensity interval training, have emerged as enjoyable and time-efficient alternatives to traditional MICT (11, 18) that could resolve the "lack of time" barrier for engaging in regular exercise (29). Interval training has been shown to elicit comparable, and at times superior, arterial responses than MICT (27); however, these observations have been made in clinical populations with compromised arterial health. In healthy adults, very few studies have examined the arterial responses to interval training, or compared its effect on arterial function and structure to MICT. To our knowledge, the effects of SIT on endothelial function have been examined in sedentary, healthy adults once (26) and only in the active lower limb. Furthermore, its effects on central stiffness are inconclusive, with studies reporting improvements (19) and no change (26).

Conventionally, brachial endothelial function is a surrogate for coronary endothelial function (1) and an independent predictor of CVD risk (14). Despite its predictive value, endothelial function in the brachial artery cannot be extrapolated to or from active lower limb arteries, as limb-specific differences have been observed (35). Previous studies have either examined the effects of MICT in both the nonactive upper limb and active lower limb (33, 36), or compared the effects of MICT and SIT, but only in the active lower limb (26). Our laboratory previously demonstrated that, in healthy young men and women, 6 wk of MICT or traditional Wingate-based SIT similarly improved endothelial function in the popliteal artery, but we did not assess the nonactive brachial artery (26). Furthermore, in our laboratory's previous work, neither MICT nor SIT elicited changes in popliteal diameter; however, time course studies suggest that training first induces functional changes, followed by structural remodeling that returns function to baseline levels (21, 36).

No studies to date have directly compared the effects of MICT and SIT in both the nonactive and active peripheral arteries of sedentary, healthy men. Therefore, the aim of this study was to comprehensively examine the effects of 6 and 12 wk of MICT and low-volume SIT on brachial and popliteal artery endothelial function and diameter, and central and lower limb arterial stiffness in sedentary, healthy men compared with nontraining controls. We hypothesized that MICT and SIT would augment endothelial function in both the nonactive brachial artery and active popliteal artery after 6 wk of training,

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but that endothelial function would return to baseline levels by 12 wk. We further hypothesized that MICT and SIT would elicit comparable increases in brachial and popliteal diameter, and comparable improvements in arterial stiffness, that would manifest by 12 wk of training.

METHODS

Participants

Twenty-seven sedentary but otherwise healthy men between the ages of 19 and 44 yr were recruited through poster advertisement in and around McMaster University. Twenty-five participants completed the study, as one subject in each of the training groups withdrew from the study for unrelated reasons. The training-induced aerobic capacity and metabolic adaptations in the same subjects have been reported elsewhere by our collaborators (12). Participants were nonsmokers, with the exception of two former smokers in the control (CTL) group. All participants were classified as sedentary based on an International Physical Activity Questionnaire score of less than 600 metabolic equivalents (MET)-min/wk. Exclusion criteria included any known cardiovascular, cerebrovascular, respiratory, metabolic, musculoskeletal, neurological, or renal diseases, as well as the use of steroid or hormone therapy, erectile dysfunction medication, or long-term drug prescriptions, including statins. This study was approved by the Hamilton Integrated Research Ethics Board and conformed to the Declaration of Helsinki. Written, informed consent was obtained from the subjects before participation.

Study Design

Following an initial screening visit to verify eligibility, participants' body mass index (kg/m^2) and aerobic fitness were determined using anthropometric measures of height (m) and weight (kg), and an incremental peak oxygen uptake ($\dot{V}O_{2peak}$) test on a cycle ergometer, respectively. Participants were assigned to a MICT ($n = 10$), SIT ($n = 9$), or nontraining control group (CTL, $n = 6$) based on age, body mass index, and $\dot{V}O_{2peak}$ to ensure equal distribution of these variables between groups at baseline. All assessments of $\dot{V}O_{2peak}$ were conducted on an electronically braked cycle ergometer (Lode Excalibur Sport, version 2.0, Groningen, The Netherlands), as described previously (13). Participants in the training groups were asked to refrain from engaging in additional forms of exercise training, while the control group was asked to remain sedentary. Training groups undertook a minimum of 30 supervised exercise sessions over a 12-wk period, with some participants completing up to 35 sessions to ensure a continued training stimulus until they could be scheduled for posttraining testing. Relevant to this publication, $\dot{V}O_{2peak}$ and vascular assessments were conducted in all groups at baseline and after 6 and 12 wk. Due to the sedentary nature of our participants at baseline, the training program incorporated a lead-in phase, with one session in *week 1*, two sessions in *week 2*, and three sessions per week thereafter, with the exception of *week 7*. Two exercise sessions in *week 7* were replaced with testing visits. Vascular testing was conducted 24–48 h after a training session for the 6-wk assessment, and on average 7 days after the last training session for the 12-wk assessment.

Training Protocols

All training was conducted in the Human Performance Research Laboratory at McMaster University. Heart rate (HR) was monitored continuously during exercise in both groups (Polar A3, Lake Success, NY). Each session began with a 2-min warm-up and ended with a 3-min cool-down. The MICT protocol was modeled after the *Canadian Physical Activity Guideline* recommendations for adults of 150 min of moderate-to-vigorous intensity aerobic activity per week (37). MICT consisted of 45 min of continuous cycling (Ergo Race, Kettler, Ense-Parsit, Germany) at ~70% maximum HR (~55% of $\dot{V}O_{2peak}$), for a total time commitment of 50 min. Mean HR for each 45-min MICT exercise bout was recorded, and workload was increased over the 12 wk to continue to elicit the same relative mean HR (70% maximum HR), thereby maintaining the same relative workload stimulus throughout the training program. SIT sessions consisted of 3 × 20-s "all out" cycling sprints (RacerMate; Velotron, Seattle, WA) against 5.0% body wt and interspersed with 2 min of active recovery at 50 W, for a total time commitment of 10 min (13). Sprint and recovery loads were set using compatible computer software (Wingate Software version 1.0; Velotron).

Vascular Assessments

Vascular assessments were conducted in the Vascular Dynamics Laboratory at McMaster University. Participants were tested in the morning after a 10-h overnight fast (no food or drink with the exception of water) and after having refrained from moderate-to-vigorous intensity activity for 24 h. All vascular assessments were conducted with the participant laying supine in a quiet, temperature-controlled room following a 10-min rest. Resting brachial blood pressure and HR were measured in triplicate using an automated oscillometric device (Dinamap Pro 100; Critikon, Tampa, FL), and the last two measurements were averaged. Resting hemodynamics were also monitored continuously throughout the testing session using a hemodynamic monitor (Finometer MIDI, Finapres Medical Systems; Amsterdam, The Netherlands) and single-lead ECG (model ML 132; ADInstruments, Colorado Springs, CO). For each visit, flow-mediated dilation (FMD) tests and resting arterial diameters were used to assess brachial and popliteal artery function and structure, respectively, while carotid distensibility and pulse-wave velocity (PWV) were used to assess arterial stiffness.

Flow-mediated dilation. FMD is an index of endothelial-dependent vasodilation, with larger dilatory responses reflecting increased endothelial function. FMD was measured simultaneously in the right brachial and popliteal arteries in accordance with current guidelines (31). Blood pressure cuffs positioned around the forearm and calf were rapidly inflated to suprasystolic pressures (200 mmHg) for 5 min to occlude blood flow (BF) to the distal vascular beds. Two identical Doppler ultrasounds (Vivid Q; GE Medical Systems, Horten, Norway) were used to measure arterial diameter before cuff inflation (30-s image at rest) and from 5 s before cuff deflation to 3 min afterwards in both arteries. Longitudinal images (duplex mode) were obtained proximal to the antecubital fossa for the brachial artery, and proximal to the popliteal fossa for the popliteal artery. An insonation angle ≤68° was used for all scans to maximize image quality (25). End-diastolic frames were analyzed using semiautomated edge tracking software [Artery Measurement System (AMS) II, version 1.141; Gothenburg, Sweden] (38) to determine arterial diameter. Resting arterial diameter was used as a surrogate for arterial structure, and absolute and relative changes in FMD were calculated using *Eqs. 1* and *2*:

$$\mathrm{FMD(mm)} = \text{peak RH diameter} - \text{resting diameter} \quad (1)$$

$$\mathrm{FMD(\%)} = \frac{\text{absolute FMD}}{\text{resting diameter}} \times 100 \quad (2)$$

Mean blood velocity (MBV) signals were also collected before cuff inflation (30 s at rest), and from 5 s before cuff deflation up to 3 min afterwards [reactive hyperemia (RH)]. For the brachial artery, an external spectral analysis system (model Neurovision 500M TCD; Multigon Industries, Yonkers, NY) was used to obtain continuous intensity weighted MBV, which was sampled using commercially available hardware (Powerlab model ML870, ADInstruments) and analyzed offline using compatible software (LabChart 7, ADInstruments). Popliteal MBV was analyzed using an available package on our ultrasound offline workstation (EchoPAC PC, version 110.0.2; GE Medical Systems). MBV

(cm/s) and arterial diameter (cm) were averaged into five-cycle rolling bins for brachial and popliteal arteries and used to calculate BF (*Eq. 3*) and shear rate (SR, *Eq. 4*).

$$\mathrm{BF(ml/min)} = (\pi r^2 \times \mathrm{MBV}) \times 60, \text{ where } r = (\text{diameter}/2) \quad (3)$$

$$\mathrm{SR}(s^{-1}) = \frac{\mathrm{MBV} \times 8}{\text{arterial diameter}} \quad (4)$$

We report the average resting BF and characterize the RH response by the peak postdeflation BF (peak RH BF), the MBV averaged to the peak RH diameter (MBV to peak), the SR area under the curve (AUC) to the peak RH diameter (SR AUC to peak), and the time to peak RH diameter (time to peak).

Common carotid artery distensibility. Common carotid artery distensibility is the relative change in artery cross-sectional area for a given change in pressure, with larger values indicating reduced stiffness. Distensibility was measured using a combination of Doppler ultrasound (Vivid Q, GE Medical Systems) and applanation tonometry (model SPT-301, Millar Instruments, Houston, TX), as previously described (22). Briefly, a 12-MHz linear array ultrasound probe was used to obtain longitudinal images of the right common carotid artery (B-mode, 22.9 fps), while a tonometer was positioned over the strongest detectable pulse of the left common carotid artery to simultaneously obtain a waveform representing intra-arterial pressure. Signals were acquired for 10 consecutive cardiac cycles. For each cardiac cycle, ultrasound images were analyzed to determine maximum (d_{max}) and minimum lumen diameters (d_{min}), and tonometry signals were used to determine the pulse pressure (PP), defined as the difference between systolic and diastolic blood pressure. Distensibility was then calculated using *Eq. 5*:

$$\mathrm{distensibility}(\mathrm{mmHg}^{-1}) = \frac{\pi\left(\frac{d_{max}}{2}\right)^2 - \pi\left(\frac{d_{min}}{2}\right)^2}{\pi\left(\frac{d_{min}}{2}\right)^2 \times \mathrm{PP}} \quad (5)$$

Pulse wave velocity. PWV is the speed of a pulse traveling along an arterial segment, with faster speeds reflecting stiffer arteries. PWV was assessed between the carotid and femoral arteries (central PWV) and between the femoral and dorsalis pedis arteries (leg PWV) and calculated using *Eq. 6*. The pulse travel distance was estimated between the two sites with an anthropometric measuring tape across the surface of the body, and 80% of the direct carotid-femoral distance was used to calculate central PWV, as recommended in the current guidelines (6). The pulse transit time was determined from pressure waveforms acquired with applanation tonometry (Mikro-Tip Catheter Transducer, model SPT-301; Millar Instruments), and digitally filtered (band pass, 5–30 Hz) to assist with the detection of the foot of each waveform, as previously described (22). PWV was analyzed in sets of 10 heart cycles and reported as the mean of 2 sets, or the median of 3 sets, if the difference between the first and second sets exceeded 0.5 m/s (6).

$$\mathrm{PWV(m/s)} = \frac{\text{pulse travel distance}}{\text{pulse transit time}} \quad (6)$$

Statistical Analyses

All data were assessed for normality using the Shapiro-Wilk test. The impact of training on brachial and popliteal endothelial function and arterial diameter and arterial stiffness indexes was examined using a two-way mixed-model ANOVA, with factors of group (MICT, SIT, CTL) and time (baseline, 6 wk, 12 wk). Significant interactions or main effects were assessed with pairwise comparisons using Fisher's least significant difference test. Where indicated, main effects were followed up with a one-way repeated-measures ANOVA. Statistical analyses were performed using SPSS Statistics (version 20.0, Chicago, IL) and GraphPad Prism (version 4.0b, La Jolla, CA). Significance was set at $P \leq 0.05$, and data are expressed as means ± SD, unless otherwise noted.

Allometric Scaling of FMD

To determine whether changes in arterial diameter over the 12 wk necessitated allometric scaling of FMD, a linear regression analysis was used to determine the slope and 95% confidence intervals (95% CI) of the relationship between the natural log of peak RH diameter ($\ln D_{peak}$, dependent variable) and resting diameter ($\ln D_{rest}$, independent predictor) across all time points (baseline, 6 wk, 12 wk). Separate regression analyses were performed for each group (MICT, SIT, CTL). Allometric scaling of FMD has been recommended if the unstandardized β-coefficient deviates from 1 and/or the 95% CI has an upper limit <1, as this indicates peak RH diameter is not increasing as a constant proportion of resting diameter (3). When indicated by these criteria, we allometrically scaled FMD by using a linear mixed model with $\ln D_{diff}$ (i.e., $\ln D_{peak} - \ln D_{rest}$) as the dependent variable, group (MICT, SIT, CTL) and time (baseline, 6 wk, 12 wk) as fixed factors, and $\ln D_{rest}$ as the covariate. Fisher's least significant difference test was used for pairwise post hoc comparisons (4). For each group at each time point, linear mixed-model estimated means (EM) were back transformed to obtain scaled FMD, $[(e^{EM} - 1) \times 100]$, and standard errors (SE) were back transformed and used to estimate standard deviations $\{[(e^{SE}-1) \times 100] \times (\sqrt{n})\}$, where n is the group sample size. Scaled FMD for individual participants were estimated using the logged diameters and linear regression unstandardized β, $\left[\frac{(lnD_{peak})}{(lnD_{rest})^{\beta}} - 1\right] \times 100$.

RESULTS

Participants

Baseline characteristics for the 25 participants who completed the study are reported in Table 1. Due to difficulty imaging some participants during the FMD test, brachial data are reported for $n = 24$ (10 MICT, 8 SIT, 6 CTL), and popliteal data are reported for $n = 22$ (8 MICT, 8 SIT, 6 CTL). Due to unobtainable arterial pressure waveform signals in one participant, distensibility, central PWV, and leg PWV data are reported for $n = 24$ (9 MICT, 9 SIT, 6 CTL). There were no group differences at baseline for any of our measures ($P > 0.05$ for all).

Training Effects

Participants in the MICT and SIT groups completed 32 ± 2 and 31 ± 1 supervised training sessions, respectively. $\dot{V}O_{2peak}$ increased similarly in both training groups by ~19% after 12 wk ($P < 0.001$ for both), but was not altered in the CTL group ($P > 0.05$).

Table 1. *Subject characteristics*

	MICT	SIT	CTL
n	10	9	6
Age (range), yr	28 ± 9 (19–44)	27 ± 7 (19–39)	26 ± 8 (19–41)
Height, cm	176 ± 10	177 ± 11	176 ± 5
Weight, kg	84 ± 20	84 ± 23	78 ± 25
BMI, kg/m^2	26 ± 6	27 ± 5	25 ± 7
$\dot{V}O_{2peak}$, ml·kg^{-1}·min^{-1}	34 ± 6	32 ± 7	32 ± 7

Values are means ± SD; *n*, no. of subjects. BMI, body mass index; $\dot{V}O_{2peak}$, peak oxygen uptake. There were no group differences at baseline.

Brachial Artery Endothelial Function

Scaled brachial FMD was similar between groups at baseline ($P = 0.13$), but group differences emerged over the 12-wk intervention (group × time interaction, $P = 0.04$; Fig. 1*A*). Scaled FMD increased with MICT from baseline to 6 wk (9.2 ± 2.4 to 11.4 ± 2.9%, $P = 0.04$), returning to baseline by 12 wk (9.5 ± 3.2%, $P = 0.80$ vs. baseline; $P = 0.12$ vs. 6 wk). In contrast, scaled brachial FMD did not change with SIT from baseline to 6 wk (7.0 ± 2.4 to 8.1 ± 2.4%, $P = 0.25$) or 12 wk (6.3 ± 2.4%, $P = 0.43$ vs. baseline), but there was a trend toward a reduction from 6 to 12 wk (−1.8 ± 2.5%, $P = 0.06$). No changes were observed in the CTL group from baseline to 6 wk (6.9 ± 2.9 to 6.0 ± 2.7%, $P = 0.47$) or 12 wk (5.8 ± 2.8%, $P = 0.38$ vs. baseline), or between 6 and 12 wk ($P = 0.86$). Between groups, MICT elicited a larger scaled FMD response than SIT and CTL at both 6 wk (two-way ANOVA, $P = 0.003$; individual group comparisons: MICT vs. SIT, $P = 0.02$; MICT vs. CTL, $P = 0.001$) and 12 wk (two-way ANOVA, $P = 0.04$; individual group comparisons: MICT vs. SIT, $P = 0.03$; MICT vs. CTL, $P = 0.02$). No training effects were observed for unscaled relative brachial FMD ($P = 0.54$), or any indexes of the brachial RH response: peak RH BF ($P = 0.19$), MBV to peak RH diameter ($P = 0.30$), SR AUC to peak RH diameter ($P = 0.96$), or time to peak RH diameter ($P = 0.50$) (Table 2).

Brachial Artery Diameter and Flow

Resting brachial artery diameter was similar between groups at baseline ($P = 0.23$) and increased over 12 wk (main effect of time, $P = 0.03$; Fig. 1*B*). MICT elicited the largest relative increase in resting diameter from baseline to 12 wk, 8%, compared with 0.5% in SIT and 3% in CTL. Follow-up analyses confirmed significant increases in resting diameter with MICT (one-way ANOVA, $P = 0.01$; individual time comparisons: baseline vs. 6 wk, $P = 0.002$; baseline vs. 12 wk, $P = 0.003$; 6 wk vs. 12 wk, $P = 0.33$), but not SIT ($P = 0.91$) or CTL ($P = 0.63$). Concomitant increases were observed in resting brachial artery BF, which was similar between groups at baseline ($P = 0.60$), but changed only with MICT (group × time interaction, $P = 0.04$; Table 2). Resting BF increased with MICT from baseline to 6 wk (+36 ml/min, $P < 0.001$) and 12 wk (+33 ml/min, $P < 0.001$ vs. baseline), with no differences between 6 and 12 wk ($P = 0.80$). No changes were observed from baseline to 12 wk with SIT (+3 ml/min, $P = 0.77$) or CTL (−6 ml/min, $P = 0.53$). Peak brachial RH diameter was similar between groups at baseline ($P = 0.17$) and increased over 12 wk (main effect of time, $P = 0.03$; Table 2). MICT elicited the largest increases in peak RH diameter, 6%, compared with 0% in SIT and 2% in CTL. Follow-up analyses confirmed significant increases in peak RH diameter with MICT (one-way ANOVA, $P = 0.004$; individual time com-

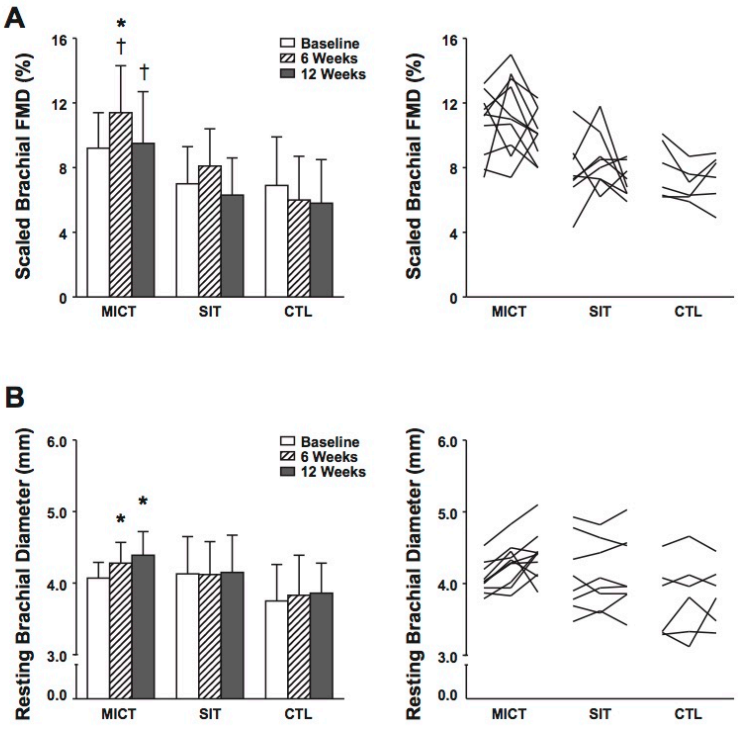

Fig. 1. Allometrically scaled brachial FMD (*A*) and resting brachial diameter (*B*) at baseline and 6 and 12 wk in MICT, SIT, and CTL groups. Group means and SDs are presented on the *left*, whereas individual data are shown on the *right*. Scaled FMD group means and SDs were back-transformed from linear mixed-model estimated means and SEs, respectively. Individual scaled FMD data were estimated using unstandardized β from linear regression analyses. Group × time interaction for scaled brachial %FMD ($P = 0.04$): *$P < 0.05$ vs. MICT baseline; †$P < 0.05$ vs. SIT and CTL at same time. Main effect of time for resting brachial diameter ($P = 0.03$, two-way ANOVA): *$P < 0.05$ vs. MICT baseline (follow-up analysis with one-way ANOVA).

Table 2. *Brachial and popliteal artery FMD characteristics*

	MICT			SIT			CTL			*P* Value
	Baseline	6 Weeks	12 Weeks	Baseline	6 Weeks	12 Weeks	Baseline	6 Weeks	12 Weeks	
Brachial artery (n = 24)										
Resting diameter, mm	4.07 ± 0.22	4.28 ± 0.29	4.39 ± 0.33	4.13 ± 0.52	4.12 ± 0.46	4.15 ± 0.52	3.75 ± 0.51	3.83 ± 0.56	3.86 ± 0.42	0.14
Peak RH diameter, mm	4.45 ± 0.29	4.70 ± 0.28	4.73 ± 0.34	4.41 ± 0.53	4.44 ± 0.41	4.41 ± 0.56	4.03 ± 0.51	4.07 ± 0.56	4.11 ± 0.38	0.11
Absolute FMD, mm	0.38 ± 0.12	0.42 ± 015	0.34 ± 0.10	0.28 ± 0.10	0.32 ± 0.09	0.26 ± 0.07	0.28 ± 0.09	0.24 ± 0.07	0.25 ± 0.09	0.60
Relative FMD, %	9.3 ± 2.9	9.9 ± 3.9	7.9 ± 2.5	6.9 ± 3.0	8.0 ± 2.8	6.2 ± 1.3	6.3 ± 1.3	6.2 ± 1.7	6.7 ± 2.5	0.54
Resting BF, ml/min	57 ± 26	93 ± 56*	90 ± 35*†	48 ± 15	53 ± 27	51 ± 19	46 ± 30	49 ± 29	40 ± 22	0.04
Peak RH BF, ml/min	422 ± 121	419 ± 155	489 ± 182	364 ± 86	456 ± 154	442 ± 161	369 ± 210	365 ± 136	353 ± 171	0.19
MBV to peak, cm/s	30 ± 7	29 ± 10	33 ± 8	29 ± 7	32 ± 8	30 ± 5	31 ± 14	28 ± 9	27 ± 11	0.30
SR AUC to peak, $\times 10^3$	30 ± 15	28 ± 16	31 ± 12	27 ± 14	27 ± 8	28 ± 9	29 ± 7	29 ± 7	27 ± 10	0.96
Time to peak, s	45 ± 10	50 ± 9	44 ± 11	43 ± 7	44 ± 11	46 ± 11	45 ± 7	50 ± 10	52 ± 9	0.50
Popliteal artery (n = 22)										
Resting diameter, mm	6.58 ± 0.93	6.36 ± 0.65	6.51 ± 0.82	6.75 ± 1.08	6.24 ± 0.95	6.37 ± 1.12	5.80 ± 0.95	5.76 ± 1.13	5.86 ± 1.01	0.38
Peak RH diameter, mm	6.82 ± 0.92	6.63 ± 0.63	6.80 ± 0.85	6.99 ± 1.10	6.51 ± 1.00	6.54 ± 1.07	6.14 ± 0.99	6.04 ± 1.12	6.16 ± 1.03	0.27
Absolute FMD, mm	0.24 ± 0.27	0.27 ± 0.25	0.30 ± 0.23	0.24 ± 0.33	0.27 ± 0.25	0.17 ± 0.14	0.34 ± 0.12	0.29 ± 0.13	0.29 ± 0.18	0.79
Relative FMD, %	3.8 ± 4.5	4.3 ± 4.1	4.6 ± 3.9	3.8 ± 4.8	4.4 ± 4.0	2.9 ± 2.7	6.0 ± 2.5	5.2 ± 2.7	5.1 ± 3.6	0.84
Resting BF, ml/min	75 ± 47	61 ± 32	83 ± 44	81 ± 43	71 ± 45	63 ± 50	48 ± 35	52 ± 36	53 ± 27	0.23
Peak RH BF, ml/min	734 ± 131	587 ± 153	737 ± 182	796 ± 240	682 ± 326	693 ± 260	565 ± 311	519 ± 326	541 ± 252	0.14
MBV to peak, cm/s	18 ± 6	10 ± 5	16 ± 6	20 ± 10	18 ± 11	13 ± 6	15 ± 5	16 ± 5	10 ± 4	0.19
SR AUC to peak, $\times 10^3$	14 ± 11	13 ± 7	14 ± 6	13 ± 5	15 ± 8	15 ± 8	13 ± 4	11 ± 6	13 ± 3	0.86
Time to peak, s	60 ± 33	107 ± 51	88 ± 48	64 ± 37	87 ± 55	98 ± 38	77 ± 25	64 ± 44	102 ± 36	0.36

Values are means ± SD; n, no. of subjects. RH, reactive hyperemia; FMD, flow-mediated dilation; Resting BF, 30-s average blood flow at rest; Peak RH BF, peak postdeflation blood flow; MBV to peak, average postdeflation mean blood velocity up to peak RH diameter; SR AUC to peak, shear rate area under curve to peak RH diameter; Time to peak, time to peak RH diameter. P values are group × time interaction. Main effect of time for brachial resting diameter (P = 0.03, baseline < 6 and 12 wk) and brachial peak RH diameter (P = 0.03, baseline < 6 and 12 wk) are shown. Main effect of group for absolute FMD (P = 0.01, MICT > SIT and CTL) is shown. *P < 0.001 vs. MICT baseline. †P < 0.01 vs. SIT and CTL at 12 wk.

parisons: baseline vs. 6 wk, $P < 0.001$; baseline vs. 12 wk, $P = 0.004$; 6 wk vs. 12 wk, $P = 0.70$), but not SIT ($P = 0.86$) or CTL ($P = 0.82$).

Popliteal Artery Endothelial Function and Diameter

Training effects were not observed for any of the popliteal artery measures: absolute FMD ($P = 0.79$), relative FMD ($P = 0.84$), resting BF ($P = 0.23$), peak RH BF ($P = 0.14$), MBV to peak RH diameter ($P = 0.19$), SR AUC to peak RH diameter ($P = 0.86$), time to peak RH diameter ($P = 0.36$), resting diameter ($P = 0.38$), or peak RH diameter ($P = 0.27$) (Table 2). We did not allometrically scale popliteal FMD, since popliteal diameter did not differ between groups at baseline ($P = 0.21$) or change over the 12 wk.

Arterial Stiffness and Resting Hemodynamics

No interactions or main effects were observed for central arterial stiffness (carotid distensibility, $P = 0.50$; central PWV, $P = 0.28$) or lower limb arterial stiffness (leg PWV, $P = 0.52$) (Table 3). Similarly, resting systolic blood pressure ($P = 0.31$), diastolic blood pressure ($P = 0.26$), mean arterial pressure ($P = 0.12$), and HR ($P = 0.85$) did not change with training (Table 3).

DISCUSSION

This is the first study to comprehensively compare the effects of MICT and SIT in both nonactive and active peripheral arteries in sedentary, healthy men. MICT induced changes in the brachial artery that were not observed with SIT, while neither training program induced changes in the active popliteal artery. These findings suggest that brachial artery responses to the intense, but brief and intermittent, nature of SIT may follow a different time course not captured by our 6- and 12-wk assessments, or may not occur at all.

Brachial Artery

In the brachial artery, MICT elicited changes in endothelial function and arterial diameter that were not mirrored with SIT. Allometric scaling unmasked group differences in endothelial function that were not detected in the analysis of unscaled

Table 3. *Arterial stiffness and resting hemodynamics*

Variable	MICT			SIT			CTL			*P* Value
	Baseline	6 Weeks	12 Weeks	Baseline	6 Weeks	12 Weeks	Baseline	6 Weeks	12 Weeks	
Distensibility, $\times 10^{-3}$ mmHg^{-1}	4.6 ± 0.7	4.8 ± 1.0	4.5 ± 1.2	4.6 ± 1.0	4.3 ± 1.0	4.9 ± 1.3	5.3 ± 1.3	5.0 ± 0.6	5.2 ± 1.4	0.50
Central PWV, m/s	6.8 ± 0.7	6.5 ± 0.8	6.6 ± 1.1	6.6 ± 0.9	6.7 ± 0.8	6.7 ± 0.5	6.5 ± 0.6	6.9 ± 0.8	6.4 ± 0.6	0.28
Leg PWV, m/s	7.1 ± 1.2	7.0 ± 1.1	6.9 ± 1.2	6.8 ± 1.0	7.0 ± 1.1	7.2 ± 0.8	6.7 ± 0.7	7.6 ± 1.3	7.5 ± 0.6	0.52
SBP, mmHg	112 ± 8	107 ± 9	111 ± 9	116 ± 8	111 ± 10	112 ± 8	110 ± 13	109 ± 11	109 ± 11	0.31
DBP, mmHg	67 ± 5	65 ± 6	66 ± 5	68 ± 3	67 ± 5	68 ± 7	67 ± 6	70 ± 9	69 ± 8	0.26
MAP, mmHg	85 ± 4	83 ± 5	84 ± 5	87 ± 3	85 ± 5	86 ± 5	84 ± 7	86 ± 6	86 ± 8	0.12
HR, beats/min	64 ± 11	61 ± 11	63 ± 8	64 ± 10	62 ± 10	62 ± 9	61 ± 12	60 ± 9	63 ± 10	0.85

Values are means ± SD. PWV, pulse wave velocity (central: carotid to femoral, leg: femoral to dorsalis pedis); SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; HR, heart rate. P values are group × time interaction. No interactions or main effects were observed.

relative FMD. The 2.2% increase in scaled FMD observed with MICT is comparable to the 1.7–3.2% increases previously reported in healthy men by studies that used protocols of similar intensity and duration (5, 8, 36). With no accompanying changes in RH BF, the increased scaled FMD observed with MICT is likely not the result of an augmented shear stimulus, but rather a reflection of training-induced improvements in brachial endothelial function. The greatest increases in brachial FMD have been reported after 2 wk of endurance training, with a gradual return to baseline levels after 4−6 wk (5, 36). Our 6-wk assessment is at the end of the previously observed window for capturing endothelial function responses in young healthy individuals; consequently, we may have missed peak FMD improvements. Interestingly, our MICT group had a baseline relative brachial FMD of 9.3%, which is larger than previously reported baseline (2.2, 5.8, and 5.9%), and training-induced peak FMD responses (3.9, 8.6, and 9.1%) (5, 8, 36). As such, it is possible functional responses to MICT progressed slower or lingered longer, enabling us to still see significant increases at our 6-wk assessment. Given the trend for reductions in scaled brachial FMD in our SIT group from 6 to 12 wk, it is unclear whether SIT may have elicited increases in brachial FMD before the 6-wk assessment, whether it would have elicited significant reductions if training were extended past the 12-wk assessment, or whether it simply does not alter brachial FMD. The direction and time course of the brachial FMD response to SIT in sedentary, healthy adults warrants further investigation.

Mechanistically, peripheral artery function changes when the endothelium repeatedly experiences elevated levels of shear stress (16). While it is technically challenging to measure shear stress during exercise, our findings suggest that MICT might provide a greater shear stimulus compared with low-volume SIT. The lack of response we observed in brachial artery endothelial function with SIT is in contrast to previous reports of interval training eliciting comparable, or superior, responses to MICT (27). This discrepancy may be the result of differences in the populations studied and the interval training models utilized. In the meta-analysis by Ramos et al. (27), interval training induced superior endothelial function changes compared with MICT in persons with elevated CVD risk (postinfarction, coronary artery disease, hypertension, metabolic syndrome, obesity, type 2 diabetes, and postmenopausal women), and presumably with impaired baseline endothelial function. Exercise training is known to elicit greater functional improvements in persons with cardiometabolic disorders (2); therefore, interval training may be a potent stimulus in impaired, but not healthy, vasculature. Additionally, interval training models previously used in clinical populations have had work intervals that were longer in duration and lower in intensity (i.e., 4 × 4 min at 90–95% peak HR; 10 × 1 min at 80–104% peak power output; 4–6 × 1 min at 80–85% $\dot{V}O_{2peak}$) compared with the model used in the present study. Therefore, it may also be that our 3 × 20-s SIT model was too brief to induce a sufficient shear stimulus, and thus functional changes, in persons without baseline impairments in endothelial function.

Despite a main effect of time in resting brachial diameter, the greatest increases were observed in MICT, whereas changes in SIT and CTL were small and within our laboratory's established day-to-day variability (3% coefficient of variance; unpublished data). Our findings are in agreement with Sawyer et al. (28), who recently reported a similar 4.9% increase in brachial artery diameter with 8 wk of a comparable MICT protocol, but no change in diameter with 10 × 1-min high-intensity interval training in obese adults. No changes in resting brachial diameter have been previously reported with lower limb training in sedentary, healthy men (8, 10, 33, 36); however, increases in vasodilator capacity were observed after an 8-wk combined cycling and treadmill program (36). Vasodilator capacity may be a more robust index of arterial remodeling, as resting diameter reflects changes in structure and/or vascular tone (34). Nevertheless, blood pressure and HR remained steady across visits, suggesting sympathetic tone was unaltered by training, and that the MICT-induced increase in resting brachial diameter likely reflects structural remodeling. This is further supported by the concomitant increase we observed in resting brachial BF with MICT, as a larger conduit artery diameter is able to facilitate more BF through the artery. Contrary to our hypothesis, SIT did not elicit any changes in resting diameter. SIT-induced structural remodeling may occur at a much slower rate than with MICT, or the brief and intermittent nature of low-volume SIT may not be sufficient to induce structural changes in the brachial artery.

Popliteal Artery

An unexpected finding in this study was that neither MICT nor SIT elicited changes in the active popliteal artery. Our laboratory previously showed that 6 wk of MICT and traditional Wingate-based SIT similarly improved relative FMD in the popliteal artery, without changing arterial diameter (26). Others have shown training-induced arterial remodeling after 4−8 wk in the popliteal artery (36), and after 8 and 12 wk in the femoral artery (10, 33). Thus we anticipated that low-volume SIT involving 1 min of intense intermittent exercise would elicit comparable responses to MICT, and that extending training to 12 wk would enable us to observe changes in arterial diameter. Compared with previous studies that successfully induced training responses, our participants had a larger resting popliteal diameter (6.4 mm vs. 5.6 mm and 4.9 mm) (26, 36). The popliteal artery is not uniform throughout its length, and normative values suggest we imaged the proximal (6.9 ± 0.9 mm) or midlevel (6.8 ± 0.8 mm), whereas previous studies imaged the smaller distal level (4.9 ± 0.6 mm) (39). The mechanisms by which the heterogeneity in size, and perhaps structural morphology along the artery, impact endothelial function are poorly understood. Nevertheless, popliteal diameter and relative FMD are negatively and moderately correlated ($r = -0.48$, $P = 0.03$) (32). This may elucidate why, compared with other studies, relative FMD in our training participants was smaller at baseline (3.8 vs. 5.0 and 6.2%) and did not change with training (±0.9 vs. +1.7 and +3.3%) (26, 36). These findings suggest that baseline popliteal diameter not only influences the FMD response, but it may also impact the magnitude of training effects in the FMD response. With limited data and discrepancies between studies, more research is required to determine the degree to which training responses depend on baseline diameter and/or the level along the popliteal artery, and whether responses differ between MICT and SIT.

Arterial Stiffness

Traditional endurance training has been shown to improve various indexes of stiffness in sedentary, healthy adults (9, 17, 20, 26, 30). Fewer studies have investigated the effects of SIT (or high-intensity interval training), and all have used protocols involving 20–40 min/session (7, 19, 26). Exercise training studies ranging from 6 days to 16 wk have reported significant reductions in central PWV of 0.3−0.5 m/s (7, 9, 19, 20). In the present study, despite comparable baseline values and a similar 0.3 m/s reduction after 6 wk of MICT, we did not observe any training effects on central PWV. Our ability to detect significant changes may have been limited by the interindividual variability in the change from baseline to 6 wk, which was twice as large as the mean difference (0.3 ± 0.6 m/s). Consistent with our central PWV results, and past findings (26), we did not observe changes in carotid artery distensibility, a localized index of central stiffness. Furthermore, 6 and 12 wk of MICT or low-volume SIT did not change leg PWV. In sedentary, healthy adults, previous observations of training effects on lower limb stiffness are limited and conflicting. Our findings are in agreement with Hayashi et al. (17) who reported no change in leg PWV after 16 wk of brisk walking/jogging. However, decreases in leg PWV were previously observed after 6 days of a 2-h cycling program (9), suggesting that changes in lower limb stiffness may require more intensive training stimuli. Training effects may also depend on baseline stiffness levels, as leg PWV was lower in the present study, 7.0 vs. 9.7 and 9.8 m/s (9, 17), suggesting that our participants already had reduced lower limb stiffness before commencing the training program. Overall, our findings suggest that 12 wk of traditional MICT or low-volume SIT did not induce changes in central or lower limb arterial stiffness in sedentary, healthy men.

Limitations and Strengths

This study assessed resting arterial diameter, which we acknowledge is neither a direct nor comprehensive indicator of arterial structure. Despite observing increases in resting and peak RH diameters in the brachial artery, these measures do not reflect an artery's true peak dilatory capacity, and thus are not ideal surrogates for examining arterial remodeling. Future studies assessing structural responses to exercise training are encouraged to induce peak arterial dilation using a combination of nitroglycerin administration, forearm ischemia, and ischemic exercise (24). As our laboratory previously observed changes in popliteal endothelial function, but not arterial diameter, following 6 wk of MICT and traditional Wingate-based SIT (26), the time points selected in the present study allow us to make comparisons to, and build on, our previous findings. However, earlier and more frequent assessments would have enabled us to capture peak functional changes with MICT and any changes elicited by SIT that may have been missed, as well as establish a time course for potential brachial artery responses to low-volume SIT. Additionally, since we observed MICT-induced changes in endothelial function and diameter in the brachial artery, it would have been interesting to assess arterial stiffness in the nonactive upper limb (i.e., carotid-radial PWV). A major strength of this study is our comprehensive comparison of the effects of MICT and low-volume SIT on the arteries of healthy, untrained adults, as well as our inclusion of a nontraining control group.

Conclusions

We demonstrated that 6 and 12 wk of traditional MICT were superior to low-volume SIT at eliciting changes in the nonactive brachial artery. Despite resulting in similar improvements in aerobic capacity, MICT induced increases in brachial endothelial function and arterial diameter that were not apparent with low-volume SIT involving a total of 1 min of "all out" exercise per session. Arterial responses to the intense, but brief and intermittent, SIT stimulus may follow a different time course not captured with our 6- and 12-wk visits, or may not occur at all. Future studies should incorporate earlier and more frequent assessments to determine the time course of potential arterial responses to low-volume SIT. Investigations of other interval training models may also elucidate the specific characteristics of stimuli required to elicit functional and structural arterial responses in healthy adults.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

N.S., J.B.G., M.J.G., and M.J.M. conceived and designed research; N.S. performed experiments; N.S. analyzed data; N.S. and M.J.M. interpreted results of experiments; N.S. prepared figures; N.S. drafted manuscript; N.S., J.B.G., M.J.G., and M.J.M. edited and revised manuscript; N.S., J.B.G., M.J.G., and M.J.M. approved final version of manuscript.

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CHAPTER 6: DISCUSSION

6.1 DISCUSSION OVERVIEW

This thesis set out to investigate the influence of differential sex hormone profiles, particularly estradiol, as well as the time-efficient 3x20-s low-volume SIT protocol on brachial artery endothelial function in healthy young adults. Prior to undertaking these investigations, however, we established best practices for FMD data analysis and demonstrated a high-degree of intra-individual consistency in the FMD response pattern (Chapter 2). Specifically, we introduced visual data screening as a useful tool for ensuring observed FMD responses and alterations are accurate and physiological. Through our investigations of the potential influence of estradiol on endothelial function, we demonstrated that brachial artery FMD remains stable across a NAT or OCP cycle despite changes in estradiol concentrations (Chapter 3). Moreover, we concluded that higher estradiol concentrations did not enhance endothelial function in premenopausal women compared to men at rest or acutely following exercise. Indeed, women were observed to have lower resting brachial artery FMD responses than men in more than one study (Chapters 3 and 4). Through our investigations of the potential influence of SIT on endothelial function, we demonstrated that brachial artery FMD was unchanged following either a single exercise session or 12 weeks of exercise training (Chapters 4 and 5). Combined, these findings suggest that the 3x20-s low-volume SIT protocol may be an insufficient stimulus for eliciting acute and/or chronic changes in brachial artery endothelial function in healthy young adults. This general discussion integrates the main findings of this thesis and provides an

overarching interpretation in light of what is currently known in the literature. Where applicable, limitations are addressed and future directions for advancing this body of work are recommended.

6.2 CONSIDERATIONS FOR ADVANCING FMD DATA ANALYSIS

To date, the variability reported in the literature between repeat FMD measurements in healthy young adults has ranged widely from 10-84% (10, 20, 31, 37, 41, 48, 49, 52). Greater adherence to FMD guidelines has been associated with improved reproducibility, but adherence has only been able to explain ~36% of the variance observed between studies (27). As current FMD guidelines focus on subject preparation and data acquisition (54), some of the unexplained variance in FMD reproducibility between studies could be attributed to the lack of standardization in data analysis practices. Internal laboratory observations revealed that the variability between repeated FMD tests can be much higher than those reported in Chapter 2 (45-50% *versus* 10-25%, respectively) if operators are inexperienced and/or do not follow the practices recommended in Chapter 2. It should be noted that these observations were made between different data sets and do not reflect improved variability within a single data set. Nevertheless, processes for screening data and recognizing potential analysis errors may help to improve FMD reliability and accuracy and should be addressed in future guidelines.

To our knowledge, this thesis is first to demonstrate that the RH dilatory response pattern, while variable between individuals, is highly consistent within an

individual. In Chapter 2, we proposed that visual data screening could improve the detection of edge-tracking errors that may otherwise go unnoticed but that could alter the reported FMD parameters considerably. For instance, a boundary detection error in resting diameter of ~0.3 mm may seem trivial but can artificially double the FMD response, as exemplified in Chapter 2, and is equivalent in magnitude to the increases in arterial diameter associated with 12 weeks of endurance training, as observed in Chapter 5. Detecting such errors could prevent the reporting of erroneous conclusions and the ascribing of physiological adjustments in endothelial function without foundation. Understandably, a valid concern regarding visual data screening is that it may bias the analysis process. We suggest that the principle of intraindividual consistency of FMD response pattern can be leveraged to improve analysis accuracy without compromising the requirement for unbiased assessments. Visual data screening only requires an operator to know which resting and RH images belong to the same FMD test and which FMD tests belong to the same individual. Thus, regardless of whether future guidelines support visual data screening, the blinding of operators to a participant's visit and/or group should be promoted as standard practice for FMD analysis. It should be noted that blinding was not used in this thesis and is regarded as a limitation; however, our findings contrasted our hypotheses and so are unlikely to have been influenced by operator bias. Nevertheless, visual data screening in conjunction with operator blinding could prove to be a highly effective strategy for

improving FMD reliability, minimizing operator bias, and reducing the risk of spurious findings.

Allometric scaling is another component of FMD data analysis that was noted in Chapter 2 and used frequently in this thesis. Conventional reporting of relative FMD is known to be impacted by baseline artery size such that relative FMD responses underestimate endothelial function for larger arteries and overestimate endothelial function for smaller arteries (2). Allometric scaling has been purported to remove the dependency of FMD on arterial diameter, thereby reflecting endothelial function more accurately (3–5). Indeed, the advocators of allometric scaling, Atkinson and Batterham, have shown that previously reported group differences in relative FMD are eliminated when allometric scaling is used to adjust for group differences in arterial diameter (4). Therefore, this thesis used allometric scaling to adjust for baseline differences in arterial diameter between men and women (Chapters 3 and 4) and exercise-induced increases in arterial diameter following 12 weeks of exercise training (Chapter 5). In both cases, we observed that allometric scaling unmasked group differences that were not apparent with analysis and reporting of absolute or relative FMD.

Although allometric scaling may reflect endothelial function more accurately than conventional relative FMD, it is not without its limitations. The statistical analysis associated with allometric scaling is much more involved than determination of a simple percent change, and without a clear understanding of how to perform the analysis, researchers risk reporting incorrect findings ensuing

from statistical errors. It is also not yet clear if, and how, adjusted ratios for individual participant data can be expressed as a percentage for conventional interpretation (38, 40). Last, and perhaps most importantly, to date, only the conventional expression of brachial artery FMD has been shown to correlate with invasive assessments of coronary artery endothelial function (53) and to predict future cardiovascular events (58). It is currently unknown if the FMD response adjusted for baseline diameter (allometrically scaled) still confers health information, and if so, what magnitude of change is clinically and physiologically relevant. It is also unclear how to statistically determine the test-retest repeatability for scaled FMD. Therefore, comparisons made in this thesis between allometrically scaled FMD and the 1-1.5% change in relative FMD deemed clinically and physiologically relevant (32, 56), and/or to our laboratory's established test-retest repeatability for relative FMD, may not be valid. As a result of the limitations surrounding its implementation and interpretation, allometric scaling has not been widely adopted in FMD research, despite having clear benefits. Future FMD guidelines should address the role of allometric scaling in FMD research. If allometric scaling is endorsed, clear and thorough instructions for its proper use should accompany the guidelines.

6.3 ENDOTHELIAL FUNCTION IS NOT INFLUENCED BY ESTRADIOL

In this thesis, brachial artery FMD remained stable across a menstrual and OCP cycle despite changes in estradiol. Although previous studies have suggested

that changes in brachial FMD are associated with changes in estradiol (1, 16, 29, 30, 57), some studies did not directly assess this relationship (1, 30, 57), while others failed to observe a significant correlation (16, 29). In Chapter 3, we demonstrated that changes in estradiol across a menstrual cycle are not correlated with changes in FMD. Similarly, in Chapter 4, we demonstrated that estradiol is not correlated with FMD, or the post-exercise change in FMD, in men and women tested in a high-estradiol phase. This lack of relationship, coupled with similar observations of unchanged FMD during high-estradiol phases (12, 33, 39, 46, 50, 51), suggests that estradiol-mediated dilation in premenopausal women may be tightly regulated across a menstrual cycle. ERα expression has been shown to increase by 30% from menstruation to ovulation (19), thus it is possible brachial artery FMD is dependent on ERα expression rather than estradiol concentrations *per se*. These differences in receptor expression could potentially explain why some studies have observed increases in FMD across the menstrual cycle while others have not. In the current thesis, women were tested in the mid-follicular phase and not near ovulation. Unfortunately, it is difficult to determine which of the previous studies that assessed FMD near days 12-14 of a menstrual cycle captured ovulation as most studies did not test for ovulation or report the average menstrual cycle length, and the length of the follicular phase is known to be highly variable (7). Moreover, ERα expression alone may not be able to explain all the differences between previous studies. Shorter variants of ERβ lack a functional ligand domain but have been shown to preferentially dimerize with ERα thereby

inhibiting its activity (43). Evidently, the signalling mechanisms regulating estradiol-mediated dilation are complex and should be considered when assessing FMD across a menstrual cycle.

With regards to our study of women using combined monophasic OCPs in Chapter 3, blood samples confirmed that endogenous estradiol was suppressed during the active pill phase, but EE bioavailability could not be determined. Nevertheless, brachial artery FMD did not change across a pill cycle and did not differ from naturally cycling women. Interestingly, prolonged use of a second, but not a third or fourth, generation pill was associated with reduced endothelial function. The androgenicity of second generation progestins is directly associated with side effects like acne, hirsutism, and weight gain (34), but its unfavourable effects on endothelial function are less clear. ER expression has been shown to be down-regulated with a levonorgestrel releasing intrauterine system, but gradually returned to baseline levels 6-12 months post-insertion (35). In cultured endothelial cells, levonorgestrel has also been shown to inhibit estradiol-mediated increases in eNOS expression (59). Additionally, second generation OCPs are known to increase blood pressure (23) and alter lipid profiles (17, 24), both of which could be contributing to the negative relationship with endothelial function, with extended use. Nevertheless, in our cohorts, endogenous progesterone and third and fourth generation progestins do not appear to be negatively affecting endothelial function.

Our conclusion that estradiol is not influencing endothelial function is further supported by our comparisons between premenopausal women and age-matched

men (Chapters 3 and 4). In Chapter 3, allometrically scaled brachial artery FMD was actually *lower* in naturally cycling women and women using OCPs compared to men, regardless of cycle phase. In Chapter 4, naturally cycling women were once again observed to have lower endothelial function than men, despite being tested during the follicular phase of the menstrual cycle. One possible explanation for the lower endothelial function in women is that they were less fit than men. While this may have been true for Chapter 3, as fitness was not assessed objectively, it does not explain the recurring finding in Chapter 4 when men and women were matched for relative cardiorespiratory fitness per fat-free mass. An alternative explanation is that the lower smooth muscle function observed in naturally cycling women in Chapter 3 may have confounded the FMD findings. Unfortunately, endothelium-independent function was not assessed in Chapter 4 as we did not want the residual effects of nitroglycerin to impact or confound our exercise stimulus and post-exercise measurements. Undoubtedly, other sex- and/or gender-based differences (i.e. differences in cell signaling regulation or type of physical activity men and women engage in, respectively) may be influencing the endothelium-dependent and/or endothelium-independent responses.

6.4 ENDOTHELIAL FUNCTION IS NOT INFLUENCED BY LOW-VOLUME SIT

To date, previous research investigating vascular responses to interval exercise has focused heavily on HIIT protocols, many of which are not that much more time efficient than traditional MICT. Several variations of the “1-min on, 1-min

off" paradigm have been investigated (6, 8, 11, 18), as well as Wingate-based SIT (28, 47), with both models suggesting that high/supramaximal intensity exercise performed in intervals can have beneficial effects on endothelial function. In this thesis, we were interested in investigating the 3x20-s protocol, which is currently one of the lowest volume and most time efficient SIT protocols that has been shown to elicit other physiological benefits (22, 42). In Chapters 4 and 5, we demonstrated that 3x20-s all-out cycling sprints did not enhance the brachial artery FMD response in heathy young adults either 1 h post-exercise or following 12 weeks of training. Although our findings collectively suggest that this low-volume protocol may be insufficient to enhance the already healthy endothelium of young adults, there are several factors that should be considered.

Our exercise stimulus involved supramaximal intensity effort, but 20-s intervals are quite brief and 3 bouts are near the minimal that has been shown to elicit improvements in fitness and metabolic markers (21, 22, 42). An important question to consider, then, is what about low-volume SIT can make it a sufficient or insufficient stimulus for eliciting changes in endothelial function? The main mechanism by which exercise is purported to have direct effects on the endothelium is the shear stress stimulus (25, 26). Using Doppler ultrasound to measure brachial artery blood flow, and the resulting shear stress, in the brachial artery during a 20-s supramaximal sprint is technically challenging to execute and was not conducted for the studies in this thesis. Consequently, it is currently unknown what effect low-volume SIT may be having on brachial artery shear

stimulus. Nevertheless, research in cultured endothelial cells has suggested that very rapid increases in shear stress are important for increasing NO production (15, 36). Specifically, eNOS has been shown to dissociate from inhibitory transmembrane proteins when the onset of shear stress is sudden (within 0.5 s) but not when it is steady (over 30 s) (15). Thus, from a vascular standpoint, the effectiveness of SIT may be dependent on how fast an individual can reach supramaximal intensity at the start of each cycling sprint, supposing of course, that this translates to a rapid increase in brachial artery shear stress. Moreover, eNOS dissociation was observed to occur within 7 s of a sudden increase in shear, with maximal dissociation occurring after 15 s and maximal reassociation after 45-60 s (15). Relating these findings to our 3x20-s model, it could be suggested that the 20-s cycling sprints are of sufficient duration to activate eNOS but that 3 bouts may have been insufficient to elicit acute or lasting changes in endothelial function. It may also be that recovery intervals of at least 45-60 s are needed to allow time for eNOS to rebind to the plasma membrane before the next cycling sprint elicits a subsequent shear stimulus. Thus, perhaps an alternate SIT model whereby the 2-min recovery intervals are reduced in order to allow for additional sprints, while still maintaining a 10 min total time commitment, may be more effective in terms of eliciting changes in endothelial function. The minimum number of cycling sprints required to effectively maximize the shear-mediated signaling of NO production warrants further investigation.

To date, the proposed time courses for the acute (13) and chronic (55) FMD response to exercise are based heavily, if not entirely, on traditional endurance exercise. Although we assessed FMD at relatively standard time points (acute: 1 and 24 h, training: 6 and 12 wk), it is possible SIT elicited changes in the endothelium that simply did not align with the timing of our assessments. Relative to the brief and condensed nature of SIT (i.e. summed total of 1 min of intense exercise within a 10 min total time commitment), our 1 h post-exercise assessment may no longer be 'acute'. Brachial artery FMD was observed to increase by ~8% immediately after an 8x20-s protocol (9), but it is unknown how long it remained elevated as FMD was not reassessed at subsequent time points. Although our 3x20-s protocol may have also elicited changes in FMD immediately after exercise, early responses are challenging to interpret as they may be confounded by exercise-induced changes in sympathetic activity, blood flow/shear stress, arterial diameter, and/or oxidative stress (44).

Regarding the training response, more frequent assessments over the duration of a training period are needed to confirm that brachial artery FMD is indeed unchanged with SIT. It is possible that arterial responses to SIT follow a condensed or extended time course relative to the established time course for MICT. On the one hand, the intensity of SIT may elicit a stronger stimulus than MICT, causing changes in FMD to occur sooner. On the other hand, the brevity of SIT may require more exercise sessions, and thus more time, before changes in FMD are observed. Although exercise training is simply a series of recurring acute

exercise sessions, the relationship between the acute and chronic (training) vascular responses is not well understood. The classical physiological *hormesis* concept suggests that challenging a system acutely can, with repeated exposure, induce long-term protective effects (45). However, it was recently shown that while FMD was not changed after an acute MICT session, post-exercise changes in FMD were still moderately correlated with resting FMD after 2 weeks of training ($r=0.63$, $p=0.002$) (14). Moreover, the hormesis concept fails to explain situations whereby endothelial function is enhanced, rather than impaired, immediately after exercise, unless of course these early increases in the acute FMD response are confounded by other factors as alluded to earlier. In this thesis, our findings of unchanged endothelial function following a single SIT session or 12 weeks of training were consistent, but our studies were not designed to examine the direct relationship between the acute and chronic FMD responses. Future investigations of the training response to SIT should incorporate an acute FMD assessment, so as to be able to assess the relationship between the acute and chronic FMD responses.

6.5 CONCLUSIONS

The studies presented in this thesis suggest that endothelial function, as measured by brachial artery FMD, is tightly regulated in healthy young adults. Brachial artery FMD appears to be unaffected by changes in estradiol across a menstrual or OCP cycle. This work suggests that it may not be necessary to control for menstrual cycle phase and/or OCP use when assessing brachial artery FMD in

premenopausal women, which would certainly make it more feasible to include women as research participants in future studies where FMD is an outcome measure. However, mechanistic studies are needed to elucidate the regulation of estradiol-mediated signaling and its effects on brachial artery FMD across a menstrual and OCP cycle. Sex differences in both endothelium-dependent and endothelium-independent function also warrant further investigation. The acute and chronic brachial artery FMD responses were also unaffected by the 3x20-s low-volume SIT protocol we applied in both a single session (acute) and repeat session (training) fashion. Investigations of the minimal number of cycling sprints required to elicit an acute change in FMD, the time course of potential changes, as well as the relationship between the acute and chronic FMD responses would all be valuable extensions of the current work. As our investigations were conducted in healthy young adults, it would also be interesting to examine whether low-volume SIT is a more potent exercise stimulus in persons with impaired endothelial function. Future investigations of the potential influence of estradiol and/or low-volume SIT on brachial artery FMD would greatly benefit from incorporating assessments of relevant regulatory mechanisms. Lastly, future FMD research could perhaps also benefit from standardized screening tools for data analysis. Despite the limitations and the fact that there is ample work for future investigations, the combined results of the current work provide substantial technical and theoretical advances in the understanding of the regulation of endothelial function in healthy young humans.

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APPENDIX A: COPYRIGHT PERMISSIONS

(Chapter 5)

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Title: Changes in brachial artery endothelial function and resting diameter with moderate-intensity continuous but not sprint interval training in sedentary men

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